LEARNING AND MEMORY

A COMPREHENSIVE REFERENCE



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FOREWORD

A comprehensive reference work on learning and memory could not be better timed than this. During the second half of the twentieth century, the study of learning and memory moved from a descriptive science largely based on the pioneering behavioral analyses of Pavlov, Thorndike, Watson, Skinner, Kamin, Rescorla, and Wagner to a new mechanistic science of mind that combines these brilliant behavioral studies with an analysis of the underlying neural mechanisms, first in a regional manner by Milner, Tulving, Mishkin, Squire, Schachter, and Morris, then on the cellular level, and finally on the molecular level.

The challenges that now face the field are outlined by the five great pioneers in the study of memory – the editor-in-chief Jack Byrne and the editors of these four extraordinary volumes: *Learning Theory and Behavior*, edited by Randolf Menzel; *Cognitive Psychology of Memory*, edited by Henry Roediger; *Memory Systems*, edited by Howard Eichenbaum; and *Molecular Mechanisms of Memory*, edited by David Sweatt. The challenge faced by the contributors to these volumes was to combine the molecular mechanisms with the other three levels in order to provide a coherent, systematically and intellectually satisfying understanding of learning and memory. This is central to the new science of mind. Since memory is the glue that holds our mental life together, the topics covered by these four volumes are central to and paradigmatic for all aspects of the neurobiology of mental life, which has as its goal the understanding of all mental processes in neurobiological terms. Indeed, it is the plasticity of the brain that is the key to understanding the continuity of all mental function. The goal for each of these four volumes was to bridge the subdisciplines concerned with the various forms of memory into a coherent science. The chapters of each of these volumes succeed admirably in doing just that. As a result, this rich and rewarding reference work will serve as a superb framework for the decades ahead, a reference that will provide both the student and the working scientist with the intellectual background necessary to understand and function effectively in the study of learning and memory.

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PREFACE

Learning and Memory: A Comprehensive Reference is the most authoritative set of volumes ever produced on learning and memory and represents the state of the science in the early 21st century. The study of learning (the process of acquiring new information) and memory (retention of that information for future use) has intrigued philosophers and writers for centuries because our memories and plans for the future consolidate who we are, and disruption of these processes dramatically interferes with our daily lives. The fascination with learning and memory is not limited to the humanities, but has been the subject of intense scientific research. Psychologists are concerned with elucidating the features of learning and memory processes and systems, neurobiologists seek to determine the neuronal mechanisms of learning and memory, and neurologists and psychiatrists focus on research and treatment of failures or disruptions in learning and memory.

The study of learning and memory represents a scientific field that has matured at all levels – from the discovery of the protein chemistry and molecular biology of the cellular events underlying learning and memory, through the delineations of the properties and functions of neuronal networks, to formulating and testing the psychological and behavioral neuroscientific theories of learning and memory. In addition, many basic research findings have applied implications on such diverse fronts as education, legal issues hinging on eyewitness testimony, learning disorders in children, memory disorders following brain damage, and declines in memory in older adults.

The volumes in this *Comprehensive Reference* are the result of a meeting in London in July of 2005 where the editors planned the massive work of consolidating all facets of the study of learning and memory. We collected nearly all the topics (albeit from many different disciplines and directions) that we considered constituted scientific approaches to learning and memory and proceeded to parcel the topics into four volumes, resulting in *Learning Theory and Behavior* edited by Randolf Menzel; *Cognitive Psychology of Memory* edited by Henry Roediger III; *Memory Systems* edited by Howard Eichenbaum; and *Molecular Mechanisms of Memory* edited by David Sweatt. This was a formidable task, not only because of the richness and diversity of the subject matter, but also because we needed to logically place topics in the appropriate volume. Although some of the decisions may seem arbitrary, and indeed there is overlap both within and between volumes, each editor ended up with a set of coherent topics that they could organize and introduce in a logical manner.

With approximately 40 chapters per volume, it is no surprise that the editors cover an unusually wide range of intellectual territory or that there is a difference in interpretation by some authors. The organization is a significant editorial challenge and investment in and of itself. However, it is the editor's selection of authors, and the ensuing scholarship on learning and memory from different perspectives, that make this series unique. Authors were identified and invited based on their expertise on a particular topic, and their contributions represent a marvelous compendium of research in learning and memory. The chapters in this series not only represent scientific strength and breadth, but also range from learning at the synaptic level to a systems level approach, and include studies of remarkable learning capabilities in a variety of invertebrates and vertebrates, including human beings.

The first volume in the series, *Learning Theory and Behavior* edited by Randolf Menzel, consists of 38 chapters and sets the tone for the interdisciplinary and comparative approach to the study of learning and memory. He introduces the volume by emphasizing both the value and the limitation of the comparative approach in natural and laboratory settings, stressing that we need information from the behaving animal as well as the neuronal

structures in order to understand the processes involved in information storage and retrieval. Several chapters review progress from using animal models, including worms, molluscs, insects, rodents, birds, and nonhuman and human primates. In addition, concepts such as planning, decision-making, self-awareness and episodic-like memory, usually reserved for human beings, are discussed at several taxonomic levels. The final chapters take an engineering perspective and describe synthetic approaches, including modeling neuronal function and developing a concise theory of the brain.

The second volume, *Cognitive Psychology of Learning* edited by H. Roediger, is comprised of 48 chapters on various aspects of cognitive ability and the underlying neuroscience. The basics of attention, working memory, forgetting, false memories, remembering vs. knowing, the process of recognition, and episodic memory are covered. In addition, topics that are often not included in "memory" volumes deservedly receive attention here, e.g., learning of concepts and categories, learning of perceptual and motor skills, language learning, and implicit learning. This volume also covers memory processes throughout the human lifespan and includes chapters on individual differences in memory ability, both subnormal (learning disabilities) and supranormal (performance of mnemonists and experts in particular domains). Finally, chapters on applied aspects of memory research, dealing with such topics as eyewitness identification in the legal system and applications of research to educational issues, are included.

Volume 3, edited by H. Eichenbaum, consists of 29 chapters which represent a "progress report" on what we know about memory systems and their relationship to different parts of the brain. *Memory Systems* returns to a comparative approach of learning and memory. This volume introduces the concepts of multiple memory systems, and many chapters discuss in extensive detail the different features of declarative memory and their underlying brain structures. Procedural learning in humans and other animals is addressed, and a short section details the involvement of hormones and emotions on memory retention or loss. Finally, changes in memory systems associated with aging, disease processes, and drug use are addressed.

The final 42 chapters in Volume 4, *Molecular and Cellular Mechanisms of Memory* edited by J.D. Sweatt, represent a review of the state of the science of what we know at the systems, cell, and molecular levels on learning and memory formation, as well as providing a look at the emerging and future areas of investigation. Once again, this volume covers an impressive amount of information derived from studies at many taxonomic levels, from molecular associative learning mechanisms, through an array of studies on synaptic plasticity, to the cell level of fear conditioning.

The centrality of learning and memory to our daily lives has led to intense analysis by psychologists and neurobiologists for the past century, and it will undoubtedly remain at the forefront of research throughout this new century as well. It is our intention that this set of volumes will contribute significantly to the consolidation of this field, and it is meant as a resource for scientists and students interested in all facets of learning and memory. No other reference work covers so wide a territory and in so much depth.

Learning and Memory: A Comprehensive Reference would not have been possible without the tremendous work of the Editorial Board, who identified the topics and their authors, and reviewed each contribution. Special thanks also go to Johannes Menzel, Senior Acquisitions Editor at Elsevier, for supporting the project and Andrew Lowe and Laura Jackson, Production Project Managers, and Joanna De Souza, Developmental Editor, for ensuring that the production schedule was maintained.

John H. Byrne

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4.01 Introduction and Overview

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4.01.1 Introduction

This fourth volume of the *Comprehensive Handbook of Learning and Memory* delves deeply into the cellular and molecular mechanisms mediating lasting changes in behavior, changes that occur in response to environmental signals. This volume is indeed the most comprehensive description in existence concerning the genetics, biochemistry, and cell biology of memory formation. Cumulatively, the reviews in this volume describe an impressive range of processes underlying memory, from atom-level resolution in some paradigms to cell circuit-level mechanisms in others, and essentially all points in between. Moreover, the phylogenetic range of the contents is equally impressive, with descriptions of memory processes in animal systems on a continuum from one of the simplest, *Caenorhabditis elegans*, to the most complex, humans.

It is striking that while the chapters in this volume deal with specific cellular and molecular mechanisms, there are in most chapters strong and direct tie-ins to behavior in the living animal. This appealing aspect of these studies arises in large part from three historical developments, in my opinion. First, many of the invertebrate systems were specifically chosen because the animal lent itself to bridging from behavior to cells to molecules. Thus tying the molecules and cells to the behavior was built into the experimental design of these studies from the outset. A second development was the advent of the technical capacity to genetically engineer mice through means of homologous gene recombination. This allowed those interested in the molecular basis of vertebrate memory to bridge from molecule to behavior within a single animal and ushered in a new era in neurobiology. The final development is more sociological than technical. Many of the leading investigators in the area of molecular and cellular mechanisms of memory actually started out as behavioral psychologists. Therefore, tying molecular and cellular mechanisms directly back to the behaving animal always was an intellectual emphasis for them. This established a culture within the subdiscipline that placed a priority on interdisciplinary studies bridging from molecules to behavior.

In the current era, we also are compelled to always consider the relevance of our basic neurobiological studies to the human condition. Advances over the last 20 years in our understanding of the basic molecular and cellular biology of memory have laid the foundation for a capacity to develop new treatments for human diseases of learning and memory. This is not an abstract principle but rather a declarative statement concerning important recent advances in the field. Descriptions of these recent advances are contained in many chapters, and sections of chapters, in this volume. These descriptions are not split out

into a separate translational or disease section of the volume, but rather are distributed throughout in places appropriate to their specific intellectual milieu. I find this a more satisfying concept for organization of the volume, and an accurate representation of the ongoing cross talk between human and basic science studies of learning and memory.

A final comment is that in many ways this volume is a snapshot of the state of scientific understanding of the neurobiology of learning and memory at the beginning of the twenty-first century. Thus, besides being a valuable resource for contemporary scientists, I believe this volume will provide a useful historical reference point as well. I am only emboldened to make this statement because of the very many outstanding scholars and scientists who have contributed their individual chapters to this work and because of the exceptionally important discoveries by the many scientists that they cite therein.

4.01.2 Organization

The organization of the volume is fairly straightforward considering the complexity of the topic at hand (see **Figure 1**). Part 1, the introduction and overview component, is this chapter and Chapter 4.02. I am very grateful to Eric Kandel, Craig H. Bailey, Angel Barco, and Robert D. Hawkins for their contribution in writing Chapter 4.02. I commissioned Eric and his colleagues to write a personal overview of contemporary discoveries and approaches in the learning and memory field. I expected that they would deliver a very readable and interesting review concerning the overall topic of the molecular and cellular biology of memory formation, describing a diverse set of model systems and approaches. They certainly hit the mark in this regard. Unbeknownst to them, I also anticipated that they might write an excellent bridge chapter that would serve to help guide a knowledgeable reader into the much more detailed following chapters in the book. When I received and read their chapter I was delighted that they had achieved this as well. I strongly encourage anyone looking for a conceptual starting point for considering the detailed molecular and cellular basis of memory, as described in this overall volume, to begin by reading Chapter 4.02.

Part 2 of the volume, comprising Chapters 4.03–4.19, covers model systems-level and cellular-level approaches to investigating learning and memory. This part can be broadly subdivided into five sections (more specific descriptions of the contents of each section will be given): Section 1 deals with nonassociative learning mechanisms. Section 2 discusses associative learning and memory, as investigated in invertebrate model organisms. Section 3 makes an important transition and describes studies of associative and spatial memory in vertebrates. Section 4 reviews topics related to memory disruption, and section 5 describes types and forms of plasticity at the cellular and synaptic level that underlie memory formation and storage.

The last section of Part 2, describing cellular mechanisms and synaptic plasticity, also serves as a foundation for and transition to Part 3 of the volume. Part 3 covers molecular-level approaches and emerging areas of discovery. This second half of the book has five sections as well: Section 1 deals with the *N*-methyl-D-aspartate (NMDA) receptor and its immediate biochemical targets. Section 2 describes genomic and postgenomic signaling. Section 3 covers the important areas of synaptic structure and signaling, and section 4 covers plasticity of cell structure and retrograde synaptic signaling. Section 5, the final section, is admittedly based on a subjective assessment on my part. This section highlights what I consider to be several emerging areas of investigation that will have a large impact on our thinking about cellular and molecular mechanisms of memory formation in the near future.

Having delivered this brief overview, in the remainder of this chapter I will describe more specifically the contents of Parts 2 and 3 of the volume (see **Figure 1**).

4.01.2.1 Part 2A: Systems-Level Approaches

4.01.2.1.1 Nonassociative learning

In this first major section of the book, we will start by covering the simplest forms of learning, that is, nonassociative forms of learning and memory. Studies of these simple forms of learning and memory have yielded great insights into the enormous complexity of information acquisition and storage in the nervous system, when approached at the cellular and molecular levels. In Chapter 4.03, one of the pioneers of this type of work, Jack Byrne, provides a review of studies of sensitization and habituation using invertebrate model systems.

This overview chapter is then followed by a description of sensitization and habituation in the *C. elegans* model system by Cathy Rankin, who pioneered the adaptation of this organism for use in

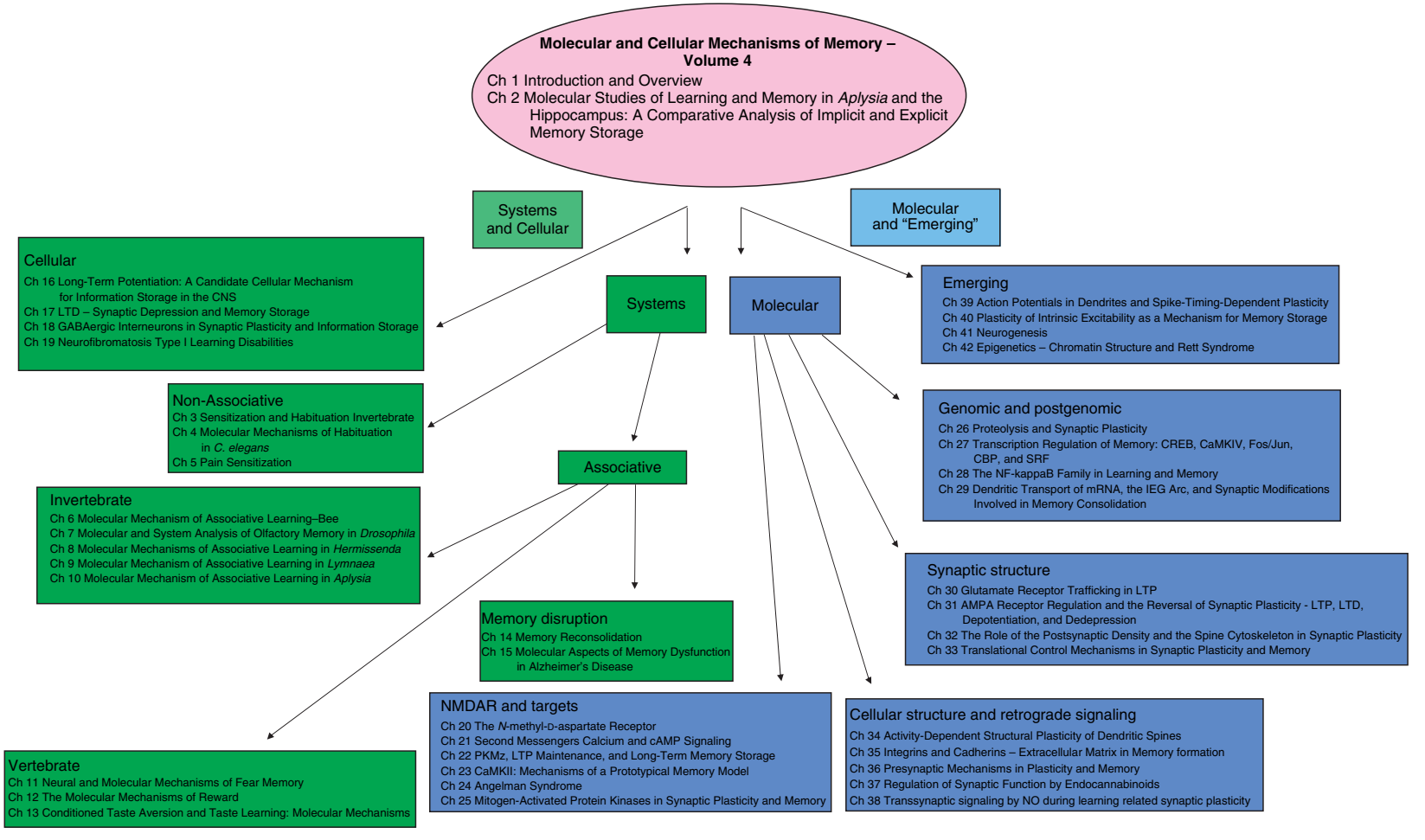


Figure 1 Contents and organization of Volume 4 of the *Comprehensive Handbook of Learning and Memory*. This volume covers molecular and cellular mechanisms of memory formation. This figure describes the overall organization and major topic areas of the volume.

behavioral studies. Studies of this sort in *C. elegans* have a particularly important context, for this organism stands apart from all others in that its entire anatomical structure and developmental program have been determined at the cellular level. Thus behavioral studies in *C. elegans* have the potential to bring an unprecedented level of certainty to understanding the neural circuitry underlying specific behaviors. In addition, *C. elegans* is a powerful genetic model system in its own right, allowing the potential to combine anatomy, genetics, and behavior in a unique fashion, as is illustrated by the studies in Chapter 4.04.

Nonassociative learning in vertebrates, especially humans, tends to not receive the same level of attention among learning and memory neurobiologists that higher-order forms of learning receive. However, sensitization and habituation are also of great relevance in humans in the clinical setting. Chapter 4.05 describes pain sensitization in vertebrate model systems, highlighting interesting parallels between molecular mechanisms of vertebrate associative learning (that will be described later in the volume) and mechanisms of nonassociative learning in these same animals. In addition, this chapter reminds us of the great significance of studying nonassociative learning and memory in the modern context of translational biomedical research.

4.01.2.1.2 Associative learning and memories of contingency

The next major section transitions us to more complex forms of learning and memory, that is, associative forms wherein an animal learns a predictive or contingent relationship between two environmental signals. Of course, this associative relationship must be represented at the molecular level in some fashion in the nervous system, and the memory for the relationship must be stored there as well. Thus, the associative learning section explores the fascinating question of how contingencies and associations are represented at the molecular and cellular level in the nervous system, and how unique molecular events produced by associative stimuli trigger lasting cellular changes manifest as memory.

4.01.2.1.3 Associative learning in invertebrate models

We first explore these questions in a series of chapters describing studies of associative conditioning in invertebrates. Each of five chapters gives an overview of a particular organismal system, describing the animal, its ethologically relevant behaviors and capacity for forming associations, the underlying circuitry, and the known cellular and molecular mechanisms contributing to altered behavior in response to associative environmental stimuli. These chapters thus provide a useful overview of the different organisms and approaches, and more detailed information about cellular and molecular mechanisms.

We are fortunate to have leading investigators for each of five unique model systems contributing to this section. In Chapter 4.06, Uli Mueller and his colleagues describe the honeybee system. Chapter 4.07 provides us with a review of learning and memory studies in the granddaddy of all genetic model systems, *Drosophila*, from Thomas Preat and his group. In Chapter 4.08, Terry Crow and his collaborators review the *Hermissenda* system, and in Chapter 4.09 George Kemenes introduces us to *Lymnaea*. Finally in this section, in Chapter 4.10 Jack Byrne describes *Aplysia* associative conditioning and reviews the unique molecular and cellular components of associative learning and memory in this system.

In editing and reviewing these chapters, I noted several interesting commonalities across these systems, which I would like to comment on in passing. First, these are interesting systems to read about, because I suspect that many readers will be introduced to these systems for the first time through this volume. Second, considerable cleverness is evident on the part of the scientists using these systems, in adapting ethologically relevant behaviors in the animal to the laboratory setting, and in undertaking very detailed molecular and cellular studies in conjunction with that approach. Finally, the types of associations these relatively simple animals are capable of making is astonishing in many instances. Many of you reading about these organisms for the first time will be surprised at the complex environmental contingencies that slugs and bugs are capable of learning.

4.01.2.1.4 Associative learning in vertebrate models

The next section moves up the phylogenetic tree to explore associative learning and memory in vertebrate systems. As this volume focuses on molecular and cellular mechanisms, all three of these chapters describe studies utilizing rodents. In Chapter 4.11, Glenn Schafe and Joe LeDoux lend their synaptic selves to the effort of describing rodent fear conditioning and its underlying cellular and molecular basis.

Their chapter describes the impressive body of literature that has been able to take studies of this particular behavior into the realm of specific cellular and molecular mechanisms, in the anatomically complex vertebrate central nervous system (CNS). These studies have allowed the delineation of mechanisms that encode a complex environmental contingency and allow the manifestation of appropriate behavioral change. In Chapter 4.12, Eric Nestler and Catharine Winstanley describe studies of the flip side of behavioral conditioning, reward-reinforced behaviors. They present an overview of reward systems in the vertebrate CNS, and describe how these systems can be co-opted to reinforce addictive behaviors. Once again, the level of understanding of the cellular and molecular mechanisms at work in these processes is quite impressive. Finally, in Chapter 4.13 in this section Kobi Rosenblum describes taste learning and conditioned taste aversion in rodents. The cortically based systems at work here, as well as the quasi-declarative nature of taste memorization, make this a fascinating system for study. Indeed, it is likely that taste learning and memory are among the most sophisticated types of information processing that rodents are capable of.

4.01.2.1.5 Memory disruption

The robustness and long-lived nature of memory are two of the most impressive aspects of the phenomenon, and indeed these attributes compelled many neuroscientists to be interested in learning and memory in the first place. Against this backdrop, the possibility of loss of previously established memories, and the loss of the capacity for new memory formation, are particularly compelling for many of us to consider. In Chapter 4.14, Cristina Alberini and Stephen Taubenfeld discuss the need for memories that have already been formed to undergo a complex process of re-establishment after every instance of recall, a phenomenon referred to as memory reconsolidation.

In Chapter 4.15, Lennart Mucke, Jeannie Chin, and Eric Roberson describe molecular mechanisms underlying the most debilitating disease of memory in existence, Alzheimer's disease (AD). This is one of the most prominent areas of biomedical research extant in the United States and Europe at present. I also would like to highlight this chapter by Mucke and colleagues for another reason. Their chapter sits at a position in this volume immediately preceding roughly 20 subsequent chapters that detail the cellular and molecular underpinnings of memory. A striking attribute of their chapter is the large number of cellular and molecular processes that are disrupted in AD, processes that are subsequently discussed in a basic science context in further chapters of this volume. I note this for two reasons. First, it is impressive and somewhat discouraging that so many of the molecular mechanisms implicated in normal memory formation are potentially disrupted in AD. Second, on a more practical side Chapter 4.15 serves as a valuable cross-reference for the biomedical relevance of understanding the detailed cellular and molecular processes described in the final sections of this volume.

4.01.2.2 Part 2B: Cellular-Level Approaches

In Chapter 4.16 of the volume, we transition from larger systems-level analyses involving whole organisms to descriptions of more specific cellular and molecular processes involved in learning and memory. I chose to make this transition with a chapter describing long-term potentiation (LTP), perhaps the cellular keystone of higher-order vertebrate memory formation. Directly indicative of that fact, and of the seminal contribution made by Tim Bliss and Terje Lomo in their discovery of LTP, every chapter after this transition point makes some reference to LTP, to a greater or lesser extent depending on the specific topic under review. Thus Chapter 4.16 provides a foundation for all subsequent chapters in this volume. More significantly, Bliss and Lomo's discovery of LTP provides an essential foundation for the modern understanding of the molecular and cellular basis of vertebrate memory formation.

The undoubted importance of LTP notwithstanding, synaptic long-term depression (LTD) plays crucial roles in vertebrate CNS function and memory formation as well. In Chapter 4.17, Christian Hansel and Mark Bear describe LTD, the conceptual mirror image of LTP. They also provide a nice coverage of the functional and behavioral significance of LTD and highlight that the phenomenon is not simply a cellular antagonist of potentiation, but rather a mnemonic mechanism in its own right.

The importance of plasticity of inhibitory synapses and cells has historically been under-appreciated. In Chapter 4.18, Chris McBain and his colleagues describe both the plasticity of inhibitory cells and synapses, and the importance of plasticity of inhibitory circuits in memory and cognitive function. To reinforce this message, in Chapter 4.19 Alcino

Silva and his collaborators describe learning and memory deficiencies associated with neurofibromatosis type 1 and describe their intriguing series of studies that identified disruption of inhibitory synapse function as a basis for human learning and memory deficits in this disorder. These two complementary chapters close out the systems and cellular section of the volume and set the stage for the detailed molecular descriptions of the final part of the volume.

4.01.2.3 Part 3A: Molecular-Level Approaches

Starting with Chapter 4.20, we begin to dissect the complex molecular systems that subserve learning, memory, and memory storage. The molecular complexity of memory is indeed awe-inspiring and not to be taken lightly. One must remember that the molecular machinery of vertebrate memory may underlie the most complex biological process in existence, at least that is concretely identifiable at this point. Chapters 4.20–4.38 describe individual components of this machinery in a comprehensive and organized fashion.

These chapters proceed in an order roughly approximating the order of molecular information flow in a neuron participating in the formation of a memory. Thus we start with a description of the NMDA subtype of glutamate receptor and its immediate molecular targets in the cell in Chapters 4.20–4.25. The next section, Chapters 4.26–4.29, describes signaling to the nucleus and the genome contained therein and reviews transcriptional and posttranscriptional mechanisms involved in memory formation. Both the immediate targets of the NMDA receptor and the altered transcriptional readout impinge upon the synapse to effect altered cell function and signaling, a critical step in the plasticity that underlies memory. Chapters 4.30–4.33 describe the translational and posttranslational mechanisms that underlie altered synaptic function in memory. Finally, it is clear that transcriptional, translational, and posttranslational mechanisms all ultimately mediate altered cell structure and communication in memory formation. These alterations involve both the pre- and postsynaptic compartments, and encompass the physical and molecular structure of both the neuron and the extracellular space. These components of the memory machinery are described in Chapters 4.34–4.38.

4.01.2.3.1 The NMDA receptor and its immediate targets

What better place to start a description of the molecular basis of memory than with the NMDA receptor itself? After all, it is both a molecular coincidence detector and a known key component of vertebrate memory formation. Chapter 4.20 describes NMDA structure and function and highlights that the NMDA receptor is in fact a huge molecular machine in its own right. In Chapter 4.21, Dan Storm and Kristin Eckel-Mahan describe two of the immediate effectors of the NMDA receptor, as well as other plasticity-related receptors: calcium and cyclic adenosine monophosphate (cAMP).

The next three chapters then discuss two memory molecules that are capable of converting a transient signal into a lasting effect in the cell – protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase II (CaMKII). Chapter 4.22, by Todd Sacktor, describes the roles of PKC and especially of the PKMzeta isoform in synaptic plasticity and memory. Chapter 4.23 brings us a description by Roger Colbran of the ways and means by which CaMKII can be regulated and persistently activated to serve as an information storage device. Chapter 4.24 by Ed Weeber and his colleagues highlights the clinical relevance of studies of CaMKII regulation, by focusing on Angelman mental retardation syndrome and disruptions of hippocampal CaMKII regulation in this human learning and memory disorder.

Finally in this section, Ray Kelleher describes the mitogen-activated protein kinase (MAPK) pathways and their roles in synaptic plasticity and memory formation. Chapter 4.25 covers one of the essential signal integration pathways in plasticity and memory formation, a pathway that also is a prototype regulator of gene transcription and protein translation. The description of this pathway helps us transition to the next section of the volume, dealing with transcriptional regulation and mRNA trafficking.

4.01.2.3.2 Genomic and postgenomic signaling

Genomic signaling in the context of synaptic plasticity and memory formation involves two basic components: Getting a signal to the transcription regulatory machinery to alter expression of the appropriate gene targets, and getting the newly transcribed mRNAs to the right places in the cell. Chapters 4.26–4.30 deal with various fascinating aspects of these processes.

Chapter 4.26 by Ashok Hegde describes a critical gene target in *Aplysia* sensitization and synaptic

facilitation, a ubiquitin C-terminal hydrolase. Ashok's chapter describes an interesting molecular system that demonstrates an important role for protein degradation in plasticity and memory, and also involves an interesting interplay of transcriptional and posttranslational regulation in order to generate a lasting signal in a neuron.

The next two chapters describe important transcription factors in plasticity and memory, those proteins that help directly translate a genome-level signal into the appropriate direct transcriptional change. In Chapter 4.27, Sheena Josselyn and Christy Cole describe transcription regulation by cAMP response element binding protein (CREB) and CREB-associated pathways, highlighting the first transcription factor to be clearly linked to synaptic plasticity and memory formation. In Chapter 4.28, Molly Meffert describes a pathway recently introduced to the plasticity and memory field, nuclear factor kappa B (NFκB). While NFκB signaling is fairly new material for most neurobiologists, in fact this pathway has long been one of the premier pathways for study in immune system transcriptional regulation.

Once changes in gene expression are triggered in the nucleus, the products of those changes must find the right site within the neuron for their residence. In Chapter 4.29, Oz Steward discusses the prototype marker for neuronal mRNA trafficking in the mammalian CNS, Arc. Studies of Arc trafficking have shed important new light on the existence and mechanisms of specific mRNA targeting in neurons, especially in the context of activity-dependent cellular plasticity and the mechanisms that tell targeted molecules where to go in the neuron, a process referred to as synaptic tagging.

Targeted molecules end up in specific sections of the neuron and at specific synapses. Thus we have completed a conceptual loop through the neuron – from the NMDA receptor at a synapse, to the nucleus, and back to a targeted synapse. Therefore in the next section we return to a discussion of synaptic mechanisms and receptors. However, it is important to remember that plasticity at synapses occurs not only (nor even predominantly) in response to genome-originating signals, but also in response to locally generated signals. Therefore, the next section deals with the synapse and its receptors not only as targets of transcriptional regulation, but also as targets of posttranslational modification and localized alterations in protein synthesis.

4.01.2.3.3 Synaptic structure and signaling

This is a substantial section that describes several of the most popularly studied and clearly important mechanisms in synaptic plasticity and memory. Indeed, in the mammalian CNS the processes described in this section are of paramount relevance. This is because the postsynaptic compartment and its environs are both the quintessential functional compartment for receiving externally generated signals, and a known locus for change at the molecular level in plasticity and behavioral memory.

In Chapter 4.30, Michael Browning and his colleagues describe mechanisms of glutamate receptor trafficking and regulation, focusing on those mechanisms operating during synaptic plasticity and memory. Rick Huganir and Hey-Kyoung Lee follow in Chapter 4.31 by reviewing a fascinating set of mechanisms controlling bidirectional regulation of glutamate receptor function during synaptic potentiation and depression. These two chapters provide a nice overview of the mechanism for dynamic regulation of glutamate receptor function in synaptic plasticity and memory.

Of course, glutamate receptors and their many associated signaling molecules sit within a specialized synaptic machine – the postsynaptic density (PSD). In Chapter 4.32, Mary Kennedy reviews the structure and function of the PSD and brings to bear sophisticated kinetic modeling approaches to help us understand how this machine achieves its effects. In Chapter 4.33, Eric Klann and Nahum Sonnenberg describe the necessity of protein synthesis for synaptic plasticity and memory, with an emphasis on localized dendritic protein synthesis being involved in these processes.

4.01.2.3.4 Plasticity of cellular structure and retrograde signaling

Molecular changes associated with memory formation are not limited to a single postsynaptic compartment, but rather encompass associated presynaptic and structural changes as well. Moreover, even in the case where a single postsynaptic site might undergo alteration, its presynaptic partner can change in concert. In Chapter 4.34, Lucas Pozzo-Miller and Christopher Chapleau describe activity-dependent structural changes in dendritic spines as an example of neuronal structural change in plasticity and memory. In Chapter 4.35, Ron Davis reviews the role of the extracellular matrix in the maintenance of synaptic structural change, focusing

specifically on integrins and cadherins. Chapter 4.36 by Craig Powell and Pablo Castillo describes presynaptic mechanisms in plasticity, covering both plasticity of the presynaptic terminal and evidence that changes at this locus are involved in various forms of memory. In considering how signals may get from the postsynaptic compartment to the presynaptic compartment, David Lovinger enlightens us with Chapter 4.37, describing regulation of synaptic function by endocannabinoids. Finally for this section, Bob Hawkins reprises the retrograde signaling theme by reviewing the role of nitric oxide in synaptic plasticity (Chapter 4.38).

4.01.2.4 Part 3B: Emerging Areas

The final section of the volume covers a somewhat disparate set of topics that nevertheless have two things in common. First, the topics of this section, in my opinion, are emerging areas of emphasis in the plasticity and memory field, topics that have the potential to give us fundamentally new perspectives on several aspects of CNS and neuronal function underlying behavioral change. Second, these mechanisms have in common that they affect or involve the entire neuron.

In Chapter 4.39, Jack Waters and Fritjof Helmchen describe back-propagating action potentials in dendrites and their capacity to regulate both the magnitude and direction of synaptic plasticity. This is followed by Chapter 4.40, where Jack Byrne and Ricccardo Mozzachiodi describe plasticity of intrinsic cellular properties, such as altered excitability, as a locus of plastic change. Neurogenesis has emerged as a viable mechanism contributing to memory formation in the adult, in complete contrast to the dogma of only a few years ago. In Chapter 4.41, Rusty Gage, Sebastian Jessberger, and James Aimone review adult neurogenesis and its mechanisms of control and the implications of this system for a potentially new cellular basis of memory. Finally, in Chapter 4.42 Jonathan Levenson and Marcelo Wood describe recent discoveries implicating epigenetic mechanisms of long-term regulation of gene expression in plasticity and memory formation.

These provocative topics may force us to consider that the entire neuron may be a locus of the engram and a site of information storage in the CNS. Thus, while synapse specificity is a powerful attribute in terms of specifying connections in a behavior-mediating circuit, synapse-specific changes may be embedded within a neuron that has had its basic properties changed in a cell-wide fashion as well. Both processes may act in concert to enable learning and memory, and the four chapters of this final section presage this potential paradigm shift in our thinking concerning vertebrate memory formation.

Acknowledgements and Thank-Yous

The Editors at Elsevier Publishing have been great to work with on this project. Executing a book series of the scope and magnitude of the *Comprehensive Handbook of Learning and Memory* is no small feat. In this vein, I would like to thank the Elsevier publishing group for deciding to invest in this project and for giving me and the other editors the opportunity to participate. I in particular wish to thank Johannes Menzel for his support and encouragement, and Joanna DeSouza for the immense amount of work she put in on organizing the series. I also am very appreciative to Vicki Hixon, who works with me at the McKnight Brain Institute at UAB, for all the tremendous help she gave me, and always with a smile. My wife, Kim Strifert, supported me throughout, from London to Houston to Memory Lane (see **Figure 2**).

I also am very grateful to Jack Byrne for inviting me to participate as a volume editor and contributor to the series. The series is both visionary and timely, and Jack has provided great leadership as editor. I sincerely appreciate the opportunity to participate. Jack, Randolph Menzel, Howard Eichenbaum, and Roddy Roediger are all not only outstanding scientists but also fine colleagues, and I thank them all for their many suggestions and for the valuable



Figure 2 Memory Lane is a state of mind, a fascinating topic of scientific inquiry, and a small road outside of Trussville, Alabama, USA. The editor of this volume dwells on all three.

brainstorming that they did not only for the overall series but for my volume as well.

Most important is recognizing the tremendous debt that I owe to the authors of the individual chapters in this volume. I have been consistently and thoroughly impressed at the quality of the chapters that the authors provided for this book. I thank them to begin with for even agreeing to provide a chapter at all. I thank them tremendously and in every instance for the superb quality of the material that they produced – a reflection of their professionalism, integrity, and scholarship.

4.02 Molecular Studies of Learning and Memory in *Aplysia* and the Hippocampus: A Comparative Analysis of Implicit and Explicit Memory Storage

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4.02.1 Introduction

Modern behavioral and biological studies have revealed that memory is not a unitary faculty of the mind but consists of distinct families of mental processes that can be grouped into at least two general categories, each with its own rules (Polster et al., 1991; Squire and Zola-Morgan, 1991). Explicit or declarative memory is the conscious recall of knowledge about people, places, and things and is particularly well developed in the vertebrate brain. Implicit or nondeclarative memory is memory for motor and perceptual skills as well as other tasks and is expressed through performance, without conscious recall of past experience. Implicit memory includes simple associative forms of memory, such as classical and operant conditioning, and nonassociative forms, such as sensitization and habituation. Explicit and implicit memory have been localized to different neural systems within the brain (Milner, 1985; Polster et al., 1991; Squire, 1992). As first shown by Brenda Milner in her neuropsychological studies of the patient H.M., explicit memory is critically dependent on structures in the medial temporal lobe of the cerebral cortex, including the hippocampal formation. Implicit memory is a family of different processes that are represented in a number of brain systems including

the cerebellum, the striatum, the amygdala, and in the simplest cases, the sensory and motor pathways recruited during the learning process for particular perceptual or motor skills. As a result, implicit memory can be studied in a variety of simple reflex systems, including those of higher invertebrates, whereas explicit memory is best studied in mammals.

Two experimental model systems have been extensively studied as representative examples of these two forms of memory storage: sensitization in the marine snail *Aplysia californica* as an example of implicit memory, and spatial memory formation in rodents as an example of explicit memory. We use them here as points of comparison to consider similarities and differences in implicit and explicit memory storage.

4.02.2 Short-Term, Intermediate-Term, and Long-Term Forms of Storage Mechanisms

Recent studies of simple forms of implicit memory in higher invertebrates and more complex forms of explicit memory in mammals suggest that changes in the strength and structure of synaptic connections underlie these diverse forms of memory storage (Kandel, 2001). For both implicit and explicit memory, two general types of storage mechanisms have been described: short-term memory lasting minutes and long-term memory lasting days, weeks, or longer. This temporal distinction is reflected in specific mechanisms for the synaptic plasticity that underlie each form of behavioral memory as well as specific molecular requirements for each of these two forms of synaptic plasticity. The short-term forms involve the covalent modifications of preexisting proteins by a variety of kinases and are expressed as alterations in the effectiveness of preexisting connections. In contrast, the long-term forms require *de novo* gene expression and the synthesis of new mRNAs and proteins. Moreover, the long-term forms often are associated with the growth of new synaptic connections. For both implicit and explicit memory storage, the synaptic growth is thought to represent the final and self-sustaining change that stabilizes the long-term process. In addition to short- and long-term memory, there are, for most types of learning, a family of intermediate processes that last one or more hours and often require translation but not transcription. These can be produced by various behavioral training protocols or in simplified neuronal systems using repeated or prolonged stimulation.

In this chapter, we discuss and compare critical synaptic sites and the underlying cellular and molecular mechanisms of short-term, intermediate-term (**Figure 1**), and long-term (**Figure 2**) memory storage that have been identified by neurobiological studies of elementary forms of implicit memory in *Aplysia* and explicit memory storage in rodents.

4.02.2.1 Implicit Memory: Sensitization and Classical Conditioning of the Gill-Withdrawal Reflex in *Aplysia*

The central nervous system (CNS) of *Aplysia* contains only approximately 20,000 large and frequently identifiable nerve cells, clustered into nine major ganglia. The ability to identify many of the individual neurons of this nervous system and record their activity has made it possible to define the major components of the neuronal circuits of specific behaviors and to delineate the critical sites and underlying mechanisms used to store memory-related representations.

The cellular and molecular mechanisms contributing to implicit memory storage have been most extensively studied for the gill- and siphon-withdrawal reflex of *Aplysia* (Carew and Sahley, 1986; Byrne and Kandel, 1996; Kandel, 2001). As is true for other types of defensive reflexes, the gill- and siphon-withdrawal reflex can be modified by several different forms of implicit learning. We begin by focusing on *sensitization,* a form of learned fear, evident as an elementary form of nonassociative learning of this defensive behavior. When a light touch is applied to the siphon of *Aplysia,* the animal responds by withdrawing its siphon and gill. This response can be enhanced or sensitized when the animal is presented with a noxious (fear-inducing) stimulus such as a tail shock. As is the case with other forms of memory, the memory for sensitization of the withdrawal reflex is graded as a function of training: A single tail shock produces short-term sensitization that lasts for minutes, whereas repeated tail shocks given at spaced intervals produce long-term sensitization that can last for several weeks (Castellucci et al., 1986). The reflex also exhibits classical conditioning, an associative form of learning. Here the siphon stimulus is presented in a paired fashion just before the tail shock so that the animal learns about the predictive relationship between the two stimuli. Enhancement of the withdrawal reflex is greater and longer lasting with paired training (classical conditioning), compared with unpaired training or training with the tail shock alone (sensitization) (Carew et al., 1981, 1983; Antonov et al., 2001).

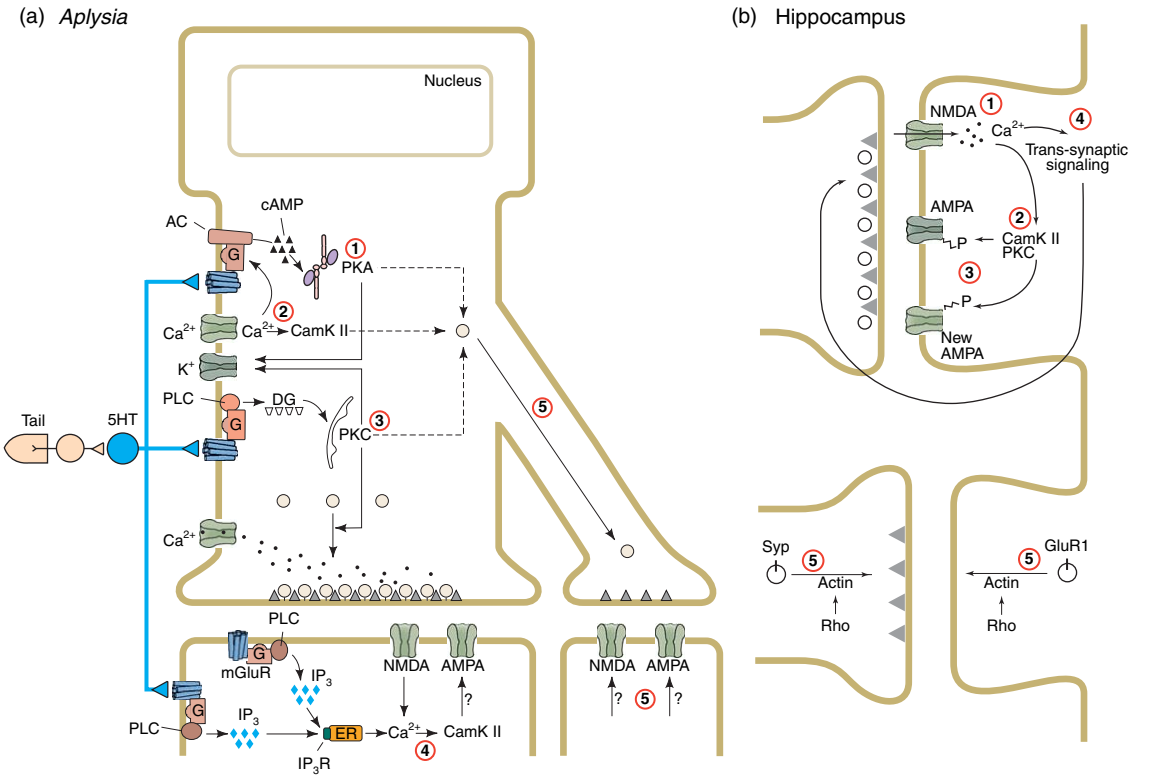


Figure 1 Mechanisms of short- and intermediate-term memory formation in *Aplysia* and hippocampus. (a) *Aplysia.* Different forms of short- and intermediate-term synaptic plasticity contributing to learning and memory in *Aplysia* involve different combinations of pre- and postsynaptic molecules including (1) presynaptic cyclic adenosine monophosphate–dependent protein kinase, (2) presynaptic Ca^{2+} and CamKII, (3) presynaptic protein kinase C, (4) postsynaptic Ca^{2+} and CamKII, and (5) recruitment of pre- and possibly postsynaptic molecules to seed potential new synaptic sites. (b) Hippocampus. Early-phase long-term potentiation in the CA1 region of hippocampus involves (1) Ca^{2+} influx through postsynaptic *N*-methyl-D-aspartate (NMDA) receptor channels, (2) activation of protein kinases including CamKII and PKC, (3) increased conductance of existing adenosine monophosphate (AMPA) receptor channels and membrane insertion of new AMPA receptors, (4) the engagement of trans-synaptic signaling, which can enhance presynaptic transmitter release, and (5) recruitment of pre- and postsynaptic molecules to seed potential new synaptic sites. See the text for details.

The relative simplicity of the neuronal circuit underlying these behavioral modifications – including direct monosynaptic connections between identified mechanoreceptor sensory neurons and their follower cells (Castellucci et al., 1970) – has allowed reduction of the analysis of short- and long-term memory for sensitization and classical conditioning to the cellular and molecular level. This monosynaptic sensory to motor neuron connection, which is thought to be glutamatergic (Dale and Kandel, 1993; Trudeau and Castellucci, 1993; Conrad et al., 1999), can be reconstituted in dissociated cell culture. Here the tail shocks are replaced with brief applications of serotonin (5-HT), a modulatory transmitter normally released by sensitizing stimuli in the intact animal (Glanzman et al., 1989; Mackey et al., 1989; Marinesco and Carew, 2002). This simplified *in vitro* model system reproduces what is observed during behavioral training. A single brief application of 5-HT produces a short-term change in synaptic effectiveness (short-term facilitation, or STF), whereas repeated and spaced applications of 5-HT produce changes in synaptic strength that can last as long as the cells survive in culture (long-term facilitation, or LTF) (Montarolo et al., 1986). Facilitation is also larger and longer lasting if the presynaptic sensory neuron fires action potentials just before the application of 5-HT, analogous to classical conditioning (Eliot et al., 1994a; Schacher et al., 1997; Bao et al., 1998).

4.02.2.2 Explicit Memory: Spatial Memory in Rodents

Mice have a well-developed capability for certain types of explicit memory. In particular, the brain of rodents is

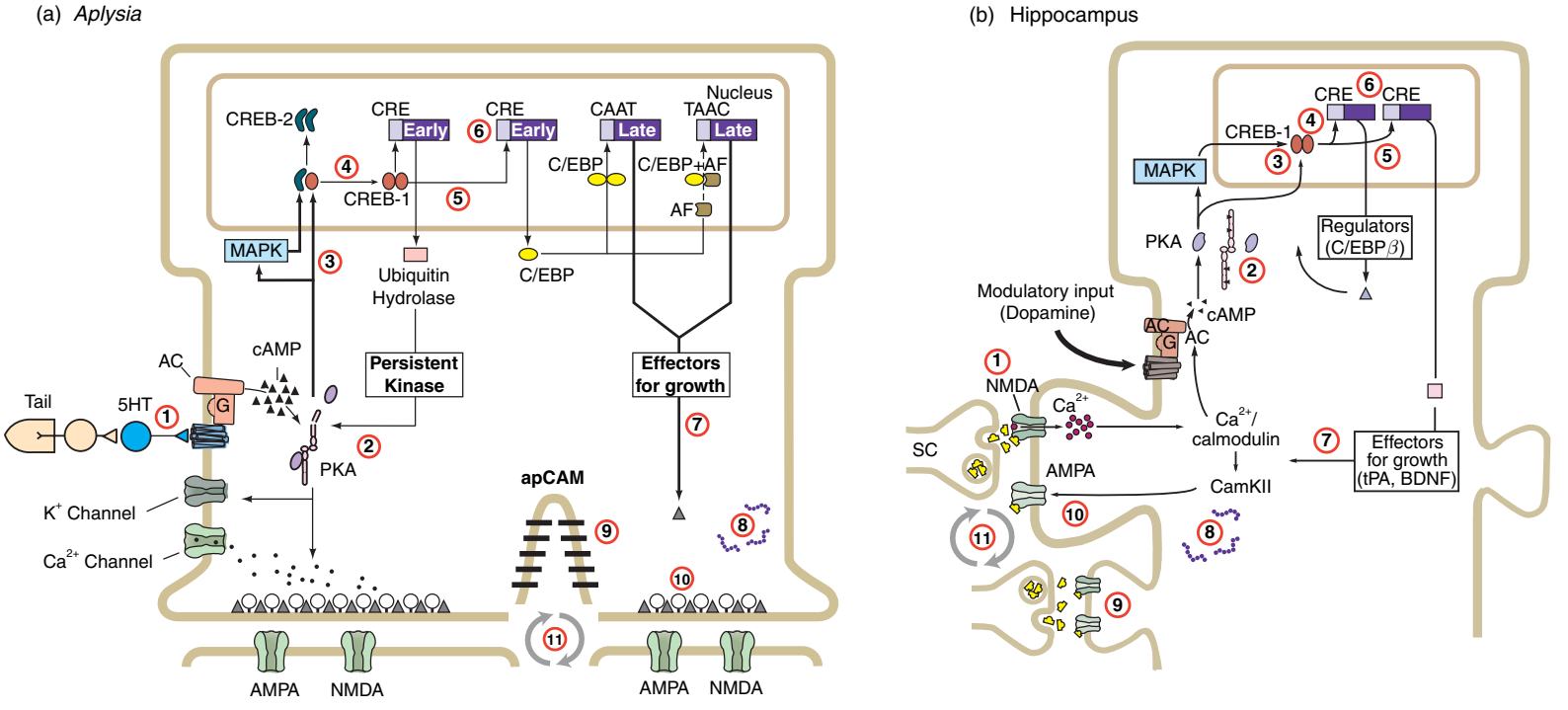


Figure 2 Mechanisms of long-term memory formation. Long-term synaptic plasticity contributing to learning and memory in both *Aplysia* (a) and hippocampus (b) involves a sequence of cellular and molecular mechanisms including (1) neurotransmitter release and short-term strengthening of synaptic connections, (2) equilibrium between kinase and phosphatase activities at the synapse, (3) retrograde transport from the synapse to the nucleus, (4) activation of nuclear transcription factors, (5) activity-dependent induction of gene expression, (6) chromatin alteration and epigenetic changes in gene expression, (7) synaptic capture of newly synthesized gene products, (8) local protein synthesis at active synapses, (9) synaptic growth and the formation of new synapses, (10) activation of preexisting silent synapses, and (11) self-perpetuating mechanisms and the molecular basis of memory persistence. The location of these events, which may act in part to stabilize some of the changes that occur during short- and intermediate term plasticity, moves from the synapse (1–2) to the nucleus (3–6) and then back to the synpase (7–11). Molecular details are discussed in the text.

highly adapted for navigating complex spatial environments. The hippocampus, a key subcortical structure in the mammalian brain, is particularly important for spatial learning and memory and represents a significant percentage of the forebrain in rodents. A remarkable feature of the hippocampus is its regularity along its cross-sectional axis, presenting large arrays of neurons aligned in distinct layers, namely, the CA1, CA3, and dentate gyrus fields. Four major pathways connect these three regions and the entorhinal cortex. This highly organized cellular anatomy has facilitated the electrophysiological analysis of these pathways both *in vivo* and *in vitro*. The phenomenon of long-term potentiation (LTP), currently thought to be the cellular correlate or, at least, a requirement for explicit memory formation in the hippocampus, was first described in the perforant pathway of the hippocampus of rabbits by Bliss and Lomo (1973) over 30 years ago. At about this time it was also appreciated that the hippocampal pyramidal neurons of the CA1 region, which undergo LTP in response to stimulation of the Schaffer collateral pathways, encode space: they are 'place cells.' These cells fire when the animal moves to a specific spatial location, suggesting that this brain region may contain the cellular substrate for the formation of spatial maps (O'Keefe and Dostrovsky, 1971). A large number of lesion, pharmacological, and genetic experiments have confirmed that the Schaffer collaterals, the connections between CA3 and CA1 pyramidal neurons, play a major role in the encoding and storage of spatial memories. As with *Aplysia,* it is possible to distinguish two stages during LTP in the Schaffer collateral pathway: an early, short-term stage (E-LTP), which lasts minutes and can be induced by stimulating the hippocampal slice with a single 100-Hz train of 1 s duration, and a later, long-term stage (L-LTP), which lasts much longer and can be induced by four or more repeated 100-Hz trains (Huang and Kandel, 1994; Martin et al., 2000).

4.02.3 Cellular and Molecular Mechanisms Underlying Short- and Intermediate-Term Forms of Implicit and Explicit Memory Storage

4.02.3.1 Short-Term Memory Involves Covalent Modifications of Preexisting Proteins and Short-Term Enhancement of Preexisting Synaptic Connections

These mechanisms were first explored in *Aplysia* (Schwartz et al., 1971; Brunelli et al., 1976; Kandel and Schwartz, 1982). 5-HT released *in vivo* during sensitization, or applied directly to cultured *Aplysia* neurons, binds to cell surface receptors on the sensory neurons and promotes the production of the diffusible second messenger cyclic adenosine monophosphate (cAMP) by activating the enzyme adenylyl cyclase (AC). This increase in internal concentration of cAMP results in a short-term increase in synaptic strength of the sensory-to-motor-neuron connection (STF). This facilitation is partially a result of the enhanced release of the transmitter glutamate by the sensory neuron onto its follower cells and is accompanied by an increase in the excitability of the sensory neuron attributable to the depression of specific sets of potassium channels, a broadening of the action potential, and an increase in Ca^{2+} influx in the presynaptic terminal (Klein et al., 1982; Castellucci et al., 1986; Dale et al., 1988; Eliot et al., 1993; Byrne and Kandel, 1996). In addition, the changes in cAMP and Ca^{2+} levels triggered by the activation of 5-HT receptors and ion channels regulate the activity of different kinases and phosphatases that control the duration and strength of the changes in synaptic efficiency, as we discuss later.

Many aspects of the basic cellular mechanisms that trigger LTP formation in the hippocampus of mammals recapitulate those found in *Aplysia,* suggesting a basic similarity in the cellular underpinnings for explicit and implicit memory. The primary excitatory neurotransmitter in this case is also glutamate. Again, second messengers are activated by synaptic stimulation, and several of these are similar in both systems. In hippocampal pyramidal neurons, synaptic release of glutamate triggers Ca^{2+} influx through *N*-methyl-D-aspartate receptors (NMDAR) and activation of several kinases including Ca^{2+}/camodulin-dependent protein kinase (CamKII), protein kinase C (PKC), and mitogen-activated protein kinase (MAPK). Spaced trains of high-frequency stimulation can also activate cAMP-dependent protein kinase (PKA) through the Ca^{2+}/calmodulin-mediated stimulation of postsynaptic adenylyl cyclase (Blitzer et al., 1995). In *Aplysia,* these second messengers mediate the transient reinforcement of synaptic connections by covalent modifications of channel closure and the enhancement of neurotransmitter release at presynaptic terminals (Martin et al., 2000; Kandel, 2001). In the hippocampus, expression of the early phase of LTP (E-LTP) involves both an increase in the number of postsynaptic alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate receptors (AMPARs) in the plasma membrane and the phosphorylation of specific subunits

and consequent modification of the biophysical properties of the channel (Malenka and Bear, 2004). E-LTP can, with certain stimulation parameters, also involve an increase in presynaptic transmitter release (Voronin and Cherubini, 2003).

4.02.3.2 Different Protocols Engage Different Combinations of Second Messenger Mechanisms

Studies of facilitation of the sensory-to-motor-neuron synaptic connection in *Aplysia* first demonstrated that even slightly different variations in the learning paradigm or in the pattern of synaptic stimulation recruit different second messenger mechanisms alone or in combination. This insight first emerged in a comparison of sensitization and dishabituation, two related forms of nonassociative learning that differ depending on the state (rested or depressed) of the reflex (Byrne and Kandel, 1996; Antonov et al., 2005). Sensitization results in the strengthening of a reflex response. On the synaptic level, this leads to the facilitation of rested synapses, which can be mimicked by a brief exposure to 5-HT. This involves activation of adenylyl cyclase and PKA in the sensory neuron, leading to reduced K^+ current, increased action potential duration, increased Ca^{2+} influx, and increased transmitter release, as described earlier. A longer exposure to 5-HT recruits, in addition, activation of PKC, which can also further increase the duration of action potentials in the presynaptic sensory neuron. Dishabituation, the strengthening of a previously habituated reflex, is reflected in the facilitation at depressed synapses. This involves PKC as well, but in this case it acts through a mechanism that is independent of spike broadening and is thought to involve vesicle mobilization. The spike broadening–independent component of facilitation may also involve Ca^{2+}/CamKII (Nakanishi et al., 1997).

In addition, as first appreciated by Ghirardi et al. (1995; see also Sutton and Carew, 2000; Sharma et al., 2003b), longer exposure to 5-HT can induce intermediate-term facilitation, which requires protein synthesis and involves MAP kinase as well as PKA or (under some circumstances) PKC.

The ability of different mechanisms to be recruited depending on experimental variables such as the pathway (Zalutsky and Nicoll, 1990), induction protocol (Grover and Teyler, 1990), saline composition (Larkman et al., 1992), and stage of development (Grosshans et al., 2002; Jensen et al., 2003) also applies to hippocampal LTP. For example, LTP in neonatal animals primarily involves PKA, whereas in more mature animals it involves CamKII (Yasuda et al., 2003).

4.02.3.3 Many Protocols Involve Pre- and Postsynaptic Mechanisms

Although the short-term mechanisms described for facilitation in *Aplysia* following sensitization or for depression following habituation have been found to be presynaptic, intermediate-term facilitation can also involve postsynaptic mechanisms including intracellular Ca^{2+} release from inositol-1,4,5-trisphosphate (IP3)-sensitive stores, activation of CamKII or PKC, and AMPA receptor insertion (Chitwood et al., 2001; Roberts and Glanzman, 2003; Jin et al., 2004, 2005; Li et al., 2005). To investigate the precise roles of pre- and postsynaptic mechanisms with intermediate protocols, Jin et al. (2004, 2005) examined facilitation induced by either a single, brief 5-HT exposure typically used to produce short-term facilitation (1 min, 50 $\mu mol\ L^{-1}$) or a more prolonged (10 min, 20 $\mu mol\ L^{-1}$) 5-HT exposure following a single pretest (rested) at sensory-motor neuron synapses in isolated cell culture. The facilitation with either protocol lasted more than 30 min compared with test alone controls, but the 10-min exposure to 5-HT produced larger facilitation than a 1-min exposure. With a 1-min exposure, bath application of an inhibitor of PKA (KT5720) or injection of a peptide inhibitor of PKA into the presynaptic sensory neuron reduced the facilitation, as did bath application of an inhibitor of CamKII (KN93) or presynaptic injection of a peptide inhibitor of CamKII (CamKII 281-309). By contrast, bath application of an inhibitor of PKC (Go6983) or injection of 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA) into the postsynaptic motor neuron had no significant effects. None of the bath-applied inhibitors affected homosynaptic depression or basal synaptic transmission. These results suggest that, with a short application of 5-HT that produces short-term facilitation, presynaptic PKA and CamKII play critical roles, and that PKC and postsynaptic Ca^{2+} are not involved.

With the 10-min 5-HT protocol that is thought to produce intermediate-term facilitation, bath application of an inhibitor of PKA (KT5720) or presynaptic injection of the PKA inhibitor did not have a significant effect, but bath application of inhibitors of either PKC (Go6983) or CamKII (KN93) reduced the facilitation. The facilitation is mediated in part by presynaptic mechanisms, since injection of a peptide

inhibitor of PKC (PKC 19-31) into the presynaptic sensory neuron also reduced the facilitation by 10-min exposure to 5-HT (although presynaptic injection of a peptide inhibitor of CamKII did not.) However, it also is mediated in part by postsynaptic mechanisms, since injection of either BAPTA or CamKII 281-309 into the postsynaptic motor neuron also reduced facilitation. By contrast, postsynaptic injection of PKC 19-31 did not reduce the facilitation. These results indicate that with a longer 5-HT application presynaptic PKC plays an important role (as it does with dishabituation), and postsynaptic Ca^{2+} and CamKII are also important, suggesting that not only the specific kinases involved but also their site of action may depend on the duration of 5-HT exposure. Thus, whereas facilitation with a short application of 5-HT is presynaptic, facilitation with a longer 5-HT exposure involves both presynaptic (PKC) and postsynaptic (Ca^{2+} and CamKII) mechanisms.

These findings are not restricted to dissociated cell culture but are also seen in a reduced preparation of the behaving animal. Facilitation of sensory-motor neuron excitatory postsynaptic potentials (EPSPs) in a semi-intact preparation during actual behavioral sensitization also involves different mechanisms and sites depending on the training protocol (Antonov et al., 2005, 2006). Short-term sensitization produced by a single tail shock leads to synaptic facilitation, which involves presynaptic PKA and CamKII that produce transient spike broadening as well as some longer-lasting mechanisms of facilitation. Intermediate-term sensitization produced by a train of four shocks also involves presynaptic PKA and CamKII and, in addition, involves postsynaptic Ca^{2+} and CamKII, which are recruited with a slight delay after the shock. After that delay, the pre- and postsynaptic mechanisms both contribute and are more than additive.

Classical conditioning (like intermediate-term facilitation induced by 5-HT) involves not only pre- but also postsynaptic mechanisms. Thus, facilitation of sensory-motor neuron EPSPs during classical conditioning in a semi-intact preparation can be blocked either by injecting a peptide inhibitor of PKA into the sensory neuron or by injecting BAPTA into the motor neuron (Antonov et al., 2003), providing the strongest evidence to date that either activity-dependent facilitation or Hebbian LTP contributes to synaptic plasticity underlying behavioral learning. In addition, conditioning is accompanied by increases in evoked firing and membrane resistance of the sensory neuron, and those presynaptic effects are also blocked either by injecting an inhibitor of PKA into the sensory neuron or by injecting BAPTA into the postsynaptic motor neuron. These results suggest that the pre- and postsynaptic mechanisms are not independent but, rather, interact through retrograde signaling.

At least under some conditions pre- as well as postsynaptic mechanisms have also been found to contribute to early-phase LTP in hippocampus (Arancio et al., 1995, 1996, 2001; Malgaroli et al., 1995; Ryan et al., 1996; Zakharenko et al., 2003; Ninan and Arancio, 2004; Wang et al., 2005; Lu and Hawkins, 2006; Ninan et al., 2006). As in *Aplysia,* in several cases inhibitors injected into the pre- and postsynaptic neurons have more than additive effects (Arancio et al., 2001; Wang et al., 2005; Lu and Hawkins, 2006), suggesting that the pre- and postsynaptic mechanisms of LTP are not independent but, rather, act synergistically.

The sensory-to-motor-neuron synapses of the gill- and siphon-withdrawal reflex of *Aplysia* also exhibit plasticity that underlies another elementary form of nonassociative learning – habituation. Habituation is probably the most ubiquitous form of learning in animals, including man. It is a process whereby an animal learns through repeated exposure that the consequences of a weak stimulus are neither noxious nor rewarding. As a result, and in contrast to sensitization, the animal learns to ignore the stimulus. For example, *Aplysia* will initially respond to a weak tactile stimulus to the siphon with a brisk withdrawal of the gill and siphon. But with repeated stimulation, the animal learns to ignore the stimulus and exhibits progressively smaller reflex responses.

As is the case with sensitization, the memory for habituation can exist in both a short-term and long-term form. A single training session of 10 stimuli produces a memory that lasts for 10 or 15 min (Pinsker et al., 1970). In contrast, four repeated training sessions of 10 stimuli each produce a memory that persists for at least 3 weeks (Carew et al., 1972). Both the short-term and long-term behavioral modifications are reflected by a decrease in the strength of the sensory-to-motor-neuron connection. The synaptic depression resulting from habituation is homosynaptic; it results, as does the behavior, from a change in activity in the same pathway that is excited by the stimulus that elicits the reflex. At the cellular level, the homosynaptic depression that underlies short-term habituation results from a decrease in the number of transmitter quanta released per action potential from the presynaptic terminals of the sensory neurons (Castellucci and

Kandel, 1974), and this is the result of a reduced Ca^{2+} influx (Klein and Kandel, 1980).

During homosynaptic depression, the reduction in transmitter release produced by a single stimulus is already evident in response to the second stimulus and can persist for 10–15 min. This suggests that the presynaptic molecular events responsible for the decrease in transmitter release are set in motion by a single stimulus and have been completed by the time the second stimulus is given. To explain how this decrease in transmitter release might occur, a modeling study by Gingrich and Byrne (1985) predicted that the pool of releasable transmitter quanta might be depleted by habituation. This hypothesis was tested directly by Bailey and Chen (1988c), using the electron microscope to visualize the presynaptic terminals of sensory neurons that had been altered by short-term habituation. They found that short-term habituation did not alter the number of sensory neuron presynaptic terminals, the number of transmitter release sites (active zones) within the presynaptic terminals, or the size of active zones. Nor did it alter the total number of synaptic vesicles in a presynaptic terminal. Rather, there was a dramatic reduction in the number of vesicles that were docked at release sites within the active zones, and thus there were fewer packets of transmitter ready to be released. Subsequent experiments further indicated that, in addition to the reduction in the number of synaptic vesicles docked at the active zone, habituation might also interfere with a mechanism directly coupled to the release process (Eliot et al., 1994b; Armitage and Siegelbaum, 1998). Combined, these studies suggested that habituation leads to the selective depletion of synaptic vesicles from the active zone and a consequent failure to mobilize overlying vesicles in the presynaptic terminal. To overcome habituation, therefore, a dishabituating stimulus would first have to mobilize vesicles into sensory neuron active zones (Hochner et al., 1986).

4.02.3.4 Redistribution of Synaptic Components and Early Microstructural Modifications

Recent imaging studies have revealed that even the early phase of hippocampal LTP can also be accompanied by concomitant pre- and postsynaptic alterations in the structure of the synapse. Tens of minutes after the induction of LTP, there is an outgrowth of new pre- and postsynaptic processes (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Ninonenko et al., 2003), and even earlier (minutes), there are increases in spine size (Matsuzaki at al., 2004), clusters of postsynaptic glutamate receptors (Shi et al., 1999), and clusters of presynaptic vesicle-associated proteins and sites where the pre- and postsynaptic clusters colocalize (Antonova et al., 2001). Any of these early structural changes could also be a 'tag' that must be stabilized by protein synthesis for more enduring plasticity (see section 4.02.4.4).

Similar to the clustering of synaptic proteins during LTP, intermediate-term facilitation in *Aplysia* is accompanied by the enrichment of empty sensory neuron varicosities with synaptic vesicles, leading to the rapid presynaptic activation of silent synapses (Kim et al., 2003). However, these intermediate-term changes do not persist for 24 h unless they are stabilized by additional molecular events (including the machinery for translational activation) recruited during the long-term process. The early (hours) stages of LTF are also accompanied by clustering of postsynaptic proteins including the *Aplysia* homologs of NMDA and AMPA receptors (Li et al., 2004). It is not yet known whether intermediate-term facilitation is accompanied by similar postsynaptic changes, but it seems likely that it is. However, presynaptic microstructural changes can occur during even earlier phases of learning-related synaptic plasticity in *Aplysia:* homosynaptic potentiation induced by moderate tetanic stimulation of the presynaptic neuron is accompanied by rapid (less than 10 min) aggregation of the vesicle-associated protein synaptophysin into new clusters or puncta (Jin et al., 2003), as occurs during early-phase LTP in hippocampal neurons and intermediate-term facilitation by 5-HT in *Aplysia.*

The rapid increases in clusters or puncta of presynaptic (synaptophysin) and postsynaptic (GluR1) proteins at the onset of LTP in hippocampal neurons are dependent on NMDA receptor activation and actin polymerization (Antonova et al., 2001). Maintenance of the increases for 30 min does not require protein synthesis, but maintenance for 3 h does (Antonova et al., 2001, 2005). Time-lapse imaging of synaptophysin–green fluorescent protein (GFP) revealed that the puncta are formed by aggregation of material from a more diffuse background level, as is also thought to occur for GluR1 (Shi et al., 1999). That aggregation may involve two types of molecules that can regulate actin: RhoA and VASP. For example, the rapid increases in puncta of both pre- and postsynaptic proteins are blocked by two inhibitors of RhoA, toxin B and Y27632, and immunocytochemical studies have shown that VASP is located at synapses and is phosphorylated and activated both presynaptically and

postsynaptically during potentiation (Wang et al., 2005). Thus, the redistribution of synaptic proteins appears to involve similar molecular pathways (regulation of actin by RhoA and perhaps also VASP) on both sides of the synapse.

Activity-dependent remodeling of the actin network is also involved in the formation of new varicosities and the enrichment of presynaptic proteins during the early stages of LTF in *Aplysia* (Hatada et al., 2000; Udo et al., 2005). In that case, actin is regulated by a different Rho GTPase, Cdc42. In addition, homosynaptic potentiation in *Aplysia* is blocked by presynaptic (but not postsynaptic) injection of phalloidin, which binds actin (Jin et al., 2003), suggesting that actin could also be involved in the rapid increase in puncta of synaptophysin during the potentiation.

Collectively, these results suggest that even the early phases of learning-related synaptic plasticity can already engage a coordinated sequence of pre- and postsynaptic functional and structural changes that may ultimately lead to the formation of new synapses, as occurs during synapse formation in development (Sanes and Lichtman, 1999; Cohen-Cory, 2002). Like synapse formation during development, these processes probably involve a variety of transsynaptic messengers. Recent evidence suggests that nitric oxide (NO) is one of several messengers involved in both the functional and structural changes during the early phases of hippocampal LTP.

4.02.4 Cellular and Molecular Mechanisms Underlying Long-Term Forms of Memory Storage

As mentioned above, the inhibition of transcription or translation does not affect short-term memory but blocks the formation of long-term memory in a variety of model systems, suggesting that the stabilization of memory traces depends on *de novo* gene expression (Kandel, 2001).

4.02.4.1 Gating Signals at the Synapse: A Balance between the Activities of Protein Kinases and Phosphatases

Synaptic stimulation of *Aplysia* sensory neurons leads to a local increase in cAMP and the activation of PKA by causing the catalytic subunits of this enzyme to dissociate from the regulatory subunits. The catalytic subunits can then phosphorylate different substrates in the synaptic terminals, such as potassium channels and proteins involved in exocytosis, leading to enhanced transmitter release during short-term memory. When synaptic stimulation reaches a given threshold or is repeated a number of times, it causes a persistent increase in the level of cAMP and leads to longer-lasting forms of synaptic plasticity. At the molecular level, this more robust pattern of stimulation causes the catalytic subunit of PKA to recruit p42 MAPK. Both then move to the nucleus, where they phosphorylate nuclear targets including other kinases that, in turn, can phosphorylate transcription factors and activate gene expression required for the induction of long-term memory (Bacskai et al., 1993; Martin et al., 1997b; Purcell et al., 2003).

In rodents, inhibition of PKA and MAPK does not affect E-LTP at Schaffer collateral synapses, but these kinases are required for L-LTP (Frey et al., 1993; English and Sweatt, 1996, 1997; Abel et al., 1997). The role of PKA seems to be different in hippocampal neurons than during LTF formation in *Aplysia* sensory neurons. In the hippocampus, PKA does not translocate to the nucleus and plays only a synaptic role: It can phosphorylate different targets, such as the GluR1 subunit of AMPAR (Lee et al., 2000), and it favors the induction of LTP by counteracting the activity of protein phosphatases (Abel et al., 1997; Winder et al., 1998). Finally, it also tags the synapse enabling the consolidation of the long-term process (Barco et al., 2002). In contrast, the role of MAPK appears to be more conserved, and its activation and translocation to the nucleus are also required, at least for forskolin- or brain-derived neurotrophic factor (BDNF)-mediated L-LTP (Martin et al., 1997b; Patterson et al., 2001).

In addition to protein kinases, synaptic protein phosphatases also play a key role in regulating the initiation of long-term synaptic changes. Various protein phosphatases, such as PP1 and calcineurin, oppose the local activity of PKA and act as inhibitory constraints on memory formation. Thus, an increase in calcineurin activity causes defects in long-term memory and L-LTP (Mansuy et al., 1998; Winder et al., 1998), whereas a reduction has the opposite effect (Malleret et al., 2001). Similarly, a reduction in PP1 activity also improves memory in mice (Genoux et al., 2002). Recent experiments in cultured *Aplysia* neurons indicate that calcineurin may also act in this organism as a memory suppressor for sensitization (Sharma et al., 2003a). Therefore, in both systems a

balance between phosphatase and kinase activities at a given synapse gates the synaptic signals that eventually reach the nucleus and can regulate both memory storage and retrieval (Abel et al., 1998).

4.02.4.2 Gating Signals at the Nucleus: Triggering *de Novo* Gene Expression

One of the features that fundamentally distinguish the storage of long-term memory from short-term cellular changes is the requirement for the activation of gene expression. Recently, Thompson et al. (2004) have found that, in the *Aplysia* sensory-motor neuron culture preparation, 5-HT stimulation that produces LTF triggers the nuclear translocation of importins, proteins involved in carrying cargos through nuclear pore complexes. Similarly, in hippocampal neurons, NMDA activation or LTP induction, but not depolarization, leads to translocation of importin (Thompson et al., 2004). The future identification of the molecular cargoes of importin and its signaling role in the nucleus are likely to increase our understanding of how transcription-dependent memory is regulated.

Studies in *Aplysia* revealed the participation of the cAMP/PKA-signaling pathway and the transcription factor, cAMP response element binding protein (CREB), in transcriptional activation by synaptic stimulation. During LTF in *Aplysia* sensory neurons, PKA activates gene expression via an *Aplysia* CREB1 (ApCREB1). In 1990, Dash et al. first demonstrated a role for CREB in LTF by microinjecting CRE oligonucleotides into sensory neurons cocultured with motor neurons (Dash et al. 1990). These decoy oligonucleotides inhibit the function of ApCREB1 by directly binding to this protein, thereby preventing its binding to CRE sites in regulatory regions that actvate expression of cAMP-responsive genes. Whereas injection of the CRE oligonucleotide had no effect on STF, it selectively blocked LTF. Studies by a number of laboratories have now revealed that different members of the CREB family of transcription factors participate in the molecular switch that regulates LTF formation (Bartsch et al., 1995, 1998; Lonze and Ginty, 2002; Barco et al., 2003). Both the CREB activator ApCREB1 and the repressor ApCREB2 contribute to this process. The formation of LTF requires the activation of ApCREB1 by PKA and the concomitant downregulation of ApCREB2 by MAPK (Guan et al., 2003). Injection of anti-ApCREB2 antibodies into *Aplysia* sensory neurons causes a single pulse of 5-HT, which normally induces STF lasting minutes, to evoke LTF that lasts several days (Bartsch et al., 1995). Conversely, injection of pApCREB1 phosphorylated by PKA can by itself trigger facilitation lasting 24 h, and this facilitation can be stabilized by a single pulse of 5-HT (Bartsch et al., 1998; Casadio et al., 1999).

These studies revealed that the transition from STF to LTF requires the simultaneous removal of transcriptional repressors and activation of transcriptional activators. Transcriptional repressors and activators can interact with each other both physically and functionally. Guan et al. (2002) used chromatin immunoprecipitation techniques to examine directly the role of CREB-mediated responses in long-term synaptic integration in the nucleus of *Aplysia* sensory neurons. They found that both facilitatory and inhibitory modulatory transmitters alter promoter occupancy by activator or repressor CREB isoforms and subsequently affect nucleosome structure bidirectionally through acetylation and deacetylation of histone residues in chromatin.

The complete set of genes regulated by a transcription factor in a specific cell type is still not known. In *Aplysia* sensory neurons, the activity of ApCREB1 leads to the expression of several immediate-response genes, such as ubiquitin hydrolase, that stabilizes STF (Hegde et al., 1997) and the transcription factor CCAAT-box-enhanced binding-protein (C/EPB), whose induction has been shown to be critical for LTF (Alberini et al., 1994). This induced transcription factor (in concert with other constitutively expressed molecules such as ApAF [Bartsch et al., 2000]) activate a second wave of downstream genes that can ultimately lead to the growth of new synaptic connections. These genes represent only a few of the family of gene products generated by CREB activity.

The participation of the cAMP/CREB pathway appears to be a general feature of long-term memory formation throughout the animal kingdom. The first genetic screenings designed to identify learning mutants in *Drosophila* revealed two interesting mutants, *dunce* and *rutabaga,* with specific defects in memory formation (Dudai et al., 1976; Duerr and Quinn, 1982) that were subsequently shown to affect genes in the cAMP signaling pathway (Byers et al., 1981; Waddell and Quinn, 2001). Experiments in transgenic flies have confirmed that the balance between CREB activator and repressor isoforms is critical for long-term behavioral memory. Thus, overexpression of an inhibitory form of CREB (dCREB-2b) blocked long-term olfactory memory but did not alter short-term memory (Yin et al., 1994; Perazzona et al., 2004). Indeed, most of the upstream signaling cascade leading to CREB activation

appears to be conserved through evolution, and many aspects of the role of CREB in synaptic plasticity described in invertebrates have also been observed in the mammalian brain. However, the role of CREB in explicit forms of memory appears to be more complex than in implicit forms of memory in invertebrates (for reviews, see Lonze and Ginty, 2002; Barco et al., 2003).

In mammals, CREB has been shown to regulate the expression of more than 100 genes, but it is still not clear how many of these putative downstream genes are actually regulated during learning and required for memory storage (Mayr and Montminy, 2001; Lonze and Ginty, 2002). The current list of target genes is heterogeneous and includes genes with very diverse functions, from regulation of transcription and metabolism to genes affecting cell structure or signaling. Many CREB targets, such as c-fos, EGR-1, or C/EBP, are themselves transcription factors, whose induction may trigger a second wave of gene expression. The availability of the complete mouse and human genome, the availability of the *Aplysia* neuronal transcriptome (Moroz et al., 2006), the development of new bioinformatics tools for their analysis, and the recent application of new unbiased, genome-wide screening approaches has begun to reveal the gene profiles regulated by CREB under different physiological conditions (Conkright et al., 2003; Euskirchen et al., 2004; Impey et al., 2004; Barco et al., 2005; Zhang et al., 2005). Although we have focused on CREB-dependent gene expression because of its conserved role in memory formation through evolution, other transcription factors, such as ApAF and C/EBP in *Aplysia* and SRF, C/EBP, c-fos, EGR-1, or NF-$\kappa\beta$ in mice (Tischmeyer and Grimm, 1999; Albensi and Mattson, 2000; Izquierdo and Cammarota, 2004; Ramanan et al., 2005) are also likely to contribute to the transcriptional regulation that accompanies long-lasting forms of synaptic plasticity.

The epigenetic marking of chromatin, by histone modifications, chromatin methylation, and the activity of retrotransposons, may have long-term consequences on transcriptional regulation of specific gene *loci* involved in long-term synaptic changes, and thus adds a new layer of complexity to our view of how nuclear function and synaptic activity affect one another (Guan et al., 2002; Hsieh and Gage, 2005; Levenson and Sweatt, 2005). The contribution of histone tail acetylation, a modification that favors transcription and is associated with active *loci*, was first revealed for LTF formation by Guan et al. (2002) in *Aplysia*. In addition to finding that facilitatory and inhibitory stimuli alter, bidirectionally, the acetylation stage and structure of promoters driven by the expression of genes involved in the maintenance of LTF, such as C/EBP, this study also demonstrated that enhancing histone acetylation with deacetylase (HDAC) inhibitors facilitates the induction of LTF. HDAC inhibitors have now been shown to enhance L-LTP in the Schaffer collateral pathway of mammals and memory formation in hippocampus-dependent tasks (Alarcon et al., 2004; Korzus et al., 2004; Yeh et al., 2004; Levenson et al., 2005). Conversely, mice with reduced histone acetyltransferase activity have deficits in both long-lasting forms of memory and LTP (Bourtchouladze et al., 2003; Alarcon et al., 2004; Korzus et al., 2004; Wood et al., 2005). These results indicate that critical chromatin remodeling occurs during the formation of long-term memory, and that these nuclear changes are required for the stable maintenance of memory storage.

4.02.4.3 Local Protein Synthesis

In addition to transcription in the nucleus and protein synthesis in the cell body, long-term memory also requires a second site of local protein synthesis at the synapse. A number of distinct mRNAs have been localized in the axons of *Aplysia* and in the dendrites of rodent hippocampal neurons (for review, see Steward and Schuman, 2001, 2003). The molecular mechanisms that target these mRNAs to the synapse are largely unknown, but some are carried by the kinesin motors, the key anterograde transport machinery (Puthanveettil and Kandel, 2006). Some of these mRNAs are thought to involve the recognition of *cis*-acting elements in their 3′ untranslated region by specific RNA-binding proteins that interact with the cytoskeleton. Once transported to the dendritic compartments, these mRNAs are translated only after docking at active synaptic sites, a process frequently referred to as synaptic or local protein synthesis. Recent studies suggest that regulation of local protein synthesis plays a major role in the control of synaptic strength at the sensory-to-motor-neuron connection in *Aplysia* and during L-LTP in the hippocampus. For example, Casadio et al. (1999) found that long-term, synapse-specific facilitation induced by 5-HT requires local protein synthesis for the stable maintenance of learning-induced synaptic growth. In the hippocampus, the induction of LTP in the Schaffer collateral pathway is accompanied by the transport of polysomes from dendritic shafts to active spines of CA1 neurons, suggesting a critical role for local protein synthesis in the morphological changes associated with LTP

(Ostroff et al., 2002), and local inhibition of protein synthesis blocks L-LTP in the Schaffer collateral pathway (Bradshaw et al., 2003; Cracco et al., 2005). The control of translation at the synapse is likely to be complex and involve several different mechanisms, including different types of mRNA transport and docking, cytoplasmic poly-adenylation, mTOR (which is the target of the selective protein synthesis inhibitor rapamycin; Cammalleri et al., 2003; Purcell et al., 2003), and the phosphorylation of different translation factors (see recent review by Sutton and Schuman, 2005). Many of the molecules contributing to the regulation of this process are required for both LTF in *Aplysia* and L-LTP in the hippocampus, including BDNF (which promotes local protein synthesis; Aakalu et al., 2001; Purcell et al., 2003), mTOR, and the cytoplasmic polyadenylation element binding protein (CPEB), which activates dormant mRNAs (Huang et al., 2002; Si et al., 2003a). The role of local protein synthesis in the capture and stabilization of synapse-specific forms of long-term plasticity is discussed in the following section.

4.02.4.4 Moving Back to the Synapse: Capture of Activity-Induced Gene Products

Following the sending of a retrograde signal to the nucleus and the subsequent transcriptional activation, newly synthesized gene products, both mRNAs and proteins, have to be delivered by kinesin-mediated fast axonal transport (Puthanveettil and Kandel, 2006) specifically to the synapses whose activation originally triggered the wave of gene expression. To explain how this specificity can be achieved in a biologically economical way given the massive number of synapses in a single neuron, Martin et al. (1997a) and Frey and Morris (1997) proposed the synaptic capture hypothesis. This hypothesis, also referred to as synaptic tagging, proposes that the products of gene expression are delivered throughout the cell but are only functionally incorporated in those specific synapses that have been tagged by previous synaptic activity. The 'synaptic tag' model has now been supported by a number of studies in both the rodent hippocampus (Frey and Morris, 1997, 1998; Barco et al., 2002; Dudek and Fields, 2002) and *Aplysia* (Martin et al., 1997a; Casadio et al., 1999).

Studies of synaptic capture at the synapses between the sensory and motor neurons of the gill-withdrawal reflex in *Aplysia* have demonstrated that the production of CRE-driven gene products in the nucleus is not sufficient to achieve synapse-specific LTF. One also needs a PKA-mediated covalent signal to mark the stimulated synapses, and consequent local protein synthesis to stabilize that mark (Martin et al., 1997a; Casadio et al., 1999). Thus, injection into the cell body of phosphorylated CREB-1 gives rise to LTF at all the synapses of the sensory neuron, but this facilitation is not maintained beyond 24–48 h unless one of the synapses is also marked by triggering the short-term process with a single pulse of 5-HT (Casadio et al., 1999). Once marked, that synapse and only that synapse shows maintained facilitation and growth.

Experiments in the rat hippocampus by Frey and Morris have demonstrated, in turn, that once transcription-dependent LTP has been induced at one pathway, the long-term process can be 'captured' at a second pathway receiving a single train that would normally produce only E-LTP. The stimulus for the short-term process causes a transient potentiation and, in addition, marks the synaptic terminals, enabling the capture of the newly expressed gene products. The properties of synaptic capture observed for intracompartmental capture in hippocampal CA1 neurons are similar to those described in the bifurcated sensory neurons of *Aplysia* (Martin et al., 1997a). However, in mammals, where there are two dendritic compartments – apical and basal – the tag appears to be restricted to specific dendritic compartments, and additional mechanisms are required to capture across compartments (Alarcon et al., 2006). Most of the molecular details underlying these processes are still unknown, but experiments with a line of transgenic mice expressing a constitutively active CREB protein have demonstrated that PKA activity is part of the tagging signal, and that the synaptic capture of CRE-driven transcription may be sufficient to support L-LTP (Barco et al., 2002). More recent experiments in these mutants and in BDNF-deficient mice support a role for presynaptically released BDNF in the tagging of the synapse for subsequent capture of L-LTP (Barco et al., 2005). Local synthesis of proteins, which we have discussed in the previous section, has also been proposed to be involved in synaptic capture of long-term forms in both *Aplysia* and rodents (Martin et al., 1997a; Barco et al., 2002). Martin et al. (1997a) investigated the role of local protein synthesis in an *Aplysia* culture system in which a single bifurcated sensory neuron was plated in contact with two spatially separated gill motor neurons. In this system, repeated application of 5-HT to one synapse produces a CREB-mediated, synapse-specific LTF that can be blocked by the local application

of inhibitors of translation, suggesting that local protein synthesis at the synapse is also required as part of the retrograde signaling cascade for the initiation of synapse-specific LTF.

4.02.4.5 The Stable Strengthening of Synaptic Connections: Synaptic Growth, Silent Synapses, and Self-Maintenance Mechanisms

Although a number of molecular components that underlie the functional changes associated with the storage of long-term memory have been characterized, little is known about how these are regulated by and coupled to the signaling pathways that give rise to the synaptic structural changes (Bailey et al., 2004).

Activity-induced remodeling of preexisting synapses and the growth of new synapses have been found to accompany various forms of long-term memory, a phenomenon particularly well documented in *Aplysia* sensory neurons (Bailey and Kandel, 1993). Long-term sensitization has been extensively studied in this respect because it is associated with a robust growth of new synaptic connections between the sensory neurons and their postsynaptic target cells (Bailey and Chen, 1983, 1988a,b, 1989). Studies of long-term sensitization have revealed that it is accompanied by two forms of learning-related structural plasticity: (1) the remodeling of preexisting synapses, resulting in an increase in the number, size, and vesicle content of presynaptic transmitter release sites (active zones) (Bailey and Chen, 1983), and (2) a growth process that appears similar to synaptogenesis during development and leads to a pronounced increase in the total number of presynaptic varicosities per sensory neuron (Bailey and Chen, 1988a). Sensory neurons from long-term sensitized animals exhibit a twofold increase in the total number of synaptic varicosities, as well as an enlargement in the size of each neuron's axonal arbor. The increase in the size and synaptic vesicle complement of sensory neuron active zones was found to be relatively transient, whereas the changes in varicosity and active zone number were much more stable and persisted for at least 3 weeks after training, similar to the duration of the behavioral change (Bailey and Chen, 1989). These findings demonstrated, for the first time, that learning-induced structural changes could be detected at the level of identified synaptic connections known to be critically involved in behavioral modification and suggested that the growth of new sensory neuron synapses is likely to represent the final and perhaps most stable phase of long-term memory storage in *Aplysia*.

More recently, Kim et al. (2003) have examined the contribution of each class of presynaptic structural change – remodeling of preexisting synapses and the growth of new synapses – to the different time-dependent phases of LTF. They monitored both functional and structural synaptic changes continuously using time-lapse confocal imaging of presynaptic varicosities of sensory neurons labeled with three different fluorescent markers: the whole-cell marker *Alexa-594* and two presynaptic marker proteins, *synaptophysin-eGFP* to monitor changes in the distribution of synaptic vesicles within individual varicosities and *synapto-PHluorin* to monitor active transmitter release sites (Miesenbock et al., 1998). Repeated pulses of 5-HT induced two temporally, morphologically, and molecularly distinct classes of presynaptic changes: (1) relatively rapid activation of silent presynaptic terminals through the filling of preexisting empty varicosities with synaptic vesicles, which requires translation but not transcription, and (2) generation of new synaptic varicosities, which occurs more slowly and requires both transcription and translation. In addition to its role in LTF, the rapid (hours) activation of silent presynaptic terminals may also contribute to the intermediate phase of synaptic plasticity and memory storage (Ghirardi et al., 1995; Mauelshagen et al., 1996; Sutton et al., 2001). These findings, the first to be made on individually identified presynaptic varicosities, suggest that long-term changes in synaptic effectiveness in *Aplysia* may involve the differential regulation of two fundamentally disparate forms of presynaptic compartment: (1) nascent (empty) silent varicosities that can be rapidly and reversibly remodeled into active transmitter release sites and (2) mature, more stable and functionally competent varicosities that may undergo a process of fission to form new stable synaptic contacts (Bailey et al., 2005).

Activation of silent synapses also plays a major role in LTP in mammals. In this case, the term *silent synapse* refers to excitatory glutamatergic synapses whose postsynaptic membrane contains NMDARs but no AMPARs (Malinow et al., 2000; Malinow and Malenka, 2002). The lack of AMPAR-mediated signaling renders these synapses inactive, or 'silent,' under normal conditions. Synaptic stimulation activates these silent synapses through the insertion of AMPARs into the postsynaptic membrane, a phenomenon sometimes referred to as AMPA-fication. Calcium/CamKII plays a critical role in this process.

Once this kinase is activated by high-frequency stimulation, it phosphorylates AMPARs or associated proteins, triggering their insertion into the postsynaptic membrane. The synapse is then no longer silent, and postsynaptic responses are, by consequence, enhanced. Conversely, synapses can be made to be silent, for example, after LTD induction, by removing AMPARs from the postsynaptic membrane (Malinow and Malenka, 2002).

In addition to changes in synaptic function, activity-induced growth and/or remodeling of synaptic connections are also important in the storage of explicit memory. However, their specific role is not as clear as in *Aplysia,* because the functional contribution of individual synapses to memory processes in the large and complex neuronal circuits of the mammalian brain is not yet well defined (see reviews by Lamprecht and LeDoux, 2004; Hayashi and Majewska, 2005; Segal, 2005). The generation and enlargement of dendritic spines has been associated with the production of LTP and synaptic activity in organotypic hippocampal slices (Matsuzaki et al., 2004; Nagerl et al., 2004), or acute slices of neonatal animals (Zhou et al., 2004), whereas these structural changes are much more subtle in the adult brain (Lang et al., 2004). In adults there is only a modest production of new spines (Zuo et al., 2005), and learning-related plasticity seems to rely more on subcellular changes than on anatomical changes. Thus, neuronal activity regulates the diffusion of molecules across the neck of dendritic spines (Bloodgood and Sabatini, 2005) and the transport of polysomes from dendritic shafts to active spines (Ostroff et al., 2002), as well as the trafficking of neurotransmitter receptors (Malinow and Malenka, 2002).

Biological molecules have a relatively short half-life (hours to days) compared with the duration of memory (days, weeks, even years). How then, in the absence of frank anatomical changes, can the altered molecular composition of a synapse be maintained for such a long time? Most answers to this elusive question rely on some type of self-sustained alteration that can somehow modulate synaptic strength. For example, Malinow and colleagues have proposed that two regulatory pathways control the insertion and removal of AMPA receptors at the synapse: The maintenance pathway is always on and controls the constant turnover of receptor subunits, whereas the constructive pathway is only turned on during LTP induction (Malinow et al., 2000; Malinow and Malenka, 2002). The activation of the constructive pathway and insertion of new AMPARs would cause the growth and/or maturation of postsynaptic densities, enabling the formation of new memories, whereas the maintenance pathway would be responsible for their stabilization (Hayashi et al., 2000; Lisman and Zhabotinsky, 2001). Another interesting model for long-term memory storage was suggested by Crick more than 20 years ago (Crick, 1984). Crick proposed that autocatalytic kinases might provide the molecular mechanism for long-lasting, self-maintained changes in synaptic function. John Lisman further developed this idea based on the autocatalytic properties of the calcium/CamKII (Lisman and Zhabotinsky, 2001).

More recently, Kandel and Si have proposed a model based on the prion-like properties of the *Aplysia* neuronal CPEB (Si et al., 2003b). *Aplysia* CPEB has two conformational states: one is inactive or acts as a repressor, and the other is active. In a naive synapse, the basal level of CPEB expression is low, and its state is inactive or repressive. However, if a given threshold is reached, CPEB switches to the prion-like state, which activates the translation of dormant mRNAs through the elongation of their poly-A tail (Si et al., 2003a). Once the prion state is established at an activated synapse, dormant mRNAs, made in the cell body and distributed cell-wide, would be translated only at the activated synapses. Because the activated CPEB can be self-perpetuating, it could contribute to a self-sustaining, synapse-specific long-term molecular change. Interestingly, a structurally similar neuronal isoform of CPEB, CPEB-3, has been found in mouse hippocampal neurons, where it is induced by the neurotransmitter dopamine (Theis et al., 2003).

These molecular mechanisms are not mutually exclusive: The synaptic translation of CamKII mRNAs can be regulated by CPEB, and the synthesis and traffic of new AMPAR subunits may require CamKII activity as well as enhanced protein synthesis (Burgin et al., 1990; Ouyang et al., 1999; Huang et al., 2002) .

4.02.5 Concluding Remarks

Cell biological and molecular studies of both implicit and explicit memory processes have revealed two major forms of storage mechanisms. The storage of long-term memory is associated with altered gene expression, as well as the synthesis of new mRNAs and proteins, and is often accompanied by changes in both the number and structure of synaptic connections. In contrast, short-term (up to about 1 h) memory and the early phases of long-term synaptic

storage in *Aplysia* and hippocampus involve covalent modification of preexisting proteins in either the presynaptic (*Aplysia*) or postsynaptic (hippocampus) neuron, leading to an alteration in the strength of preexisting synaptic connections.

The discovery of intermediate (2–3 h) forms of synaptic plasticity that involve elements of the short-term and long-term molecular mechanisms in both *Aplysia* and hippocampus have demonstrated that distinct phases that overlap these processes can be revealed by specific protocols. These new intermediate phases may also form a bridge to the morphological changes that occur during long-term plasticity: Recent imaging studies of living synapses in hippocampus and *Aplysia* have shown that even the early phases of synaptic plasticity are accompanied by microstructural alterations. These early structural changes may seed the formation of new functional synapses, which may then be further elaborated and stabilized by additional long-term training. This emerging view is likely to supplement the more established idea that early-phase plasticity exclusively involves either pre- or postsynaptic covalent modifications and suggests that these early phases of synaptic plasticity also may already involve microstructural alterations capable of contributing importantly to the transition between short-term and long-term memory storage.

Finally, as we have outlined in this chapter, one of the key unifying findings emerging from the molecular study of implicit and explicit memory processes is the unexpected realization that these distinct forms of memory, which differ not only in the neural systems involved but also in the nature of the information stored and in the role of attention in that storage, nevertheless share a common set of molecular mechanisms for their long-term representation. Thus, whereas animals and humans are capable of a wide variety of learning processes that use a number of different second messenger and signaling cascades, they may recruit the same restricted set of molecular logic for the storage of long-term memory.

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4.03 Sensitization and Habituation: Invertebrate

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4.03.1 Introduction

Survival of animals is dependent on their capacity to adapt to their environment by modifying their behavior. The experience-induced modification of behavior is a manifestation of learning, whereas memory is the retention of a learned behavior over time. A conceptual scheme that has driven the investigation of learning for the largest part of the twentieth century rests on the distinction between associative and nonassociative forms of learning. Nonassociative learning is best exemplified by habituation and sensitization. Habituation is defined as the gradual waning of a behavioral response to a weak or moderate stimulus that is presented repeatedly. Following habituation, the response may be restored to its initial state either passively with time (i.e., spontaneous recovery), or with the presentation of a novel stimulus (i.e., dishabituation). Sensitization is defined as the enhancement of a behavioral response elicited by a weak stimulus following another, usually noxious stimulus. Sensitization can also develop in response to a moderate stimulus that is presented repeatedly at relatively short intervals.

Associative learning refers to the formation of an association either between two stimuli (i.e., classical conditioning), or between a behavior and a stimulus (i.e., operant conditioning). In classical conditioning, a novel or weak stimulus (conditioned stimulus; CS) is paired with a stimulus that generally elicits a reflexive response (unconditioned stimulus and response, respectively; US and UR). After sufficient training with contingent CS-US presentations (which may be a single trial), the CS comes to elicit a learned response (conditioned response; CR), which often resembles the UR (or some aspect of it). Operant conditioning is an experimental procedure in which the behavior of an animal may be followed by either a desirable or an aversive stimulus, arranged by the

experimenter. The desirable stimulus (e.g., food) will typically increase the future occurrence of this behavior (a process called positive reinforcement). An aversive stimulus (e.g., a noxious electric shock) will tend to decrease the future probability of this behavior (a process called punishment). A behavior can also be reinforced when it becomes contingent with the removal of an aversive stimulus from the animal's environment (i.e., negative reinforcement). Thus, through the processes of operant conditioning an animal learns the consequences of its behavior.

Humans and other animals are capable of displaying more complex forms of learning than the four types described above. However, these four types are likely to constitute the building blocks for more complex forms of learning. Thus, a major goal of neurobiologists is to explain the anatomical, biophysical, and molecular processes of the nervous system that underlie simple forms of learning and memory. Specifically, what parts of the nervous system are critical for learning? How is information about a learned event acquired and encoded in neuronal terms? How is information stored, and, once stored, how is it retrieved? Most neuroscientists believe that the answers to these questions lie in understanding the ways in which the properties of individual nerve cells in general, and synaptic connections, in particular, change when learning occurs. To that end, the investigation of neuronal mechanisms of learning and memory in invertebrates has been very fruitful over the past 40 years. This chapter will focus on mechanisms of habituation and sensitization in *Aplysia* and other invertebrates. A detailed discussion of mechanisms of associative learning in *Aplysia* and other invertebrates can be found in subsequent chapters (*See* Chapters 4.02, 4.04, 4.06, 4.07, 4.08, 4.09, 4.10).

4.03.2 Habituation and Sensitization in *Aplysia*

4.03.2.1 Aplysia Withdrawal Reflexes and Underlying Neural Circuits

One animal that is well suited for the examination of the molecular, cellular, morphological, and network mechanisms underlying neuronal plasticity and learning and memory is the marine mollusc *Aplysia*. This animal has a relatively simple nervous system with large, identifiable neurons that are accessible for detailed anatomical, biophysical, and biochemical studies. Neurons and neural circuits that mediate many behaviors in *Aplysia* have been identified. In several cases, these behaviors can be modified by learning. Moreover, specific loci within neural circuits at which modifications occur during learning have been identified and aspects of the cellular mechanisms underlying these modifications have been analyzed.

Two withdrawal reflexes of *Aplysia* have been used extensively to analyze the neuronal mechanisms contributing to nonassociative and associative learning (for reviews see Hawkins and Kandel, 1984; Carew and Sahley, 1986; Byrne, 1987; Byrne et al., 1991, 1993). The first behavior is the siphon–gill withdrawal reflex. Within the mantle cavity is the respiratory organ of the animal, the gill, and protruding from the mantle cavity is the siphon (**Figure 1**). The

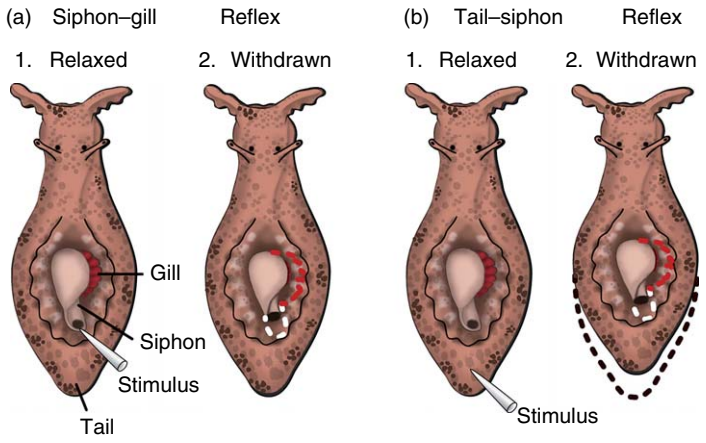


Figure 1 Siphon–gill and tail–siphon withdrawal reflexes of *Aplysia*. (a) Siphon–gill withdrawal. Dorsal view of *Aplysia* (1) Relaxed position. (2) A stimulus (e.g., a water jet, brief touch, or weak electric shock) applied to the siphon causes the siphon and the gill to withdraw into the mantle cavity. (b) Tail–siphon withdrawal reflex. (1) Relaxed position. (2) A stimulus applied to the tail elicits a reflex withdrawal of the tail and siphon.

siphon–gill withdrawal reflex is elicited when a tactile or electrical stimulus is delivered to the siphon and results in withdrawal of the siphon and gill (**Figure 1(a)**). A second behavior that has been examined extensively is the tail–siphon withdrawal reflex. Tactile or electrical stimulation of the tail elicits a coordinated set of defensive responses, two components of which are a reflex withdrawal of the tail and the siphon (**Figure 1(b)**).

A prerequisite for the analysis of the neural and molecular basis of learning is an understanding of the neural circuit that controls the behavior. The afferent limb of the siphon–gill withdrawal reflex consists of sensory neurons with somata in the abdominal ganglion. The siphon sensory neurons (SN) monosynaptically excite gill and siphon motor neurons (MN) that are also located in the abdominal ganglion (**Figure 2**). Activation of the gill and siphon motor neurons leads to contraction of the gill and siphon. Excitatory, inhibitory, and modulatory interneurons (IN) in the withdrawal circuit have also been identified, although only excitatory interneurons are illustrated in **Figure 2**. The afferent limb of the tail–siphon withdrawal reflex consists of a bilaterally symmetric cluster of sensory neurons that are located in the left and right pleural ganglia (Walters et al., 1983a). These sensory neurons make monosynaptic excitatory connections with motor neurons in the adjacent pedal ganglion, which produce withdrawal of the tail (**Figure 2**). In addition, the tail sensory neurons form synapses with various identified excitatory and inhibitory interneurons (Buonomano et al., 1992; Cleary and Byrne, 1993; Xu et al., 1994). Some of these interneurons activate motor neurons in the abdominal ganglion, which control reflex withdrawal of the siphon. Moreover, several additional neurons modulate the tail–siphon withdrawal reflex (Raymond and Byrne, 1994; Cleary et al., 1995) (**Figure 3(a1)**).

The sensory neurons for both the siphon–gill and tail–siphon withdrawal reflexes are similar and appear to be important plastic elements in the neural circuits. Changes in their membrane properties and the strength of their synaptic connections (synaptic efficacy) are associated with learning and memory. Moreover, the properties of these neurons are modulated by *in vitro* analogs of behavioral training.

4.03.2.2 Habituation

Habituation, perhaps the simplest form of nonassociative learning, refers to the gradual waning of the responses elicited by a repeatedly presented stimulus. Repeated presentation of a relatively weak stimulus will most probably lead to habituation, whereas repeated presentation of a relatively strong stimulus

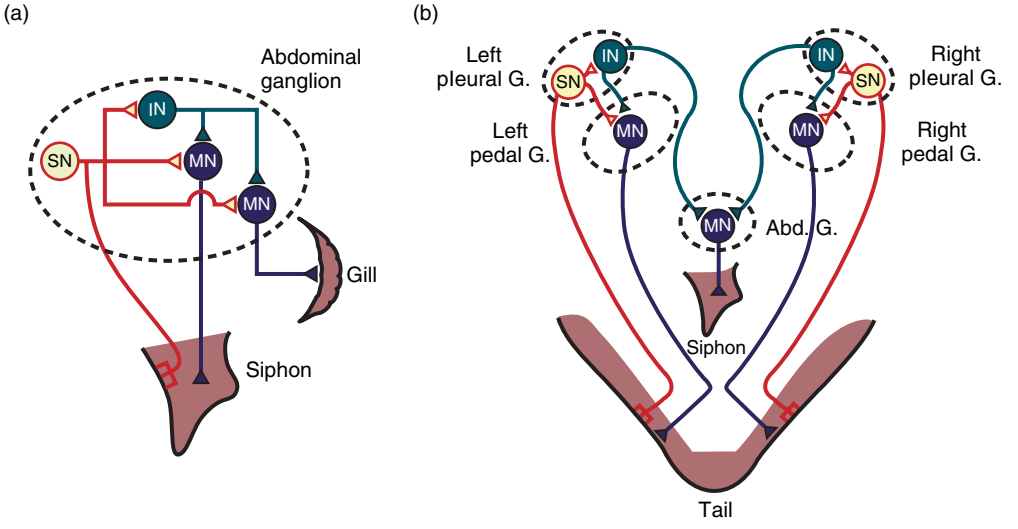


Figure 2 Simplified circuit diagrams of the siphon–gill (a) and tail–siphon (b) withdrawal reflexes. Stimuli activate the afferent terminals of mechanoreceptor sensory neurons (SN) whose somata are located in central ganglia. The sensory neurons make excitatory synaptic connections (triangles) with interneurons (IN) and motor neurons (MN). The excitatory interneurons provide a parallel pathway for excitation of the motor neurons. Action potentials elicited in the motor neurons, triggered by the combined input from the SNs and INs, propagate out peripheral nerves to activate muscle cells and produce the subsequent reflex withdrawal of the organs. Modulatory neurons (not shown here but see **Figure 3(a1)**), such as those containing serotonin (5-HT), regulate the properties of the circuit elements, and, consequently, the strength of the behavioral responses.

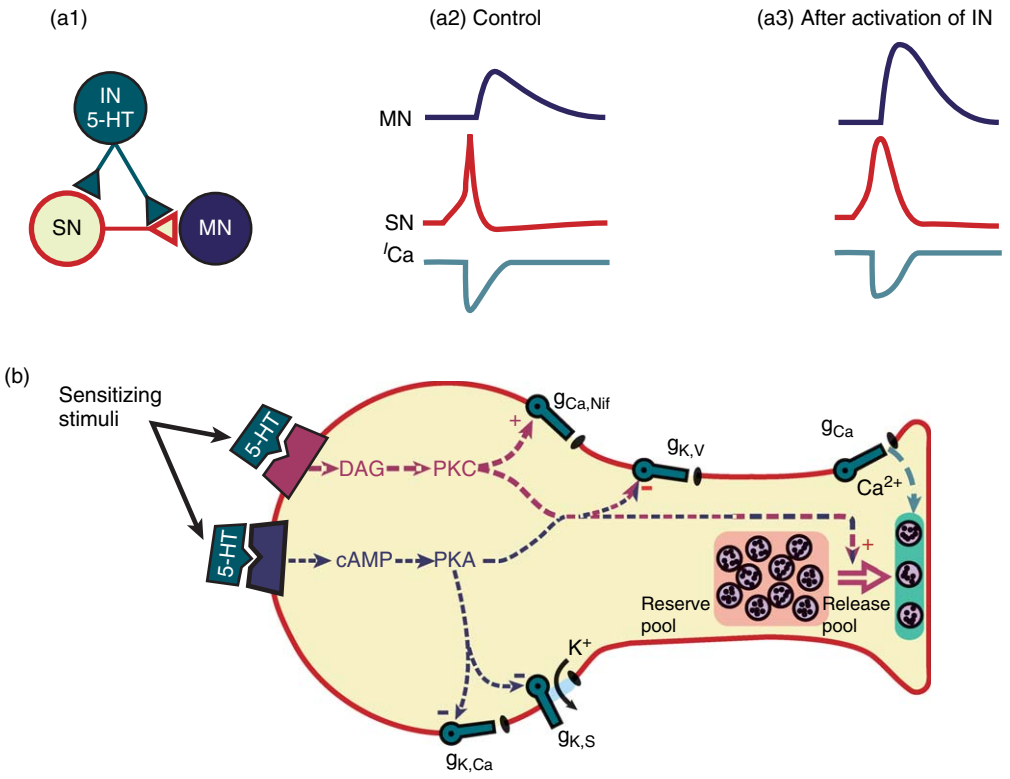


Figure 3 Model of heterosynaptic facilitation of the sensorimotor connection that contributes to short-term sensitization in *Aplysia*. (a1) Sensitizing stimuli activate facilitatory interneurons (IN) that release modulatory transmitters, one of which is 5-HT. The modulator leads to an alteration of the properties of the sensory neuron (SN). (a2, a3) An action potential in a SN after the sensitizing stimulus results in greater transmitter release and hence a larger postsynaptic potential in the motor neuron (MN) than an action potential prior to the sensitizing stimulus. For short-term sensitization the enhancement of transmitter release is due, at least in part, to broadening of the action potential and an enhanced flow of Ca^{2+} into the sensory neuron. (b) Molecular events in the sensory neuron. 5-HT released from the facilitatory interneuron (Part a1) binds to at least two distinct classes of receptors on the outer surface of the membrane of the sensory neuron, which leads to the transient activation of two intracellular second messengers: DAG and cAMP. These second messengers, acting though their respective protein kinases, affect multiple cellular processes, the combined effects of which lead to enhanced transmitter release when a subsequent action potential is fired in the sensory neuron. See section 4.03.2.3.1 for abbreviation definitions.

may lead to sensitization, which is discussed in the next section of the chapter. Habituation is generally distinguished from simple fatigue or sensory adaptation because responsiveness can be rapidly restored (dishabituated) by the presentation of a novel stimulus to the animal. The parametric features of habituation have been previously described in detail by Thompson and Spencer (1966).

Habituation shares some features with more complex forms of learning. First, habituation has a temporal gradient. Similar to most forms of learning, each trial has only a transient effect, which necessitates the presentation of multiple trials. Second, the interval at which training trials are presented is critical. Massing many trials together may lead to faster, albeit only short-lived habituation. In contrast, spacing trials too far apart may lead to little or no habituation. Therefore, an optimal intertrial interval exists, which is determined by the stimulus features and the response system. Third, the effects of habituation training are reversible. As mentioned above, they can be reversed spontaneously with the passage of time (spontaneous recovery), or they may be reversed by the presentation of a novel stimulus (dishabituation). Fourth, habituation learning is stimulus-specific. Although habituation can generalize to novel stimuli, this generalization is limited and depends on the degree of physical similarity between the trained and novel stimuli. Habituation is an indispensable form of learning. It is probably the earliest manifestation of the ability of all animals to store and retrieve the memory of a stimulus, as well as the

ability to filter out stimuli that are inconsequential. The latter is a necessary element of selective attention, which places behavior under the dynamic control of stimuli that carry important behavioral contingencies (more on attention can be found in subsequent chapters; *See* Chapters 1.13, 2.02).

A major step in the understanding of neural mechanisms of habituation was made in the early 1970s (Pinsker et al., 1970; Carew and Kandel, 1973). In a group of three seminal articles, it was reported that the siphon–gill withdrawal reflex of *Aplysia* can display habituation, that habituation was accompanied by a decrease in the spike activity of gill motor neurons in response to tactile stimulation of the siphon, and that an activity-induced decrease in the efficacy of sensorimotor synapses could be responsible for the reduced responsiveness of motor neurons and for behavioral habituation (Castellucci et al., 1970; Kupfermann et al., 1970; Pinsker et al., 1970). Starting with those early reports, behavioral habituation of withdrawal reflexes became tightly linked to homosynaptic depression of sensorimotor synapses. Since then, the vast majority of research studies that aimed at understanding the mechanisms of habituation focused on understanding the mechanisms of synaptic depression, which, similar to habituation, can appear in both short- and long-term forms.

4.03.2.2.1 Short-term depression of Aplysia sensorimotor synapses

Quantal analysis of sensorimotor synapses suggested that short-term homosynaptic depression involves primarily a decrease in presynaptic transmitter release (Castellucci and Kandel, 1974). The presynaptic nature of synaptic depression was also supported by a more recent report that repeated application of exogenous glutamate to the postsynaptic neuron did not result in depression, that blockade of postsynaptic glutamate receptors did not block depression, and that synaptic depression did not correlate with changes in the amplitude of miniature excitatory postsynaptic potentials (mEPSPs) (Armitage and Siegelbaum, 1998). To account for the depressive effect of repeated activity of sensory neurons on transmitter release, an early model of depression relied on cumulative inactivation of calcium channels and decline in the calcium entering the presynaptic terminal and triggering release (Klein et al., 1980).

Extensive parametric analysis of the kinetics of depression and recovery from depression as a function of the stimulation frequency revealed that the mechanisms must be more complex than just a decrease in calcium influx or a depletion of presynaptic vesicles (Byrne, 1982). A quantitative model of transmission at the sensorimotor synapse suggested that the inactivation kinetics of presynaptic calcium channels cannot account for the kinetics of depression; neither does simple depletion of releasable vesicles (Gingrich and Byrne, 1985). Rather, the model suggested the existence of dynamic interactions between use-dependent depletion of readily releasable vesicles and calcium-dependent mobilization of stored vesicles to supply the releasable ones. This model of synaptic depression was supported by morphological studies of the sensorimotor synapse, which indicated that the fraction of readily releasable vesicles decreased with activity, parallel to synaptic depression (Bailey and Chen, 1988b).

A subsequent study of spontaneous release from cultured sensory neurons revealed that synaptic depression was accompanied by a decrease in the frequency of mEPSPs (Eliot et al., 1994). However, the change in mini EPSP frequency did not parallel the synaptic depression in magnitude or in duration. This finding argued against the depletion of presynaptic terminals with releasable vesicles as the sole mechanism of depression, and suggested that depression may be due to a change in excitation–secretion coupling as well. However, this decrease in excitation–secretion coupling does not appear to be due to the decrease in calcium influx that had been previously suggested (Klein et al., 1980): Calcium imaging of cultured sensory neurons revealed that the calcium transients are unaffected by repetitive activity (Armitage and Siegelbaum, 1998).

Based on theoretical and statistical analyses of transmission at the sensorimotor synapse, another model of synaptic depression put forth the activity-dependent inactivation of individual release sites, proposing the transient switching off of presynaptic release machinery following an action potential (Royer et al., 2000). A similar model of synaptic depression arising from inactivation of release sites was suggested by Gover et al. (2002). Finally, the comparison of transmitter release from cultured sensory neurons stimulated by hypertonic solutions versus electrical activity revealed that both types of stimuli draw transmitter from the same presynaptic pool, and suggested that depression is mediated by both depletion of releasable transmitter and a change in excitation–secretion coupling (Zhao and Klein, 2002). Thus, the most recently proposed model of depression of sensorimotor synapses relies again on activity-dependent depletion of releasable vesicles,

acknowledging though that there must be at least one other process that contributes as well.

Despite their differences, the studies outlined in this section have two common elements. First, they all supported the presynaptic nature of depression. Second, they all employed repetitive stimulation of the synapse at intervals at least as long as 1 s. However, when the sensorimotor synapse is stimulated at 10 Hz (100-ms interval), but not at 1 Hz, depression of evoked excitatory postsynaptic potentials (EPSPs) partly results from desensitization of the postsynaptic receptors (Antzoulatos et al., 2003). Therefore, both the kinetics of synaptic depression (Byrne, 1982; Eliot et al., 1994) and the mechanisms underlying it depend on the stimulation regime.

Another form of short-term depression of *Aplysia* sensorimotor synapses can be elicited by brief exposure to the neuropeptide Phe-Met-Arg-Phe-NH_2 (FMRFa). Because activation of a third type of synapse, a modulatory one, and release of a neuromodulator are required, this form of plasticity is termed heterosynaptic. In contrast, depression arising exclusively from intrinsic activity is termed homosynaptic depression. FMRFa-immunoreactive inhibitory interneurons have been identified that innervate tail and siphon sensory neurons (Mackey et al., 1987; Small et al., 1992; Xu et al., 1994). These interneurons are activated by shock to nerves that innervate the tail, and stimulation of these neurons inhibits sensorimotor synapses. However, the extent to which activation of these FMRFa-immunoreactive neurons contributes to habituation has not been determined.

Applying FMRFa to sensory neurons leads to a hyperpolarization of the membrane potential, a decrease in the duration of the action potential, and inhibition of synaptic transmission via the modulation of potassium conductances (Abrams et al., 1984; Ocorr and Byrne, 1985; Belardetti et al., 1987; Critz et al., 1991; Pieroni and Byrne, 1992). Moreover, FMRFa directly affects presynaptic Ca^{2+} currents (Blumenfeld et al., 1990; Edmonds et al., 1990) and the release machinery itself, as indicated by a decrease in the frequency of mEPSPs (Dale and Kandel, 1990). The second messenger mediating the actions of FMRFa seems to be arachidonic acid (AA) produced by phospholipid metabolism (Piomelli et al., 1987) and its downstream metabolite 12-hydroperoxyeicosatetraenoic acid (12-HPETE) (Buttner et al., 1989). Recently, FMRFa was found to inhibit one member of the MAP kinase family, extracellular signal-regulated protein kinase (ERK), but activate another, p38 mitogen-activated protein kinase (p38 MAPK) (Guan et al., 2003; Fioravante et al., 2006). The latter probably activates a phospholipase A2 molecule, which in turn can release AA from phospholipids (Piomelli, 1991). FMRFa also engages protein phosphatases in regulating the outward potassium currents (Ichinose and Byrne, 1991), in particular protein phosphatase 1 (PP1). In other systems, p38 MAPK can activate PP1 (Westermarck et al., 2001), raising the interesting possibility that FMRFa exercises its actions on sensory neuron conductances through a p38 MAPK-PP1 pathway.

4.03.2.2.2 Long-term depression of Aplysia sensorimotor synapses

Repetitive stimulation of *Aplysia* withdrawal reflexes can lead to both short- and long-term habituation (Pinsker et al., 1970; Carew and Kandel, 1973; Stopfer et al., 1996). Short- and long-term habituation share aspects of a common mechanism, synaptic depression. However, whereas short-term synaptic depression arises primarily from transient changes in release, long-term depression has been attributed to persistent structural changes in sensory neurons (Bailey and Chen, 1988a). Extensive morphological analyses of sensory neurons from habituated animals have revealed that the number of synaptic contacts is reduced compared to controls. Moreover, the structure of presynaptic terminals is affected, with fewer synaptic vesicles and reduced size of active zones (the sites of transmitter release).

In addition, activation of sensory neurons at 2 Hz for 15 min induces prolonged (at least 80 min) homosynaptic depression (long-term depression; LTD) of isolated *Aplysia* sensorimotor synapses in cell culture (Lin and Glanzman, 1996). This form of depression relies on activation of postsynaptic *N*-methyl-D-aspartate (NMDA)-like receptors and is sensitive to postsynaptic Ca^{2+}, because infusion of the calcium chelator BAPTA into the postsynaptic motor neuron blocks induction of LTD, but not short-term synaptic depression. Similarly, prolonged habituation of the siphon-elicited gill withdrawal reflex in reduced preparations was recently shown to depend on activity of postsynaptic glutamate receptors both of the NMDA and non-NMDA type (Ezzeddine and Glanzman, 2003).

A more extensively studied form of long-term synaptic depression in *Aplysia* is elicited by repeated application of the neuropeptide FMRFa (Montarolo

et al., 1988; Guan et al., 2003). FMRFa-induced LTD requires transcription, translation (Montarolo et al., 1988; Bailey et al., 1992), but also gene silencing (Guan et al., 2002). Inducing events in FMRFa-mediated LTD include activation of p38 MAPK and recruitment of the transcription repressor CREB2 (cAMP response element binding protein) to the promoter region of genes such as *c/ebp* (Guan et al., 2003; Fioravante et al., 2006).

Little is known about the mechanisms underlying consolidation of LTD. Two genes that are regulated by FMRFa and could be important in the consolidation of LTD are sensorin (Sun et al., 2001) and *Aplysia* cell adhesion molecule (Schacher et al., 2000), even though the requirement of their regulation for LTD has not been demonstrated. Finally, expression of heterosynaptic LTD is accompanied by morphological changes, including loss of presynaptic varicosities and retraction of neurites (Schacher and Montarolo, 1991).

4.03.2.3 Sensitization

Sensitization refers to the augmentation of the behavioral response elicited by a test stimulus. Sensitization to a test stimulus can be induced in one of two ways. First, it can be induced by presentation of another, usually strong stimulus. An example of such sensitization is pseudoconditioning, where an increase in responsiveness to the CS in a classical conditioning procedure may not be due to associative learning (as in classical conditioning), but instead due to the sensitization induced by the strong US. Second, sensitization can be induced by the mere repetition of the test stimulus. As mentioned above, the repetition of a weak stimulus will lead to habituation of the behavioral response, whereas repetition of a moderate to strong stimulus may lead to sensitization. This form of sensitization can sometimes appear as a transient rise in response magnitude before habituation eventually ensues.

Both forms of sensitization have been studied in *Aplysia*, with major emphasis on the former one described above. Similar to habituation, sensitization was also attributed early on to plasticity of the sensorimotor synapse (see next section). Although habituation was attributed to homosynaptic depression of the synapse, sensitization was attributed to heterosynaptic facilitation, induced by the diffuse release of neuromodulators, such as serotonin. Also similar to habituation, sensitization can appear both in short- and long-term forms, which have been extensively studied in their neuronal analogs, short and long-term synaptic facilitation.

4.03.2.3.1 *Short-term sensitization*

In *Aplysia*, sensitization of withdrawal reflexes can be induced by electric shocks to the tail or the lateral body wall of the animal (Carew et al., 1971). Peripheral electric shock has been shown to modulate transmission at the sensorimotor synapse through heterosynaptic facilitation (Carew et al., 1971; Walters et al., 1983a,b).

Several lines of evidence suggest that serotonin (5-HT) is the neurotransmitter involved in heterosynaptic facilitation. First, 5-HT is present in *Aplysia* hemolymph, and its concentration increases in sensitized animals (Levenson et al., 1999). Recent studies also indicated that 5-HT concentration increases in several regions of the *Aplysia* CNS in response to nerve stimulation (Marinesco and Carew, 2002). Second, serotonergic cells are present (Hawkins, 1989; Nolen and Carew, 1994) and serotonergic fibers are in close proximity to sensory neurons (SNs) (Zhang et al., 1991; Marinesco and Carew, 2002; Zhang et al., 2003). Third, depletion of endogenous 5-HT by addition of a neurotoxin (5,7-DHT) blocks the ability of tail stimuli to sensitize the gill-withdrawal reflex (Glanzman et al., 1989). Along the same lines, application of the 5-HT receptor antagonist cyproheptadine blocks facilitation induced by nerve stimulation (Mercer et al., 1991). Finally, exogenously applied 5-HT mimics the actions of tail stimulation both in facilitating the strength of synaptic connections and in increasing the strength of reflex responses (Brunelli et al., 1976; Walters et al., 1983a, b; Abrams et al., 1984; Zhang et al., 1997), and nerve shock-induced 5-HT release correlates with synaptic plasticity (Marinesco et al., 2006).

The conclusions drawn from studies conducted in the 1970s and 1980s led to the formulation of a model for short-term sensitization, according to which sensitizing stimuli activate serotonergic facilitatory interneurons, releasing 5-HT and activating a serial cascade of events in the sensory neurons. The binding of 5-HT to one class of receptors on the outer surface of the membrane of the sensory neurons leads to the activation of adenylyl cyclase, which in turn, leads to an elevation of the intracellular level of the second messenger adenosine-3′,5′-monophosphate (cyclic AMP, cAMP) in sensory neurons. When cAMP binds to the regulatory subunit of cAMP-dependent protein kinase (protein kinase A, PKA), the catalytic subunit is freed and can now add phosphate groups to specific substrate proteins and, hence, alter their functional

properties (Bernier et al., 1982; Ocorr and Byrne, 1985; Pollock et al., 1985; Ocorr and Byrne, 1986; Ocorr et al., 1986; Sweatt et al., 1989). One effect of activated PKA is phosphorylation of a class of membrane channels (S-K^+ channels, named for their ability to be modulated by serotonin) and reduction of the S-K^+ conductance ($G_{K,S}$) (Klein and Kandel, 1980; Klein et al., 1982; Siegelbaum et al., 1982). Consequently, a test stimulus triggers a greater number of action potentials in the sensory neuron after sensitization. Each of these spikes is broader, leading to increased Ca^{2+} influx and enhanced transmitter release. As a result, the follower motor neuron is more intensely activated, and the behavioral response is enhanced (i.e., sensitized). Thus, it was previously believed that serotonin (5-HT) exerted all of its actions in the sensory neurons via the cAMP-mediated reduction of $G_{K,S}$.

Later studies, however, indicated that the effects of 5-HT are more complex than originally suggested. Not only $G_{K,S}$ is modulated by 5-HT, but also at least three other conductances: 5-HT increases a dihydropyridine-sensitive Ca^{2+} current ($G_{Ca,Nif}$) (Braha et al., 1990; Edmonds et al., 1990), decreases a component of the Ca^{2+}-activated K^+ current ($G_{K,Ca}$) (Walsh and Byrne, 1989), and modulates a voltage-dependent K^+ current ($G_{K,V}$) (Baxter and Byrne, 1989, 1990a; Goldsmith and Abrams, 1992; Hochner and Kandel, 1992; Sugita et al., 1994; White et al., 1994). The effects of channel modulation appear to be synergistic, favoring increased sensory neuron excitability or transmitter release. Spike broadening, which has a major impact on transmitter release, is probably due to modulation of $G_{K,V}$ rather than $G_{K,S}$, whereas $G_{K,S}$ and $G_{K,Ca}$ appear to be critical for regulating membrane excitability, with modest effects on spike duration (Baxter and Byrne, 1990a, b). The 5-HT-induced increase in $G_{Ca,Nif}$ does not appear to contribute to enhanced transmitter release, as this conductance is not directly responsible for triggering exocytosis of synaptic vesicles, although it may contribute to accumulation of presynaptic calcium during intense activity (Edmonds et al., 1990).

Two of the three 5-HT-induced effects on K^+ conductances (modulation of $G_{K,S}$ and $G_{K,Ca}$) are mediated exclusively by PKA. The effects of 5-HT on $G_{K,V}$ appear to be caused by activation of two second messenger pathways, only one of which is the cAMP pathway mentioned above (Hochner and Kandel, 1992). Serotonin also appears to act through another class of receptors to increase the level of the second messenger diacylglycerol (DAG). DAG activates protein kinase C (PKC), leading to its translocation (Sossin, 2007). PKC, like PKA, is involved in the spike-duration-dependent process of facilitation (Sugita et al., 1992, 1994). In addition, a nifedipine-sensitive Ca^{2+} conductance ($G_{Ca,Nif}$) and the delayed K^+ conductance ($G_{K,V}$) are regulated by PKC. The modulation of $G_{K,V}$ contributes importantly to the increase in duration of the action potential (**Figure 3(a3)**). Because of its small magnitude, the modulation of $G_{Ca,Nif}$ appears to play a minor role in the facilitatory process.

The role of ionic conductances in modulation of synaptic transmission is relatively well understood. Less well understood is a second process that has a profound effect on synaptic transmission, but which is independent of spike duration (spike-duration independent process; SDI). The existence of the second process was first postulated based on a mathematical model of a sensory neuron (Gingrich and Byrne, 1985, 1987). Experimental studies have provided support for the SDI process (Hochner et al., 1986; Braha et al., 1990; Pieroni and Byrne, 1992; Klein, 1993, 1994). Although the mechanism is poorly understood, the SDI process is likely to include mobilization of vesicles into a readily releasable pool. This process appears to be particularly important when the sensory neuron is depressed by previous low frequency (ISI = 10 s) stimulation (Braha et al., 1990; Ghirardi et al., 1992; Pieroni and Byrne, 1992; Klein, 1993; Sugita et al., 1997), making the SDI process an attractive candidate for dishabituation mechanisms and for maintaining synaptic strength during high levels of release (see below). The relative contribution of PKA and PKC to facilitation of previously depressed synapses varies as a function of the extent of preexisting depression. In nondepressed synapses, 5-HT produces short-term facilitation that can be blocked completely by inhibitors of PKA but is not affected by H7, an inhibitor of PKC. In contrast, as synapses become more depressed, the inhibitor of PKC becomes progressively more effective in blocking 5-HT-induced short-term facilitation (Braha et al., 1990; Ghirardi et al., 1992; Sugita et al., 1997).

Nevertheless, it has only recently become clear that, apart from dishabituation, sensitization may also involve facilitation of depressed synapses, because a moderate stimulus does not trigger a single spike in the sensory neuron, but a burst of spikes (Phares et al., 2003). By the end of this burst, the motor neuron responses depress substantially, regardless of the state of the first response. Enhancement of these depressed

responses by sensitization is likely to involve the SDI process.

Progress has been made in understanding the second-messenger cascades involved in the SDI process and identified synaptic terminal proteins as downstream targets. These targets include synapsin and SNAP-25, highly conserved synaptic proteins that appear to regulate homosynaptic depression and short-term heterosynaptic facilitation. Synapsin is localized in presynaptic nerve terminals, where it interacts with synaptic vesicles, actin and spectrin (Jovanovic et al., 1996; Matsubara et al., 1996; Hosaka et al., 1999; Zimmer et al., 2000). Because the interaction of synapsin with actin and synaptic vesicles is regulated by phosphorylation, synapsin is believed to reversibly tether synaptic vesicles in a reserve pool, thereby regulating vesicle availability and mobilization (Hilfiker et al., 1998, 1999). The *Aplysia* isoform of synapsin (apSyn) contains the same domain arrangement as other vertebrate and invertebrate synapsins (Angers et al., 2002). Several potential regulatory sites have been identified throughout the sequence of apSyn. In addition to the PKA/CAMK I consensus phosphorylation site in the A-domain, two potential MAPK sites and several PKC sites are detected. In ganglia and in cultured cells, synapsin localizes in presynaptic varicosities and forms distinct puncta, presumably due to the aggregation of protein and its interaction with vesicle membranes (Angers et al., 2002).

ApSyn is phosphorylated following application of 5-HT, which results in short-term facilitation of the sensorimotor synapse. This phosphorylation requires PKA and MAPK. Also, 5-HT results in a reduction in the number of apSyn puncta, which represents the dissociation of the protein from synaptic vesicles (and probably the cytoskeleton) upon phosphorylation. The reduction of apSyn puncta after 5-HT is dynamic and reversible, and it requires PKA and MAPK activity (Angers et al., 2002). Finally, recent results from apSyn overexpression experiments indicated that synapsin regulates basal synaptic strength, homosynaptic depression and 5-HT-induced recovery from depression (Fioravante et al., 2007).

Based on the results described above, the following model has been proposed (Angers et al., 2002): At rest, most vesicles are clustered in a filamentous protein network forming the reserve pool, and 5-HT can modulate the function of apSyn by altering its phosphorylation state via PKA and MAPK. Upon phosphorylation, apSyn molecules dissociate from the vesicles and the cytoskeleton, allowing vesicles to be mobilized to release sites when they become depleted.

Another highly conserved synaptic protein, SNAP-25, has recently been implicated in the regulation of short-term facilitation, especially in previously depressed synapses. In these synapses, PKC, rather than PKA, predominantly mediates 5-HT-induced dedepression (Ghirardi et al., 1992; Dumitriu et al., 2006) through phosphorylation of SNAP-25 (Houeland et al., 2007) and probably other, yet unidentified synaptic targets.

4.03.2.3.2 Long-term sensitization

Whereas short-term sensitization can be induced by a single brief stimulus, the induction of long-term sensitization, whose memory can persist for days to weeks, requires a more extensive training regime (e.g., repeating the sensitizing stimuli over a 1.5-h period). Compared with short-term sensitization, less is known about the cellular mechanisms underlying long-term sensitization. One simplifying hypothesis is that the mechanisms underlying the expression of long-term sensitization are the same as those of short-term sensitization, but extended in time. Some evidence supports this hypothesis. For example, similar to short-term sensitization, K^+ conductances and excitability of sensory neurons are also modified by long-term sensitization (Scholz and Byrne, 1987; Cleary et al., 1998).

Biophysical properties of neurons mediating the *Aplysia* withdrawal reflexes have been examined following long-term sensitization induced by a single 1.5-h-long training session (1-day protocol), or by four such sessions repeated at 24-h intervals (4-day protocol). Twenty-four hours following the 1-day protocol of long-term sensitization training, three biophysical properties of tail sensory neurons are altered: Neuronal excitability, the after-depolarization following long current pulses, and the after-depolarization following short current pulses (Cleary et al., 1998). In addition to the biophysical properties of sensory neurons, 1-day training affects two properties of motor neurons: The resting membrane potential is increased and the spike threshold is decreased (Cleary et al., 1998). Long-term sensitization also correlates with facilitation of the sensorimotor synapses, both after 1-day training and after 4-day training (Frost et al., 1985; Cleary et al., 1998; Wainwright et al., 2004; Antzoulatos and Byrne, 2007). Surprisingly, although short-term sensitization is associated with spike broadening in sensory neurons (see above), long-term sensitization is associated with spike narrowing in sensory neurons (Antzoulatos and Byrne, 2007). The functional effects of the spike narrowing are not clear,

but narrowing of the spike may be related to a decrease in spike propagation failures that occurs in response to high-frequency peripheral stimulation after long-term sensitization.

Another branch of the research on long-term sensitization has focused on the morphological effects of sensitization training on sensory neurons. One-day training for long-term sensitization does not induce gross structural changes in sensory neuron morphology, even though it does induce long-term changes in excitability and synaptic strength (Cleary et al., 1998). In contrast to 1-day training, 4-day training is more effective at inducing outgrowth when compared to untrained controls (Bailey and Chen, 1983; Wainwright et al., 2002;, 2004). This outgrowth includes an increase in the total arborization length of sensory neuron branches, in the number of sensory neuron branch points and varicosities, and in the number of synaptic contacts between sensory and motor neurons (Wainwright et al., 2002, 2004).

Biochemical correlates of long-term sensitization have also been examined. Most of these studies have focused on the induction phase, identifying changes in levels of the second messenger cAMP and regulation of several proteins (Barzilai et al., 1989; Eskin et al., 1989; Muller and Carew, 1998; Zwartjes et al., 1998). Progress has been made in identifying biochemical changes related to the consolidation or expression of long-term sensitization. Recently, long-term training was observed to produce enhanced uptake of glutamate (Levenson et al., 2000), the putative transmitter of sensory neurons (Antzoulatos and Byrne, 2004). This increase in uptake occurred in sensory neurons and appeared to be caused by an increased number of glutamate transporters. Although the functional role of this enhanced uptake is presently unclear, it indicates that clearance of glutamate from the cleft may be an important factor in the regulation of synaptic efficacy (Chin et al., 2002b). A change in glutamate uptake could potentially exert a significant effect on synaptic efficacy by regulating the amount of transmitter available for release, the rate of clearance from the cleft, and thereby the duration of the EPSP and the degree of receptor desensitization (Antzoulatos et al., 2003).

A substantial amount of data indicates that the induction of both short- and long-term sensitization partly share common cellular pathways (**Figure 4**). For example, both forms of sensitization activate the cAMP/PKA cascade. In the long-term form, however, activation is prolonged and sufficient to induce gene transcription and new protein synthesis (Castellucci et al., 1989; Levenson et al., 1999). This finding is consistent with the relatively long duration of the training period required for inducing long-term sensitization and the lasting duration of the effects. cAMP presumably exerts its major effects by activation of PKA (Schacher et al., 1988; Scholz and Byrne, 1988; O'Leary et al., 1995; Muller and Carew, 1998). Activated PKA translocates to the nucleus,

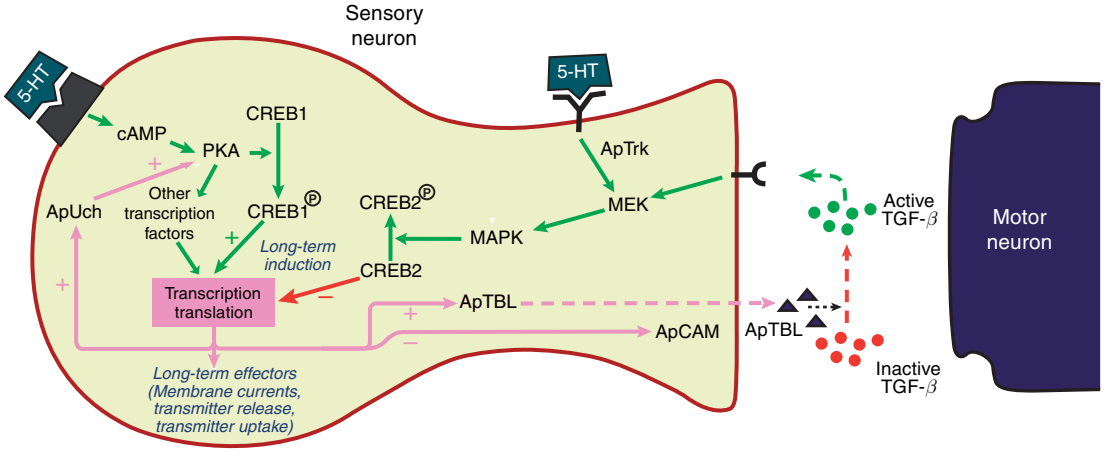


Figure 4 Simplified scheme of the mechanisms in sensory neurons that contribute to long-term sensitization. Sensitization training leads to release of 5-HT, which activates the cAMP/PKA cascade and the ERK MAPK cascade. PKA phosphorylates and activates CREB1, whereas ERK phosphorylates and inhibits CREB2. CREB1 acts as an initiator of gene transcription and CREB2 acts as a repressor of gene transcription. The combined effects of activation of CREB1 and inhibition of CREB2 lead to regulation of the synthesis of at least ten proteins, only three of which (apTBL, apUCH, apCAM) are shown. ApTBL is believed to activate latent forms of TGF-β, which can then bind to receptors on the sensory neuron and further activate MAPK. See section 4.03.2.3.2 for abbreviation definitions.

where it phosphorylates and activates the transcription factor CREB1. Activated CREB1, which is necessary for long-term facilitation (LTF), binds to the promoter region of responsive genes, and induces their expression (Dash et al., 1990, 1991; Bartsch et al., 1998; Guan et al., 2002). A prolonged increase in cAMP also leads to the induction and subsequent expression of a gene encoding the protein ubiquitin C-terminal hydrolase (Ap-Uch). This neuron-specific enzyme enhances the degradation of certain proteins including the regulatory subunits of PKA (Hegde et al., 1997). With fewer regulatory subunits of PKA to bind to catalytic subunits, the catalytic subunits are persistently active and may contribute to long-term facilitation of transmitter release (Muller and Carew, 1998).

In addition to the cAMP/PKA cascade, sensitization training and prolonged 5-HT application also activate MAPK (Sacktor and Schwartz, 1990; Sossin and Schwartz, 1992, 1993; Sossin et al., 1994; Sossin and Schwartz, 1994; Martin et al., 1997a; Sharma et al., 2003; Sharma and Carew, 2004). Prolonged application of 5-HT results in persistent phosphorylation (and subsequent activation) of MAPK though activation of a tyrosine receptor kinase-like molecule (ApTrk) (Ormond et al., 2004), cAMP (Martin et al., 1997b; Michael et al., 1998) (but see Dyer et al., 2003), and/or the neuropeptide sensorin (Hu et al., 2004b). Activated MAPK translocates to the nucleus (Martin et al., 1997b) where it may regulate gene transcription, possibly through inhibition of the transcription factor CREB2 (Bartsch et al., 1995). Since under basal conditions CREB2 acts as a repressor of gene transcription, its inhibition may lead to derepression and net gene expression in concert with CREB1 (**Figure 4**). The involvement of additional transcription factors such as ApAF (*Aplysia* activating factor) (Bartsch et al., 2000; Lee et al., 2006) and ApLLP (*Aplysia* LAPS18-like protein) (Kim et al., 2003a, 2006) in learning-induced gene expression is currently being investigated.

Recently, it has become clear that the role of transcription factors in long-term memory formation is not limited to the induction phase but may also extend to the consolidation phase. For example, prolonged treatment with 5-HT leads to the binding of CREB1 to the promoter of its own gene and induces CREB1 synthesis (Mohamed et al., 2005). The newly synthesized CREB1 appears to be necessary for LTF (Liu et al., 2008). This observation agrees well with earlier findings that the requirement for gene expression is not limited to the induction phase. The necessity of prolonged transcription and translation for LTF observed at 24 h persists for at least 7–9 h after induction (Alberini et al., 1994; O'Leary et al., 1995). These results suggest that CREB1 can regulate its own level of expression, giving rise to a CREB1 positive feedback loop that is necessary for memory consolidation.

In addition to CREB1, several other proteins are regulated during LTF. One of the newly synthesized proteins, intermediate filament protein (IFP) (Noel et al., 1993), is thought to contribute to the new growth observed after prolonged treatment with 5-HT. Increased synthesis of calmodulin (CaM) (Zwartjes et al., 1998) also occurs, but the functional significance of this effect has not been determined. The neuropeptide sensorin is also upregulated by 5-HT and is thought to contribute to the formation and stabilization of new synapses (Hu et al., 2004a,b).

Aplysia tolloid/BMP-like protein (apTBL-1) (Liu et al., 1997) is also synthesized in response to increases in cAMP. Tolloid and the related molecule BMP-1 appear to function as secreted Zn^{2+} proteases. A signal sequence at the amino terminal indicates that apTBL-1 is secreted to the extracellular space where one of its actions may be to activate members of the TGF-β family of growth factors (**Figure 4**). Indeed, in sensory neurons, TGF-β mimics the effects of 5-HT in that it produces long-term increases in synaptic strength and excitability of the sensory neurons (Zhang et al., 1997; Farr et al., 1999). Interestingly, TGF-β activates the MEK/MAPK pathway in the sensory neurons and induces MAPK translocation to the nucleus (Chin et al., 2002a, 2006), where it phosphorylates CREB1 (Chin et al., 2006). This activation could yield another round of protein synthesis to further consolidate long-term sensitization.

LTF involves not only increased synthesis but also downregulation of proteins such as the regulatory subunit of PKA (discussed earlier in this section) and a homolog of neuronal cell adhesion molecule (NCAM). Downregulation of NCAM alters the interaction of the neuron with other cells and allows the restructuring of the axon arbor (Mayford et al., 1992; Bailey et al., 1997). The sensory neuron could then form additional connections with the same postsynaptic target or make new connections with other cells.

4.03.2.3.3 Other temporal domains for the memory of sensitization

Operationally, memory has frequently been divided into two temporal domains, short-term and long-term. It has become increasingly clear from studies of a number of memory systems that this distinction

is overly restrictive. For example, in *Aplysia*, Carew and his colleagues (Sutton et al., 2001) and Kandel and his colleagues (Ghirardi et al., 1995) discovered an intermediate phase of memory that has distinctive temporal characteristics and a unique molecular signature. The intermediate-phase memory (ITM) for sensitization is expressed approximately 30 min to 3 h after the beginning of training. It declines completely prior to the onset of long-term memory. Like long-term sensitization, its induction requires protein synthesis, but unlike long-term memory, it does not require mRNA synthesis. The expression of the intermediate-phase memory requires the persistent activation of PKA (Muller and Carew, 1998; Sutton and Carew, 2000; Sutton et al., 2001).

An intermediate-term facilitation (ITF) of the sensorimotor synapse, which is produced by application of five pulses of 5-HT (an analog of sensitization training), corresponds to the ITM as it displays similar temporal dynamics and requires protein synthesis but not RNA synthesis (Ghirardi et al., 1995; Sutton and Carew, 2000). This latter feature of ITF distinguishes it from short-term facilitation, which requires neither protein nor RNA synthesis, and long-term facilitation, which requires both (see above). Depending on the induction protocol, ITF may require intermediate-term activation of PKA or PKC (Sossin et al., 1994; Sutton and Carew, 2000; Pepio et al., 2002; Lim and Sossin, 2006). These kinases can be activated for hours following prolonged treatment with 5-HT (Muller and Carew, 1998; Sutton and Carew, 2000). Finally, activation of previously silent release sites has also been implicated in ITF and could be a mechanism for memory consolidation (Kim et al., 2003b).

In addition to the intermediate-phase memory, it is likely that *Aplysia* has different phases of long-term memory. For example, at 24 h after sensitization training there is increased synthesis of a number of proteins, some of which are different from those whose synthesis is increased during and immediately after training (Noel et al., 1993). These results suggest that the memory for sensitization that persists for more than 24 h may be dependent on the synthesis of proteins occurring at 24 h and may have a different molecular signature than the 24-h memory.

Based on the experimental results reviewed above, a synthesis of the sensitization mechanisms in *Aplysia* can now be attempted. A brief sensitizing experience can affect the animal transiently (i.e., short-term sensitization), through an increase in the excitability of sensory neurons and in the efficacy of sensory neuron synapses. Short-term facilitation is achieved through spike-broadening-mediated and spike-duration-independent increases in transmitter release. These modifications, lasting up to several minutes, are mediated by phosphorylation of K^+ channels and other effector molecules, such as synapsin. More prolonged training, which typically involves multiple, appropriately timed stages of sensitization, can lead to modifications lasting 24 h or more. A single day of sensitization training leads to persistent increases in the efficacy of sensory neuron synapses and in the excitability of sensory neurons. Long-term facilitation after a single day of training does not involve gross structural changes of sensory neurons or changes in the number of synaptic varicosities. With 4 days of training, long-term sensitization is still accompanied by synaptic facilitation, but changes in sensory neuron excitability are not as prominent as they are after short-term training or after a single session of long-term training. After 4 days of training, long-term synaptic facilitation is achieved through an increase in the number of synaptic contacts. Collectively, these results indicate that facilitation of sensory neuron synapses is a ubiquitous feature of sensitization. However, the mechanisms that support the facilitation vary over time and with the extent of training. The conversion of one type of long-term expression mechanism to another is interesting, as it presumably reflects the engagement of a distinct set of genes that are part of an overall program for the expression of particularly enduring forms of long-term memory.

4.03.3 Habituation and Sensitization in Other Invertebrates

4.03.3.1 Gastropod Molluscs

4.03.3.1.1 Tritonia

To escape a noxious stimulus, the opisthobranch *Tritonia diomedea* initiates stereotypical oscillatory swimming. This escape swim can be dissected into several components, including number of cycles per swim, latency to swim onset, and swim cycle period. The various swim components can exhibit habituation, dishabituation, and/or sensitization (Frost et al., 1996). In particular, the escape swim undergoes habituation and dishabituation of the number of cycles per swim (Mongeluzi and Frost, 2000). Swimming probability can also decrease as a result of habituation (Brown,

1998). This habituation is accompanied by sensitization of the latency to swim onset (Frost et al., 1998).

The neural circuit underlying swim consists of sensory neurons, precentral pattern generating (CPG) neurons, and motor neurons. This circuit can be studied in the isolated perfused brain of *Tritonia*, where electrical stimulation of a nerve can elicit fictive swimming patterns (Dorsett et al., 1973). Habituation of fictive swimming correlates with a decrease in the cycle number and cycle period of swim motor programs (Frost et al., 1996; Brown, 1997) and appears to involve plasticity at multiple loci, including decrement at the first afferent synapse. Sensitization appears to involve enhanced excitability and synaptic strength in one of the CPG interneurons. Modulation of interneurons can be mediated by 5-HT, which has diverse effects on multiple loci of the circuit (Sakurai et al., 2006).

4.03.3.1.2 *Land snail* (Helix)

Land snails withdraw in response to weak tactile stimulation. The withdrawal behavior is mediated by a neuronal circuit involving four groups of nerve cells: Sensory neurons, motor neurons, modulatory neurons, and command neurons (Balaban, 2002). This withdrawal can be habituated or sensitized, depending on the intensity of stimulation. Habituation of the withdrawal behavior emerges from depletion of neurotransmitter at sensory cell synapses as well as heterosynaptic inhibition mediated by FMRFa-containing neurons (Balaban et al., 1991). Sensitization appears to be mediated by serotonergic modulatory cells whose spiking frequency increases following noxious stimulation (Balaban, 2002). These serotonergic cells are electrically coupled so that they get recruited and fire synchronously in response to strong excitatory input. One gene that is upregulated by external noxious input is the *Helix* Command Specific #2 (HCS2) (Balaban et al., 2001). The *HCS2* gene encodes a precursor protein whose processed products may function as neuromodulators or neurotransmitters mediating the withdrawal reactions of the snail (Korshunova et al., 2006). Application of neurotransmitters and second messengers known to be involved in withdrawal behavior result in upregulation of *HCS2* gene (Balaban, 2002).

The mechanisms underlying habituation and sensitization in the *Helix* can be further investigated by reconstructing behaviorally relevant synapses in culture. Using this approach, mechanosensory neuron-withdrawal interneuron synapses were found to display several forms of short-term synaptic plasticity such as facilitation, augmentation, and posttetanic potentiation (Fiumara et al., 2005).

4.03.3.2 Arthropods

4.03.3.2.1 *Crayfish* (Procambarus clarkii)

A crayfish escapes from noxious stimuli by flipping its tail. A key component of the tail-flip circuit is a pair of large neurons called the lateral giants (LGs), which run the length of the animal's nerve cord. The LGs are the decision and command cells for the tail-flip. The crayfish tail-flip response exhibits habituation (Wine et al., 1975) and sensitization (Krasne and Glanzman, 1986). Plastic changes induced during learning involve modulation of the strength of synaptic input driving the LGs (Edwards et al., 1999). A diminution of transmitter release with repeated activation of afferents is thought to underlie habituation (Krasne and Roberts, 1967; Zucker, 1972). An inhibitory pathway was also identified that can tonically inhibit the LGs (Krasne and Wine, 1975; Vu and Krasne, 1992, 1993; Vu et al., 1993). This putatively GABAergic (Vu and Krasne, 1993) (but see Heitler et al., 2001) inhibitory pathway also plays a major role in habituation (Krasne and Teshiba, 1995). In addition to the regulation of synaptic strength, habituation also results in decreased excitability of LGs (Araki and Nagayama, 2005). Bath application of the endogenous neuromodulators 5-HT and octopamine decrease the rate of LG habituation to repetitive sensory stimulation (Araki et al., 2005). Octopamine is also thought to at least partly mediate sensitization, because it mimics the sensitizing effects of strong stimulation on the tail-flip (Glanzman and Krasne, 1986; Krasne and Glanzman, 1986).

4.03.3.2.2 *Honeybee* (Apis mellifera)

Honeybees, like other insects, are superb at learning. For example, classical conditioning of feeding behavior can be produced by pairing a visual or olfactory stimulus with sugar solution to the antennae. Numerous studies described the molecular mechanisms underlying memory formation, which involve upregulation of the cAMP pathway and activation of PKA resulting in CREB-mediated transcription of downstream genes (Menzel, 2001) (*See* Chapter 4.06). Nonassociative learning has also been studied in the honeybee, albeit to a lesser extent. Habituation of the proboscis extension reflex can be elicited by repeatedly touching one antenna with a droplet of sugar water (Braun and Bicker, 1992) and lasts for at least 10 min (Bicker and Hahnlein, 1994). Following habituation, the proboscis

extension response can be restored spontaneously with time (spontaneous recovery) (Bicker and Hahnlein, 1994) or by stimulating the contralateral antenna (dishabituation) (Braun and Bicker, 1992). Application of tyramine, a metabolic precursor of the endogenous neuromodulator octopamine, accelerates the rate of habituation of the reflex (Braun and Bicker, 1992). Recently, activation of PKA was implicated in habituation of the reflex, but not dishabituation or spontaneous recovery, suggesting that the cellular mechanisms mediating habituation, dishabituation, and spontaneous recovery are distinct (Muller and Hildebrandt, 2002). With repeated training sessions over 2 days, long-term memory for habituation lasting for 24 h can be demonstrated (Bicker and Hahnlein, 1994). Finally, sensitization of the antenna reflex can be produced as a result of presenting gustatory stimuli to the antennae (Mauelshagen, 1993; Menzel et al., 1999).

4.03.3.2.3 Drosophila melanogaster

Because the neural circuitry in the fruit fly is both complex and inaccessible, the fly might seem to be an unpromising subject for studying the neural basis of learning. However, the ease with which genetic studies are performed compensates for the difficulty in performing electrophysiological studies (DeZazzo and Tully, 1995). A multitude of behaviors have been used as a model system to study nonassociative learning in *Drosophila*, including proboscis extension (Duerr and Quinn, 1982), thoracic bristle-elicited cleaning reflex (Corfas and Dudai, 1989), landing response (Rees and Spatz, 1989; Asztalos et al., 1993), jump-and-flight escape response (Engel and Wu, 1996), and odor-elicited startle response (Cho et al., 2004). Habituation has been demonstrated in all of these behaviors and molecular pathways underlying this form of learning have been identified and include the cAMP/PKA pathway (dunce and rutabaga mutants) (Duerr and Quinn, 1982; Corfas and Dudai, 1989; Engel and Wu, 1996; Cho et al., 2004), protein phosphatase 1 (Asztalos et al., 1993), and cGMP-dependent protein kinase (PKG) (Engel et al., 2000). In flies carrying the rutabaga mutation, which leads to diminished cAMP synthesis, habituation of the cleaning reflex is abnormally short-lived but dishabituation is unaffected (Corfas and Dudai, 1989). Moreover, in flies carrying the dunce mutation, which results in elevated cAMP levels, habituation rates of the jump-and-flight reflex are moderately increased but spontaneous recovery and dishabituation are not affected (Engel and Wu, 1996). These results reinforce the idea that the processes underlying habituation, dishabituation, and spontaneous recovery are distinct (also see the section titled 'Honeybee (*Apis mellifera*)'). Finally, in the dunce and rutabaga flies, sensitization of the proboscis-extension reflex dissipates more rapidly compared to wild-type controls (Duerr and Quinn, 1982).

4.03.3.3 Annelids

4.03.3.3.1 Leech

In the leech *Hirudo medicinalis*, nonassociative learning has been studied in several well characterized behaviors: Movements in response to light and water currents (Ratner, 1972), bending (Lockery and Kristan, 1991), shortening reflex to repeated light (Lockery et al., 1985) or tactile stimulation (Belardetti et al., 1982; Boulis and Sahley, 1988; Sahley et al., 1994), and swimming (Catarsi et al., 1993; Zaccardi et al., 2001).

In the shortening reflex of the leech, the neuronal changes underlying habituation and sensitization occur in the pathway from mechanosensory neurons to electrically coupled neurons, the S cells (Bagnoli and Magni, 1975; Sahley et al., 1994). Habituation of this reflex can reach asymptotic levels after 20 training trials and correlates with decreased S-cell excitability (Burrell et al., 2001). The reflex can be restored following application of a single noxious stimulus (dishabituation) (Boulis and Sahley, 1988). The potentiation of the shortening reflex observed during sensitization requires the S neurons, as their ablation disrupts sensitization (Sahley et al., 1994). This potentiation is mediated by 5-HT through an increase of cAMP (Belardetti et al., 1982), which also increases S-cell excitability (Burrell et al., 2001). Depletion of 5-HT disrupts sensitization (Sahley et al., 1994). Interestingly, ablation of the S cells only partly disrupts dishabituation, indicating that separate processes contribute to dishabituation and sensitization (Ehrlich et al., 1992; Sahley et al., 1994).

An additional mechanism that could potentially contribute to habituation of the shortening reflex involves depression of the synapses of touch (T) sensory neurons onto their follower target neurons. This synaptic depression has been associated with an increase in the amplitude of the T-cell after-hyperpolarizing potential (AHP) that follows their discharge (Brunelli et al., 1997; Scuri et al., 2002). The lasting increase in AHP amplitude, following low-frequency stimulation of T cells, has been attributed, in turn, to increased activity of the electrogenic Na^+ pump, and requires activation of phospholipase A2 and the downstream arachidonic acid metabolites (Scuri et al., 2005).

4.03.3.4 Nematoda

4.03.3.4.1 Caenorhabditis elegans

C. elegans is a valuable model system for cellular and molecular studies of learning. Its principal advantages are threefold. First, its nervous system is extremely simple. It has a total of 302 neurons, the anatomical connectivity of which has been described at the electron microscopy level. Second, the developmental lineage of each neuron is completely specified. Third, its entire genome has been sequenced, making it highly amenable to a number of genetic and molecular manipulations. *C. elegans* responds to a vibratory stimulus applied to the medium in which they locomote by swimming backwards. This reaction, known as the tap withdrawal reflex, exhibits habituation, dishabituation, sensitization, and long-term (24-h) retention of habituation training (Rankin et al., 1990). Laser ablation studies have been used to elucidate the neural circuitry supporting the tap withdrawal reflex and to identify likely sites of plasticity within the network. Plastic changes during habituation appear to occur at the chemical synapses between presynaptic sensory neurons and postsynaptic command interneurons (Wicks and Rankin, 1997). Analysis of several *C. elegans* mutants has revealed that synapses at the locus of plasticity in the network may be glutamatergic (Rose and Rankin, 2001). Mutation of the gene coding for the brain-specific inorganic phosphate transporter *eat-4* results in more rapid habituation compared to wild-type worms and slower recovery (Rankin and Wicks, 2000). The protein coded by *eat-4* is involved in the regulation of glutamatergic transmission and is homologous to the mammalian vesicular glutamate transporter VGLUT1 (Bellocchio et al., 2000). *Eat-4* worms also do not display dishabituation, suggesting that neurotransmitter regulation plays a role in habituation and dishabituation (Rankin and Wicks, 2000). Moreover, worms that carry a mutation in glr-1, an excitatory glutamate receptor expressed in postsynaptic command interneurons, do not display long-term memory for habituation (Rose et al., 2003). In general, the study of behavioral genetics in the worm has provided significant insights into the ways in which genes regulate behavior (Rankin, 2002).

4.03.4 Emerging Principles

As a result of research on several invertebrate model systems, some general principles have emerged. A list of these principles might include the following: (1) short-term and long-term forms of learning and memory require changes in existing neural circuits, (2) these changes may involve multiple cellular mechanisms within single neurons, (3) second messenger systems play a role in mediating cellular changes, (4) changes in the properties of membrane channels are commonly correlated with learning and memory, (5) changes in intrinsic excitability (*See* Chapter 4.40) and synaptic efficacy are correlated with short- and long-term memory, and (6) long-term memory requires new protein synthesis and growth, whereas short-term memory does not.

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4.04 Molecular Mechanisms of Habituation in *C. elegans*

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4.04.1 Introduction to Habituation

The most basic form of learning is nonassociative learning, which involves alterations in response to a single (sometimes repeated) stimulus. Habituation, dishabituation, and sensitization are the three main forms of nonassociative learning. Habituation is the simplest form of nonassociative learning and has been defined as a decrease in response to repeated or long-lasting stimulation (Groves and Thompson, 1970). This form of learning has been found in all organisms studied, from protozoa to humans. If a strong, novel stimulus is presented after the organism has been habituated to the initial stimuli, the organism will immediately recover its habituated response. This phenomenon is known as dishabituation and has been used to distinguish habituation from sensory adaptation or fatigue. Although a great deal is known about the characteristics of habituation, very little is known about the molecular mechanisms that underlie it.

Habituation allows an organism to ignore irrelevant stimuli; thus it is the basis for selective attention. If organisms did not habituate, then they would give equal attention to all stimuli in the environment and could not attend to stimuli important for survival. Further, the behavioral rules that govern habituation described by Groves and Thompson (1970) are followed in all species and systems studied. For these reasons, the mechanisms underlying this simple form of learning are likely to be highly conserved throughout evolution. To accurately study such a simple form of learning, it becomes increasingly important to reduce any other factors that could confound such study. Because of this, organisms that exhibit very simple behaviors and that can easily be studied at the cellular and genetic levels provide good model systems to discover the molecular mechanisms involved in habituation.

4.04.2 *Caenorhabditis elegans* as a Model System

Caenorhabditis elegans is a very powerful and useful model system in which to study molecular mechanisms of simple forms of learning and memory. Rankin et al. (1990) first showed that *C. elegans* are capable of a variety of simple behaviors including habituation, dishabituation, and long-term memory lasting for at least 24 h. This nematode has a small nervous system that is

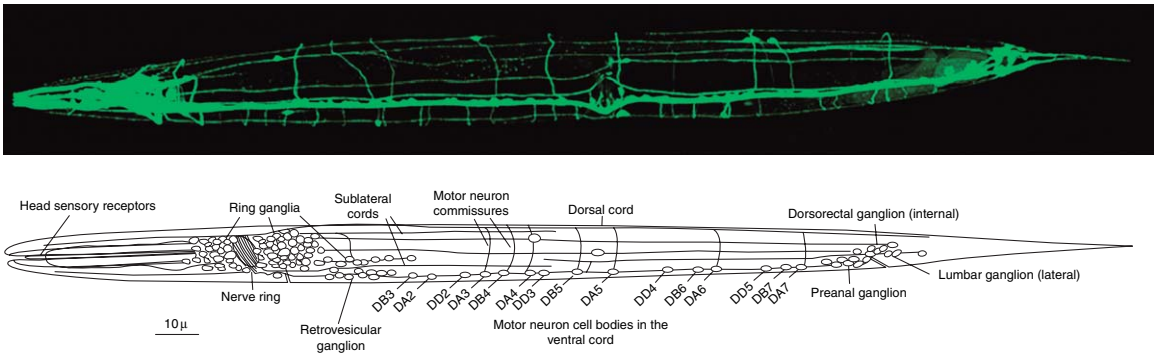


Figure 1 The nervous system of *C. elegans*. Top: *C. elegans* nervous system labeled with a pan-neuronal GFP (photo courtesy of W. Materi and D. Pilgrim). Worm is 1 mm in length. Bottom: Anatomical drawing of the nervous system of *C. elegans*. Drawing courtesy of Richard Durbin.

composed of only 302 neurons, which allows for the unique ability to map behaviors to specific identified neurons and sites of plasticity (**Figure 1**). The connectivity of the nervous system has been resolved at the electron microscope level and has produced a complete wiring diagram indicating 5000 chemical synapses and 3000 electrical synapses (White et al., 1986). Further, the entire cell lineage has been investigated and determined. Combining this knowledge, researchers have been able ablate single identified neurons and investigate the behavioral outcome (Chalfie et al., 1985). Also, since the cuticle of this nematode is transparent, the use of laser ablation of single, identified neurons has made circuit analysis possible, and the use of genetic markers such as green fluorescent protein (GFP) has allowed the analysis of changes in expression patterns of specific gene products *in vivo* (Chalfie et al., 1994). The worm's genome has been mapped and sequenced, which has led to the identification of numerous genes and gene products (Wood, 1988; Riddle et al., 1997). Also, the genetics of *C. elegans* is easy to manipulate. The use of both forward and backward genetic screens and, more recently, RNA interference techniques has led to the identification of a large number of genes that play a role in mediating various behaviors. Another major advantage of this model system is that it is relatively simple to manipulate gene expression spatially or temporally, allowing for the investigation of the roles of specific genes in targeted tissues and/or at specified time points.

4.04.3 Olfactory Habituation

The presentation of an olfactory stimulus can lead to a chemotaxic response, which is the migration toward or away from that stimulus. *C. elegans* will perform both of these behaviors; it is attracted to various ions, amines, and some volatile substances (ketones, esters, etc.), and it is repelled by acidic pH, D-tryptophan, and various volatile substances (benzaldehyde, octanol, etc.; Bargmann and Mori, 1997). Continuous or repeated presentation of such compounds can result in a decrement of the chemotaxic response (Colbert and Bargmann, 1995). Bernhard and Van der Kooy (2000) showed that this decrease in behavioral response could be mediated by two forms of olfactory plasticity: adaptation and habituation. Although adaptation can be considered a form of plasticity, it is not considered to be a form of learning because the response decrement is mediated by sensory fatigue, and the response will return to baseline levels only after sufficient time is allowed for the sensory system to recover. On the other hand, habituation to olfactory stimuli is considered a learning process because, although a similar response decrement occurs as with adaptation, when a novel or noxious stimulus is administered, dishabituation will occur.

Using solubilized Na^+ (an attractant ion), Wen et al. (1997) showed that olfactory habituation can occur in *C. elegans*. They showed that, when exposed to an attractant (75 $mmol^{-1}$ $NaCH_3COO$) for a prolonged time, the chemotaxic response to migrate toward that attractant diminished. But when worms were exposed to a much higher concentration of $NaCH_3COO$ (300 $mmol^{-1}$) for a brief period of time and then given the same behavioral assay, the habituated response returned to near baseline levels, indicating that dishabituation had occurred and that this behavioral decrement was habituation and not adaptation.

To investigate the differences between the processes of habituation and adaptation, Bernhard and Van der Kooy (2000) varied preexposure

concentrations of the volatile odorant diacetyl (DA) within a single paradigm. They found that preexposing and testing worms in high concentrations of DA induced a nonreversible decrement in chemotaxic response despite the introduction of a strong, novel stimulus. When preexposed to an intermediate concentration of DA, no decrement of response was observed. Interestingly, at very low concentrations of preexposure and testing, worms exhibited a decrement in response that could be dishabituated. Taken together, these data suggest that the processes of olfactory plasticity can be dissociated from habituation by the concentration of DA used, with adaptation requiring high concentrations of DA and habituation requiring low concentrations of DA.

Although a number of genes important for adaptation have been identified, little is known about the genetics of olfactory habituation. Wen et al. (1997) showed that mutant worms, *lrn-1* and *lrn-2,* had deficits in classical conditioning associative learning paradigms but showed no deficits in nonassociative habituation. However, more recently, Morrison and van der Kooy (2001) showed that a mutation in an alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate (AMPA)-type glutamate receptor subunit, *glr-1,* impaired both olfactory associative learning and habituation. These data suggest that the mechanisms involved in associative learning and habituation may be dissociable at the level of the *lrn-1* and *lrn-2* genes but share a common pathway that involves the *glr-1* gene.

4.04.4 Mechanosensory Habituation

4.04.4.1 Mechanosensation in *C. elegans*

Mechanosensation, which is the transduction of mechanical force into intracellular signals, allows organisms to sense touch, vibration, and other tactile stimuli. In *C. elegans,* mechanosensation has been studied in two major ways: first, touch to the body from gentle stimulation with a small hair and, second, vibration felt through the surrounding environment resulting from the administration of a mechanical tap delivered to the side of a Petri dish (Garcia-Anoveros and Corey, 1997). Most of the research on habituation has been done using the tap stimulus. The advantage of using the tap stimulus as opposed to the body touch when studying mechanosensory behavior is that the strength of stimulation can be controlled using a machine, whereas there is uncontrollable variation when delivering touch using a handheld device. In response to a tap stimulus, a worm will respond by swimming backward (a reversal), which has been termed the tap-withdrawal response.

4.04.4.2 Habituation

Using the tap-withdrawal response, Rankin et al. (1990) were the first to show that *C. elegans* is capable of nonassociative learning. When they administered a tap stimulus to the side of the Petri plate holding the worm, they observed that the distance that the worms reversed in response to the tap decreased when the stimulus was repeated at regular intervals. The response returned to baseline levels (spontaneous recovery) a few minutes following the last tap stimulus. To ensure that this response decrement was habituation and not sensory fatigue, they followed habituation training with a brief electrical shock in order to dishabituate the worms. Following shock, their response to tap increased significantly above the habituated level, indicating that the electrical shock had induced dishabituation, and the original response decrement observed was habituation and not adaptation or fatigue.

4.04.4.3 Behavioral Analyses of Short-Term Habituation

Thompson and Spencer (1966) and Groves and Thompson (1970) laid out the behavioral characteristics of habituation. These same criteria are used today to define habituation, and so far, all species studied show these same characteristics. Having a behavior well characterized is an asset when trying to determine underlying cellular mechanisms. It is important for researchers to make constant comparisons between behavior and the hypothesized cellular mechanisms in order to develop a greater understanding of the factors that govern habituation.

In their descriptions of habituation, Thompson and Spencer (1966) and Groves and Thompson (1970) missed one aspect of habituation that is common in all systems studied and can be used to find clues about possible molecular mechanisms of habituation. These early papers stated that habituation is sensitive to frequency, with high-frequency stimuli producing more rapid habituation than low-frequency stimuli, and that habituation recovers spontaneously. Both of these are correct; however, in all species studied, frequency also affects the rate of spontaneous recovery, with high-frequency

stimulation leading to more rapid spontaneous recovery than low-frequency stimulation (Rankin and Broster, 1992; **Figure 2**(**a**)). Rankin and Broster (1992) showed that in *C. elegans* this relationship of spontaneous recovery to frequency of stimulation held, regardless of the number of stimuli delivered (as long as decrement had reached asymptotic levels) and regardless of the level of habituation reached (when levels of habituation were matched between worms habituated with high and low frequencies, rate of recovery was still dependent on frequency of the habituation). This is important for two reasons. The first is that this difference is the opposite of what would be predicted by fatigue or adaptation in that, in both of those cases, the more complete the decrement the longer the recovery. With high frequency the decrement is rapid and often complete, but recovery is very rapid, while with low frequency the decrement is not complete and yet recovery takes much longer than for high-frequency stimulation. Thus, the sensitivity of spontaneous recovery to the frequency of stimulation is a second way, in addition to dishabituation, to distinguish whether a behavioral decrement is the result of habituation, or the result of sensory adaptation or motor fatigue. The second reason that the sensitivity of recovery to frequency of stimulation is important is the deductions one can draw from this about molecular

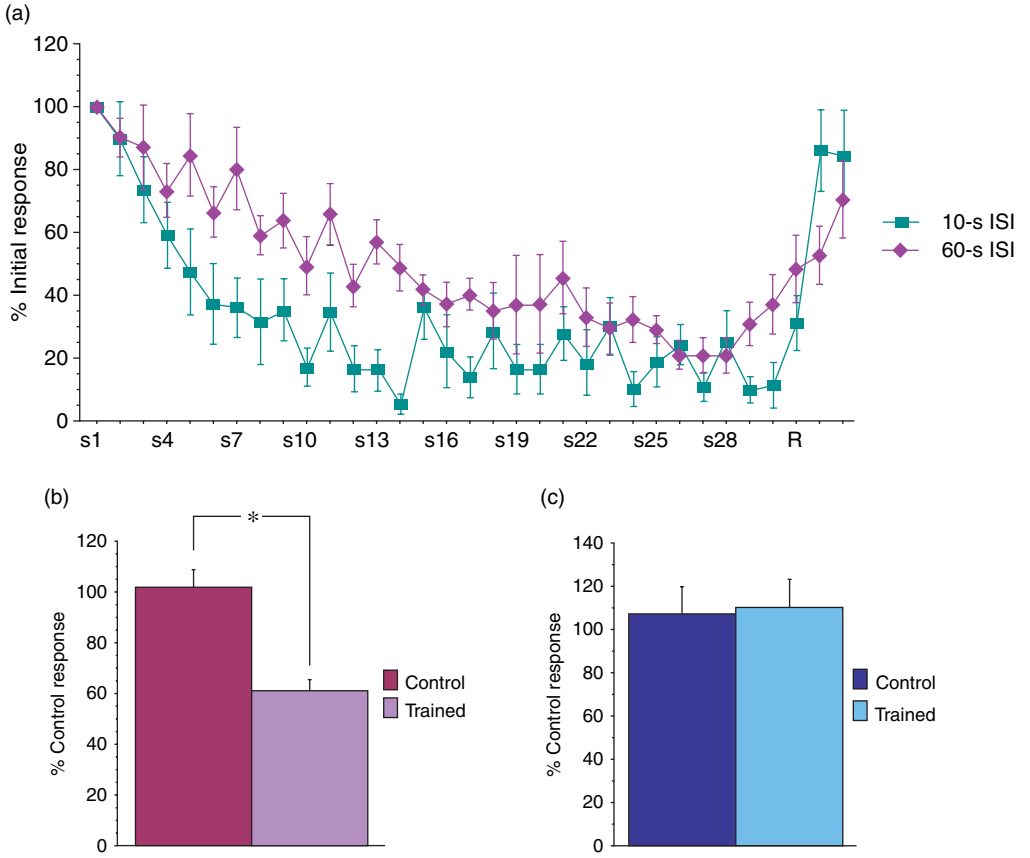


Figure 2 (a) Reversal responses of wild-type worms shown as the mean percent initial response across 30 tap stimuli with three recovery taps at 30 s, 5 min, and 10 min after habituation training. The 10-s interstimulus interval (ISI) group shows more rapid habituation and a lower asymptotic level when compared to the 60-s ISI group. The 10-s ISI group also shows faster and more rapid recovery following habituation training than the 60-s ISI group. (b) Mean percentage of control response magnitude for long-term memory for five test stimuli 24 h following habituation training given at a 60-s ISI. The significantly lower level of responding in the trained group compared to the control group indicates the retention of memory for habituation training. (c) Mean percentage of control response magnitude for long-term memory for five test taps 24 h following habituation training given at a 10-s ISI. The lack of memory observed when training is given at a 10-s ISI suggests either that 60-s ISI training selectively recruits molecular mechanisms needed to induce the formation of long-term memory for habituation training, or that 10-s ISI training recruits molecular mechanisms that block the formation of long-term memory (Butterfield and Rankin, unpublished results, 2006).

mechanisms of habituation. If an animal that has habituated to stimuli at a high frequency (i.e., short interstimulus intervals, ISIs) recovers rapidly, while an animal habituated to the same level to a low frequency recovers more slowly, this indicates that the two habituated animals are not the same, and some different processes have been activated in the neurons of the two animals to regulate recovery differently. From this observation Rankin and Broster (1992) hypothesized that habituation was not mediated by a single molecular mechanism, but that stimulation at different frequencies recruited different cellular mechanisms. The observation that long ISIs can be used to produce long-term memory for habituation, while short ISIs cannot, provides further support for this hypothesis (Beck and Rankin, 1997; **Figures 2**(**b**) and **2**(**c**)). Broster and Rankin (1994) hypothesized that, if different ISIs recruited different molecular mechanisms, then studies of genes involved in habituation should lead, at the very least, to the discovery of genes that play a role in habituation to all frequencies, genes that play a role in habituation to high frequencies, and genes that play a role in habituation to low frequencies. We have recently found support for this hypothesis and identified two genes that are involved in short-term habituation. One specifically affects habituation at high frequencies; the other specifically affects habituation at low frequencies (Rankin, unpublished data, 2006).

4.04.5 Neural Circuit

4.04.5.1 Identifying Neurons Involved in Habituation

Once the behavior was well characterized, the next step in discovering the molecular mechanisms involved in habituation to tap stimuli was to identify the neural circuit responsible for this behavior. The neural circuit underlying the behaviors of backward swimming in response to head touch and forward swimming in response to tail touch was characterized by Chalfie et al. (1985). Wicks and Rankin (1995) investigated the neural circuit underlying the tap-withdrawal response by studying the effects of laser ablating cells in the head and tail touch circuits on the response to tap and found that the response to tap involves integration of sensory input from both the head and tail. The neural circuit for the response to tap consists of five mechanosensory cells, two bilaterally paired PLM neurons that transduce tail touch, and 2 bilaterally paired ALM neurons and a single AVM neuron that transduce head touch; these sensory neurons synapse onto four pairs of command interneurons that mediate forward (AVAs and AVDs) and backward movement (AVBs and PVCs; **Figure 3**(**a**)). Ablation of all sensory cells completely abolished the response to tap (Wicks and Rankin, 1995). Ablation of only the head touch neurons (ALMs and AVM) resulted in consistent forward swimming in response to tap (termed an acceleration) in contrast to the consistent reversal responses seen in intact worms. Similarly, backward swimming was always seen in response to tap in PLM ablated (PLM–) worms. These reversals were larger than the reversals of intact animals, suggesting that in the intact worms the effect of stimulation of the tail cells by the tap competes with the effect of stimulation of the head touch cells and moderates the response size. Interestingly, ablation of AVM only resulted in a decrease in reversal frequency and magnitude of reversals and an increase in acceleration (forward swimming) frequency. In a study of the response to tap across development, it was observed that, at younger stages, worms responded by both reversing and accelerating at equal frequencies, similar to what is observed in AVM– worms (Chiba and Rankin, 1990). Since AVM is not present at hatching and only becomes fully functional in young adults, Chiba and Rankin (1990) suggested that the shift in adult worms to predominantly reversing response to tap may be mediated by the development of AVM. All these results combined indicated that the response to tap was mediated by the integration of inputs from two competing neural circuits, one driving forward movement and one driving backward movement.

4.04.5.2 Roles of Identified Neurons in Habituation

To identify the sites of changes in the pathway(s) underlying habituation of the tap-withdrawal response, Wicks and Rankin (1996b) laser ablated specific touch cells and observed any changes in habituation rate or asymptotic level. Laser ablation of the PLM sensory neurons results in consistent backward movement in response to tap. When given the short-term habituation training at a 10-s and 60-s ISI, PLM– worms showed habituation at both ISIs. The initial slope of the PLM– group was smaller than that of the intact group (**Figures 3**(**b**) and **3**(**c**)). When Wicks and Rankin (1996b) investigated the role of the touch cells of the anterior mechanosensory field (AVM and ALM), they found

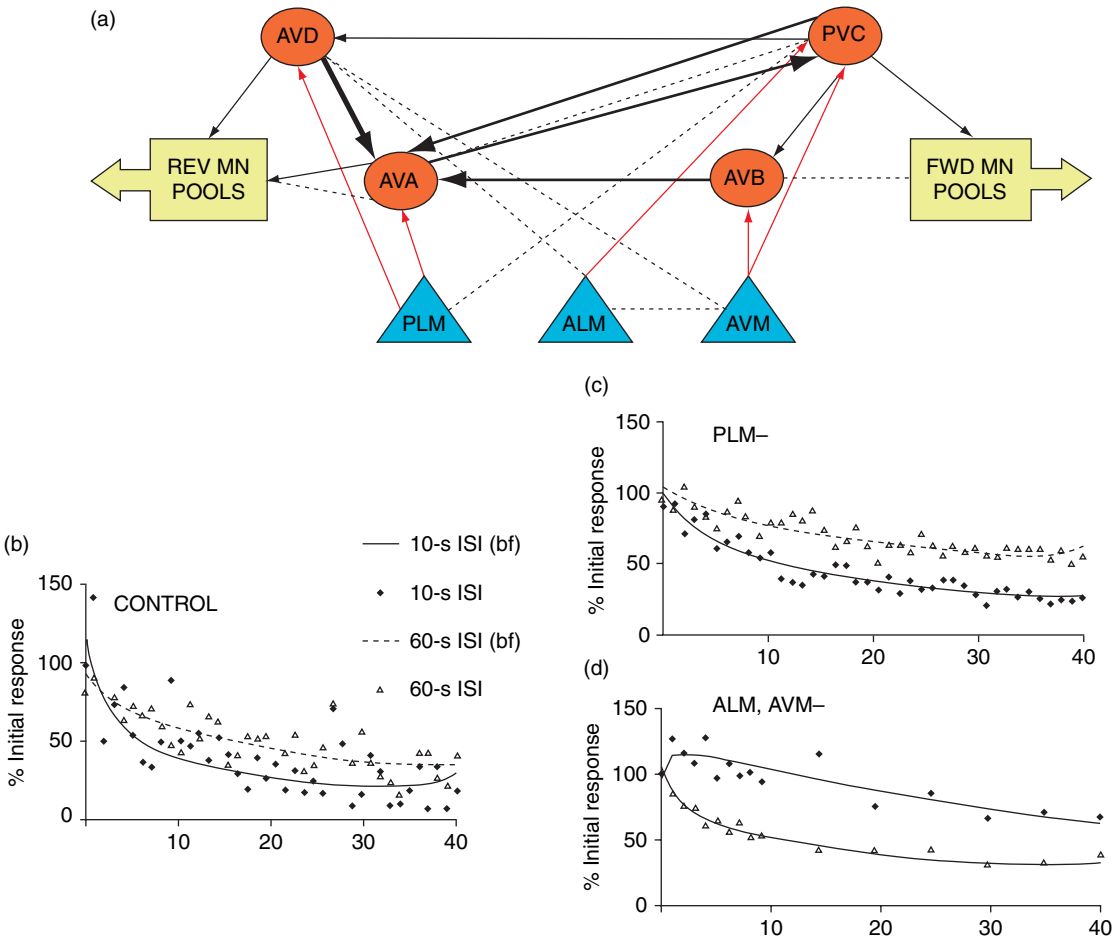


Figure 3 (a) Simplified neural circuit underlying tap withdrawal. The circuit consists of five mechanosensory neurons (*triangles*), eight command interneurons (*circles*), and two motor neuron pools (*squares*). All cells represent bilateral classes of cells except ALM, which is a single cell. The arrows and dotted lines represent chemical synapses and gap junctions, respectively. The number of synaptic contacts is proportional to the width of the arrows. The red-colored arrows indicate the synaptic connections that have been hypothesized to be the sites of plasticity that mediate habituation (Wicks and Rankin, 1997; Kitamura et al., 2001). (b, c) Scatter plot graphs fitted with best fit lines (bf) demonstrating the effect of ISI (10 s vs. 60 s) on the kinetics of habituation of the reversal response in intact animals (control) and PLM-ablated animals (PLM–). (d) Scatter plot graph showing the effects of ablation of ALM and AVM on the kinetics if habituation of acceleration responses. Wicks and Rankin (1996b) hypothesized that the responses of both the PLM– and AVM, ALM– groups combine to make up the behavior we see in the intact animal. Figure adapted from Wicks SR and Rankin CH (1996b). The integration of antagonistic reflexes revealed by laser ablation of identified neurons determines habituation kinetics of the *Caenorhabditis elegans* tap withdrawal response. *J. Comp. Physiol. A* 179: 675–685, with permission from Springer-Verlag.

results that were quite different from those of the PLM– group. Worms with ablations in the head sensory neurons accelerate forward in response to tap. They found that both ALM– and ALM, AVM– groups habituated slower at a 10-s ISI than at a 60-s ISI, and they showed an initial response facilitation prior to the decrement that was especially strong when habituated at a 10-s ISI (**Figure 3(d)**). Further, the ALM, AVM– group had a significantly higher asymptotic level at a 10-s ISI as opposed to the 60-s ISI. Finally, ablation of one of the pairs of command interneurons (PVCs), thought to modulate forward movement, resulted in worms that reversed 100% of the time; however, unlike the PLM– worms, the reversals seen in PVC– worms were not significantly different in magnitude from control worms. The results support the hypothesis of Chalfie et al. (1985) that the PLM cells make inhibitory chemical connections to the head touch circuit interneurons (AVD and AVA); these connections remain intact

following PVC ablation and continue to compete with the head sensory neuron stimulation. Taken together, the results of ablation of cells important in both the forward and backward motion pathways have shown that reversals and accelerations habituate at different rates; thus their relative contribution to the intact response varies over the course of habituation. For example, at a 10-s ISI the initial facilitation of the acceleration response competes more strongly with the reversals and the reversals seen in intact worms decrease in amplitude very quickly. This suggests that to understand the habituation of behavior it is not sufficient to study the mechanisms underlying the decrement in a single cell, but it may be necessary to understand the effect of repeated stimulation on all aspects of the neural circuits underlying the behavior.

4.04.5.3 Localizing the Site of Plasticity in the Neural Circuit

Two behaviors that share command interneurons and motor neuron pools with the tap-withdrawal response are thermal sensation and spontaneous reversing. All of these behaviors differ from tap at the level of sensory input. Wicks and Rankin (1997) hypothesized that, if the site of plasticity that mediates habituation is located at the sensory input level, then habituation to tap should not alter baseline levels of both the response to a heat probe and spontaneous reversing. On the other hand, if the site of plasticity is at the level of the interneurons and motor neurons, then these behaviors should be altered by habituation training in response to tap. Wicks and Rankin (1997) found that habituation to tap did not affect other behaviors, thus providing data to support the hypothesis that the site of plasticity lies in the touch cells and/or the synaptic connections they make onto the interneurons.

Investigation into habituation of anterior body touch has also led to increased knowledge of how habituation occurs in the neural circuit. Kitamura et al. (2001) performed gentle body touch using a hair at a 15-s ISI, using intact worms, and observed the expected kinetics of habituation with an initial, rapid decrement of reversal magnitude followed by an asymptotic level of that response. Through systematic ablations of combinations of neurons involved in the response to anterior body touch (ALM, AVM, AVD, and PVC), they found that laser ablation of both AVD interneurons resulted in significantly more rapid habituation than observed in intact animals. The ablation of any of the other interneurons had no effect on the rate of habituation, suggesting that the AVD interneurons play a critical role in the habituation to anterior body touch. Kitamura et al. (2001) also found that coablating the right ALM and the AVM neurons resulted in rapid habituation, suggesting that in these animals the chemical synapse that the left ALM makes with the right AVD and PVC interneurons is responsible for the behavior observed. Further investigation led to the conclusion that the chemical synapses between the left ALM sensory neurons and the PVC interneurons mediate the rapid habituation of the coablated worms. The fact that more rapid habituation can be attributed to at least two sites in the neural circuit suggests that each synapse in the circuit may have the potential to be the site of plasticity.

Taken together, the studies performed by both Wicks and Rankin (1995) and Kitamura et al. (2001) show that habituation to mechanosensory stimuli in intact animals involves the integration of a variety of inputs. However, the most likely site of plasticity appears to be situated at the level of the chemical synapses between the sensory neurons and their target interneurons.

4.04.6 Genetic Dissection of Short-Term Habituation

4.04.6.1 Role of Genes Involved in Glutamate Neurotransmission

A number of genes involved in glutamate neurotransmission are expressed in the touch cells and the command interneurons, suggesting that glutamatergic transmission plays a major role in the response to tap. Presynaptically, in the sensory neurons (ALM, AVM, and PLM) a homologue of a mammalian glutamate vesicular transporter, known as EAT-4, is expressed (Lee et al., 1999). Postsynaptically, in the command interneurons (AVA, AVB, AVD, and PVC) homologues of both the mammalian AMPA/Kainate-type and n-methyl-D-aspartate (NMDA) glutamatergic receptors, GLR-1 and NMR-1, respectively, are expressed (Hart et al., 1995; Maricq et al., 1995; Brockie et al., 2001). If glutamatergic transmission plays a critical role in habituation in *C. elegans,* then worms that lack, or have mutations in, one or more of these genes should have altered patterns of habituation.

Rankin and Wicks (2000) first examined *eat-4* mutants using the short-term habituation paradigm. Initially, they found that, when compared to

wild-type worms, there was no difference in the initial response to the tap stimulus. When given repeated stimulation, at both 10- and 60-s ISIs, *eat-4* worms habituated more rapidly and reached a lower asymptotic level than wild-type worms. Similarly, *eat-4* worms showed much slower spontaneous recovery following habituation training; however, the dependence on ISI was still present in both habituation and recovery kinetics (faster decrement and faster recovery from short ISI training as compared to long ISI training). This result suggests that the absence of EAT-4 disrupts one or more cellular mechanisms of habituation but leaves others (ISI dependent processes) intact. Interestingly, *eat-4* is also the first gene that has been shown to play a role in dishabituation. When given a dishabituating stimulus (an electric shock), the *eat-4* mutants did not show facilitation of the response above the habituated level, indicating that they did not dishabituate. Since *eat-4* worms still show ISI-dependent spontaneous recovery, the decrement seen in these worms is habituation and not fatigue or adaptation. Because we do not know the relationship between the molecular mechanisms of habituation and the molecular mechanisms of dishabituation, these results illustrate the importance of having more than a single way to distinguish habituation from fatigue. Using a transgenic rescue strain (DA1242) produced by Lee et al. (1999), Rankin and Wicks (2000) showed that wild-type habituation and dishabituation behaviors were also rescued. These studies with *eat-4* worms support the hypothesis that glutamatergic transmission plays an important role in habituation of the tap-withdrawal response and that the glutamate vesicular transporter is essential for dishabituation.

Because *eat-4* worms did not respond differently from wild-type in response to the initial tap stimuli but differed only after repeated stimuli, Rankin and Wicks (2000) concluded that EAT-4 (a glutamate vesicular transporter) is not required for glutamatergic transmission but, rather, is required for sustained synaptic activity. The hypothesis is that the touch sensory neurons of *eat-4* worms have fewer glutamate-filled vesicles than those of wild-type worms, and so they are quickly exhausted in response to repeated stimulation. The importance of neurotransmitter vesicles in habituation is supported by work in *Aplysia* that has shown that there are fewer synaptic vesicles in the active zones of sensory neurons from habituated animals than from the terminals of nonhabituated animals (Bailey and Chen, 1988). The regulation of the amount of glutamate in vesicles in the terminals of the sensory neurons and/or the regulation of vesicular release are the most likely mechanisms underlying the behavioral changes observed during habituation.

Since the deficits in presynaptic release of glutamate seen in *eat-4* worms alter the kinetics of habituation to tap, it was suggested that postsynaptic glutamate receptors should also play a significant role in the same behavior. Genes for four ionotropic glutamate receptors are expressed on the command interneurons: *glr-1, glr-2, nmr-1*, and *nmr-2* (Hart et al., 1995; Maricq et al., 1995; Brockie et al., 2001). *glr-1* and *glr-2* have been shown to form heteromeric receptors (Chang and Rongo, 2005), and *nmr-1* and *nmr-2* are thought to form heteromeric receptors as well. Studies to date have focused mainly on mutations in *glr-1* and *nmr-1*. When given habituation training, *glr-1* worms showed smaller initial responses to tap than wild-type animals but showed relatively normal short-term habituation to 10- and 60-s ISIs when compared to wild-type worms (Rose et al., 2002). *nmr-1* worms showed normal short-term habituation, indistinguishable from wild-type. The results thus far indicate that none of the glutamate receptor genes tested alone gives the same pattern as EAT-4–deficient worms, which suggests that habituation may be mediated postsynaptically by the activation of an, as yet, unidentified glutamate receptor or by the simultaneous activation of multiple glutamate receptors.

4.04.6.2 Other Identified Components of Habituation

Sanyal et al. (2004) showed that dopamine, which plays an important role in behavioral plasticity in many mammalian systems, also may play an important role in modulating *C. elegans* habituation. Sanyal et al. (2004) studied mutations in two genes involved in dopamine regulation and neurotransmission; *dop-1* mutants, which do not express a dopamine receptor on neurons involved in the mechanosensory circuit, show altered patterns of habituation compared to wild-type worms, as do *cat-2* mutants, which lack an enzyme that is required for dopamine synthesis. Sanyal et al. found that *dop-1* mutants and *cat-2* mutants had a more rapid decrement in reversal frequency (the number of worms that respond to each tap) than wild-type worms. However, when measuring reversal length (the dependent variable in all previous studies mentioned), there was no difference among all three groups. This result

suggests two alternative hypotheses: the first is that different mechanisms regulate the decrease in the probability of a reversal response and the size of the response; the second is that dopamine may not be involved in habituation to tap, but instead may modulate the integration of sensory stimuli from the head and tail touch circuits.

Xu et al. (2002) performed a forward genetic screen on habituation to tap and isolated *hab-1,* a mutant which habituated more slowly and responded at a higher asymptotic level than wild-type worms when tested at both 2- and 10-s ISIs. Further, the *hab-1* mutants responded like wild-type worms to the initial tap and also responded normally to a dishabituating stimulus. Unfortunately, the gene product of *hab-1* has yet to be identified. However, the observed pattern of slower habituation is opposite to the results seen with both *eat-4* and *dop-1,* suggesting that the gene product of *hab-1* may play an antagonistic role to the molecular mechanisms of glutamatergic and dopamine signaling that is hypothesized to underlie short-term habituation.

4.04.7 Analyses of Long-Term Habituation

4.04.7.1 Dependence on Protocol

Analysis of long-term memory for habituation to the tap-withdrawal response has led to increased knowledge and further understanding of the mechanisms that govern habituation behavior. Rankin et al. (1990) were the first to report long-term memory habituation to tap. Using a modified protocol from experiments with *Aplysia* (Carew et al., 1972), they were able to show that if worms were given distributed habituation training they were able to retain memory for that training for at least 24 h. To investigate the behavioral parameters that reliably produce long-term memory for habituation, Beck and Rankin (1997) examined ISI and type of training. They replicated the finding of Rankin et al. (1990) that long-term memory could be produced by distributed training, and they found that, if they used a massed training protocol where all stimuli were delivered without break periods instead of a distributed training protocol, long-term memory was not observed. In addition, they found that long-term memory could not be reliably produced from training at a 10-s ISI, whereas it was produced using a 60-s ISI. This result supports the hypothesis of Rankin and Broster (1992) that there may be specific cellular mechanisms, which are recruited by training with a 60-s ISI or inhibited by training with a 10-s ISI, to induce memory formation during habituation training (**Figures 2(b)** and **2(c)**). Taken together, these data show that long-term memory of habituation training is most reliably produced when stimuli are administered at longer ISIs in a distributed or spaced manner. This parallels observations made in studies that found that distributed training is superior to massed training for the induction of long-term memory in many species, including *Aplysia, Drosophila,* and humans (Ebbinghaus, 1885; Carew *et al.*, 1972; Tully and Quinn, 1985).

4.04.7.2 Molecular Correlates of Memory for Habituation Training

Since distributed training was essential for the induction of long-term memory for habituation training, it was hypothesized by Beck and Rankin (1995) that the mechanisms that are responsible for consolidation of the memory were most likely occurring during the interblock intervals. To assess this, Beck and Rankin (1995) used heat shock (32 °C) to block protein synthesis and disrupt these cellular mechanisms. The cellular response to heat shock that was first observed in *Drosophila* has been shown to be same in every organism studied (Schlesinger et al., 1982). The response is the termination of protein synthesis of all proteins other than a class of proteins called heat shock proteins, the production of which is significantly increased. To determine the timing of memory consolidation of habituation training, heat shock was delivered before, during, or after training. Beck and Rankin (1995) found that disruption of memory consolidation by heat shock occurred during but not before or after habituation training, which supported their hypothesis that some of mechanisms that mediate the induction of long-term memory are occurring during the interblock periods. As well, they found that neither the kinetics of habituation nor the initial response to tap was affected by heat shock treatment. These data suggest that repeated tap stimulation with intervals between blocks triggers molecular mechanisms that involve protein synthesis.

Since the research with *eat-4* mutants suggested that glutamatergic neurotransmission plays a pivotal role in short-term habituation, Rose et al. (2002) hypothesized it might also play a role in long-term habituation. To investigate this, Rose et al. (2002) used the *eat-4* mutants to test for the presence of long-term memory for habituation training using the distributed training protocol. Interestingly, even though *eat-4* mutants habituate faster and more

completely than wild-type worms, they did not retain memory for this training 24 h later. This suggests that the sustained glutamate release that is important in short-term habituation is also critical to the formation of long-term memory. Rose et al. used the *eat-4* rescue strain, DA1242, and found that DA1242 worms showed normal memory 24 h after training; this supports the hypothesis that presynaptic glutamate release is essential for the formation of long-term memory.

Because release of presynaptic glutamate appeared to be essential for the induction of long-term memory, Rose et al. (2002) hypothesized that postsynaptic glutamatergic receptors might be involved in aspects of long-term memory for habituation as well. To examine this hypothesis Rose et al. (2003) tested worms with mutations in *glr-1,* a homologue of mammalian AMPA/Kainate glutamate receptor subunit (GluR1) and worms with a mutation in *nmr-1,* a homologue of mammalian NMDA-type glutamate receptor subunit (NR1). They found that worms with a mutation in *nmr-1* showed normal long-term memory for habituation. In contrast, *glr-1* worms showed no long-term memory for habituation. To confirm the importance of glutamate receptors in the formation of long-term memory, wild-type worms were treated with DNQX, a competitive non-NMDA glutamate receptor antagonist, during training. Treatment with DNQX had no effect on short-term habituation, but it did block the formation of long-term memory (Rose et al., 2003). These data suggest that *glr-1* plays a critical role in the induction of long-term memory but is not an essential component for short-term habituation.

The role of glutamate receptors in synaptic plasticity and memory formation has been extensively studied in mammalian systems. The most prominent and well-characterized forms of synaptic plasticity that may underlie mammalian memory formation are long-term potentiation (LTP) and long-term depression (LTD). Both LTP and LTD involve changes in synaptic expression levels of GluR1-containing AMPA-type glutamate receptors and trafficking of the receptors to and from the postsynaptic membrane (Malinow and Malenka, 2002). To examine whether similar processes were involved in the induction of memory for habituation training Rose et al. (2003) tested whether habituation training affected the expression pattern of the GluR1 homologue, GLR-1. Using worms carrying chimeric receptors made up of GLR-1 tagged with GFP (GLR-1::GFP), they were able to visualize changes in punctate *glr-1* expression (Rongo and Kaplan, 1999) along the ventral nerve cord (an area that corresponds to important synaptic sites reported in electron microscopy studies; White et al., 1986). They found that, following distributed habituation training, the number of GLR-1::GFP puncta along the ventral nerve cord did not change, but the size of the puncta in trained worms was significantly smaller than in control worms (**Figures 4(a)** and **4(b)**). This result suggests that the distributed habituation training did not alter the number of synapses along the nerve cord but rather reduced the number of receptors expressed per synapse on the interneurons. Further, blocking protein synthesis by applying heat shock during training blocked the downregulation of GLR-1::GFP expression. These data suggest that, although *glr-1* does not appear to play an important role in short-term habituation, its regulation critically mediates long-term memory for habituation.

4.04.8 Summary

The evidence discussed has shown that *C. elegans* can habituate to both olfactory stimuli and mechanosensory stimuli. Little is known about olfactory habituation other than that it appears to require low doses of the chemical stimuli and is dependent on the *glr-1* gene. On the other hand, mechanosensory habituation has been studied at the level of behavior, neural components, and genes involved. Using the tap-withdrawal paradigm, the dependence upon ISI appears to mediate a large number of aspects of habituation, including the rate of habituation, the level of habituation, and spontaneous recovery from habituation. The neural circuit that mediates habituation to tap has been thoroughly investigated, and this has led to the hypothesis that the most likely sites of plasticity are the synaptic connections between the sensory neurons and the command interneurons. Thus far, the molecular components that affect habituation include presynaptic glutamate release and dopaminergic neurotransmission, and are independent of the activation of *glr-1.* However, distributed habituation training that induces long-term memory recruits other cellular processes. These processes have been shown to be protein synthesis dependent and rely heavily upon the alterations in the expression of *glr-1.*

Taken together, these data suggest that habituation is not a simple or singular process but, rather, is mediated by a complex set of events that incorporates a variety of molecular mechanisms. These events

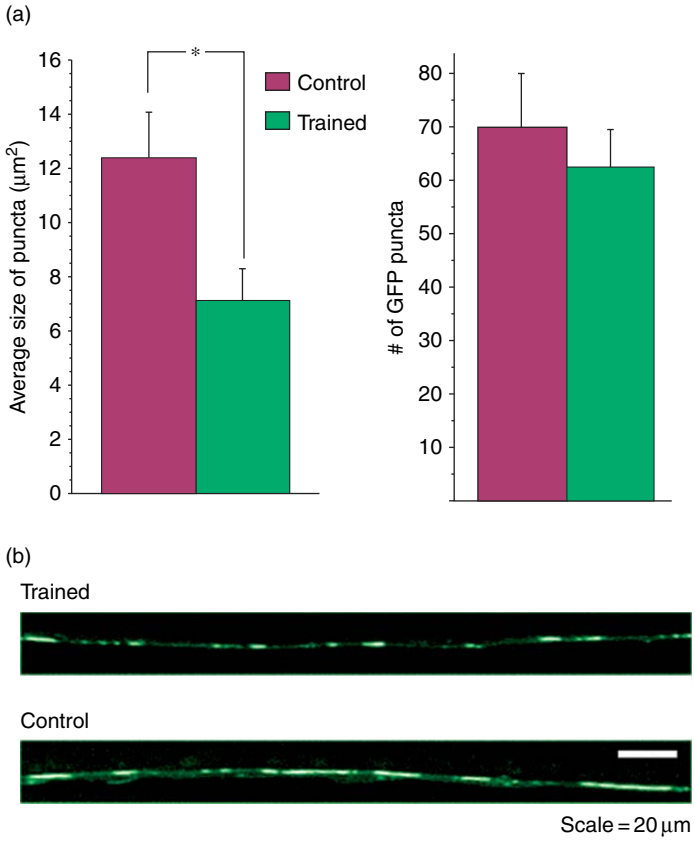


Figure 4 (a) Long-term memory for habituation training correlates with changes in GLR-1::GFP expression in the posterior ventral nerve cord 24 h after distributed habituation training. There were significantly smaller GFP puncta in the trained worms than in control worms; however, the number of puncta was not different between the two groups, suggesting that memory was reflected in a change in the average size of synapses and not a change in the number of synapses (Rose et al., 2003). (b) Confocal images of GLR-1::GFP expression in trained and control worms taken 24 h after distributed training.

integrate multiple neurotransmitters, multiple receptors, and multiple subcircuits, each mediating different aspects of habituation. Rather than there being a single mechanism of habituation, the data from *C. elegans* lead to the hypothesis that there are multiple mechanisms that can underlie this seemingly simple response decrement. Because of its well-studied genetics, physiology, and behavior, *C. elegans* is now, and will continue to be, a very powerful model system in which to elucidate the cellular events and processes that underlie key components involved in learning and memory.

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4.05 Pain Sensitization

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4.05.1 Introduction

Over 10 years ago, Allan Basbaum published a review article entitled 'Memories of pain' (Basbaum, 1996). This article highlighted the role of central nervous system (CNS) plasticity in mediating pain sensitization, and since that time research into the molecular mechanisms mediating pain sensitization has exploded, leading to a new appreciation for the complexity of pain and its sensitization. Here we will introduce the neurobiology of pain and discuss the many mechanisms of sensitization in the pain neuraxis, as well as their relation to mechanisms of learning and memory.

4.05.1.1 Defining Pain: Acute Versus Chronic Pain

Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage (Merskey, 1979). Physiological pain serves a vital survival function as it alerts organisms to the presence of damaging or potentially damaging stimuli. The importance of the protective role of pain is exemplified by the detrimental outcomes of numerous pathological conditions that are characterized by the lack of pain sensation. A common group with significant morbidity due to the lack of pain perception is diabetics with peripheral neuropathy. In these patients, unnoticed repetitive injuries to the joints can lead to permanent joint deformity. Diabetic patients with peripheral neuropathy can also develop ulcers caused by undetected excessive pressure, by rubbing against the skin on the foot, or by stepping on a sharp object (Boulton, 2004). These ulcers can erode to the bone and lead to serious infections with resultant sepsis or need for amputations. Another rare, but yet very dramatic, example of the

protective role of pain is seen in patients with congenital insensitivity to pain. In these patients, pain and temperature insensitivity is manifested in childhood and results in the occurrence of painless fractures, ulcers, burns, and self-mutilation that can lead to death at a young age (Nagasako et al., 2003).

Under normal physiological conditions, pain results from trauma, inflammation, or nerve injury, thus acting as an early warning system that protects the body from further injury. Physiological pain usually subsides as the injury heals and can be frequently diagnosed and treated. Chronic pain, on the other hand, is considered a pathological condition with little or no beneficial function. It can last for a prolonged period of time, outlast the initial pathology, and it is often accompanied by maladaptive behaviors, irritability, depression, and disruption of work and social relationships (American Pain Foundation, 2002). Chronic pain may result from ongoing causes of pain with a known pathology such as in arthritis and cancer. Alternatively, chronic pain may be caused by either an unknown pathology or an associated injury, making their treatment even more challenging.

A major but also distinct component of pain is nociception, which is the sensory process by which a noxious stimulus, such as one induced by tissue damage, is transduced to neurophysiological signals that are then transmitted to the CNS. Nociception can occur without pain perception and pain perception can occur without nociception. An example of pain in the absence of nociception is seen in the phantom limb syndrome, when patients with amputated limbs report feeling pain in the missing limb.

4.05.1.2 Chronic Pain and Synaptic Plasticity

The understanding of the neural mechanisms underlying nociception and pain perception has significantly increased in the last 2 decades. A key finding for this advancement was the realization that responses of the sensory system to a given input are not fixed, but rather change, as a result of previous neuronal activity. This means that the responses of the sensory system are highly dependent on the neural memory of the system. This neuronal plasticity, also referred to as sensitization, has been observed and studied in different parts of the pain neuraxis such as peripheral nociceptors, spinal dorsal horn neurons, rostroventral medulla, anterior cingulate cortex, and amygdala.

Maladaptive and persistent neural changes in the sensory system can occur in response to trauma, inflammation, or nerve injury and are thought to underlie chronic pain. A classic example of sensitization is seen in the dorsal horn of the spinal cord (**Figure 1**). Following tissue injury, spinal cord dorsal horn neurons, which are the first CNS processing station for pain signal from the periphery, show a decreased threshold for action potential firing, increased responsiveness to a given stimulus, and receptive field enlargement (Woolf, 1983; Woolf and Wall, 1986; Cook et al., 1987). The increased responsiveness of

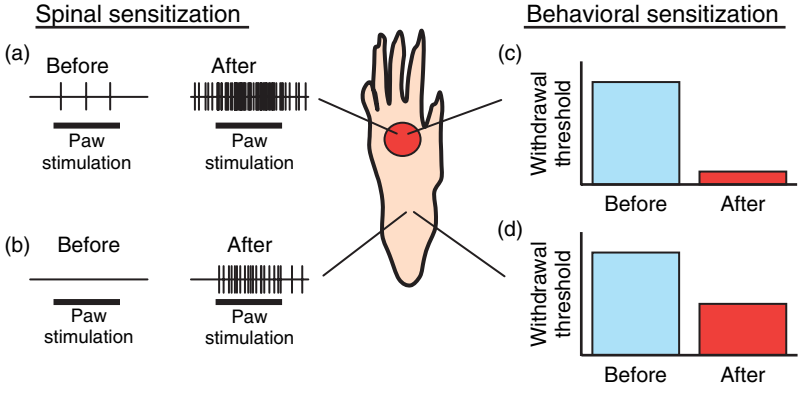


Figure 1 Diagrammatic representation of injury-induced spinal and behavioral sensitization. (a, b) Responses of a spinal cord dorsal horn neuron to paw stimulation before and after tissue injury. (a) Responses to stimulation in the injured area (red circle). After tissue injury, dorsal horn neurons exhibit increased responsiveness to a given stimulus. (b) Responses to stimulation in the noninjured area. Before tissue injury, the receptive field of the neuron did not include the stimulated area. After tissue injury, the neuron responds to stimulation in the noninjured area, demonstrating an enlargement of the receptive field (c, d) Paw withdrawal thresholds in response to mechanical stimulation before and after tissue injury. After tissue injury, withdrawal thresholds in response to mechanical stimulation of the injured (c) and noninjured areas (d) decrease.

dorsal horn neurons to a given stimulus correlates with the behavioral sensitization commonly observed after tissue injury (**Figure 1**). This pain sensitization is characterized by increased pain responses to normally noxious stimuli (hyperalgesia) and/or by pain responses to previously innocuous stimuli (allodynia). Hyperalgesia and allodynia after tissue injury can be experienced both in the injured area (primary hyperalgesia or allodynia) and in adjacent noninjured areas (secondary hyperalgesia or allodynia). Receptive field enlargement of dorsal horn neurons could therefore explain, at least in part, the phenomenon of secondary allodynia and hyperalgesia.

For years, researchers have been trying to identify neural processes that specifically mediate pathological but not physiological pain. The end goal is to develop novel and more efficient approaches for treating chronic pain conditions by decreasing maladaptive pain without affecting physiological pain, which is a vital adaptive response. Numerous studies have revealed striking similarities between the neural mechanisms underlying pain sensitization and those underlying learning and memory (Ji et al., 2003). These similarities have made the development of novel analgesic therapies even more difficult by adding yet another level of potential undesired side effects. At the same time, realization of this relationship has led to the development of a cognitive-behavioral approach to managing chronic pain that has been shown to be highly effective in helping to manage chronic pain. In this chapter, we will start by briefly discussing the advantages and limitations associated with the use of animal models for the study of pain and by summarizing basic concepts of the physiology of pain. We will then discuss the different forms of sensitization that have been reported in different areas of the pain neuraxis, including their anatomical and cellular substrates and their association with behavioral hypersensitivity that is induced by trauma, inflammation, or nerve injury. The chapter will end by comparing the neural mechanisms of pain with those of learning and memory and by discussing the implications for pain management.

4.05.2 Animal Models for the Study of Pain

The goal of pain research is to acquire new knowledge on the mechanisms, pathogenesis, diagnosis, and treatment of pain. The use of animal models of pain is essential to achieve this goal but, because of the negative nature of pain, there are a number of concerns as well as ethical, moral, and legal issues associated with the use of animals to study pain. Ethical guidelines for the investigation of experimental pain in animals have been established by the International Association for the Study of Pain (IASP) (Zimmermann, 1983). These guidelines primarily aim at ensuring that the animal is exposed to the minimal pain necessary for the purposes of the experiment. Whenever possible, experiments should be carried out in anesthetized animals utilizing transient stimuli and avoiding tissue damage. Many experiments to investigate the neural basis of pain can be pursued using these methods. However, to evaluate the mechanisms underlying persistent pain-related behaviors after tissue injury, the use of conscious animals and their exposure to tissue injury are imperative. To minimize discomfort and pain, the duration of all pain experiments should be as short as possible and the number of animals involved should be kept to a minimum. If possible, the investigator should expose the pain stimulus to himself to ensure that the experimental animal is not exposed to pain greater than humans could tolerate. Finally, to minimize discomfort, most experiments utilize techniques in which the animal has control over the intensity and duration of the pain stimulus by measuring the latency or threshold for withdrawal responses to the pain stimulus.

By definition, pain is a subjective perception of a given stimulus. Therefore, the main limitation of the use of animal models to study pain is that the investigator cannot determine with certainty the animal's perception. Animal models of pain are designed to mimic human pain conditions and employ characteristic animal behavioral responses to pain such as licking, withdrawal, and vocalization. Although not perfect, these methods allow the investigator to infer that the animal is experiencing pain. Changes in these stereotypical behaviors have long been used to measure the efficacy of experimental manipulations and pharmacological agents in reducing pain. Many of the drugs currently used to treat clinical pain have been shown to decrease nociceptive behavioral responses using animal models (Negus et al., 2006). Furthermore, neural mechanisms thought to underlie chronic pain have been observed both in human and in animal models of pain (Ji et al., 2003; Klein et al., 2004). It can therefore be concluded that animal models of pain are indeed useful for studying pathological pain.

4.05.3 Physiology of Pain

Pain processing starts with the conversion of noxious stimuli to neurophysiological signals (**Figure 2**). These neural signals are transmitted from the site of injury to the spinal cord and the brain where information about the intensity, quality, and location of the stimuli is processed. This processing is not unidirectional, however; descending pathways from the brain can significantly affect nociceptive transmission in the spinal cord, thus affecting pain perception. In this section, we will discuss the physiological basis of pain. The processes of transduction, transmission, modulation, and perception of pain will be discussed.

4.05.3.1 Pain Transduction

As in all sensory systems, the nociceptive system starts with the transduction of an external stimulus, in this case a noxious stimulus (thermal, mechanical, or chemical), to a neurophysiological signal. The transduction process occurs in nociceptor terminals that are located in the skin, muscles, and other end-organ tissues. Nociceptors are peripheral nerve endings of primary sensory neurons that selectively respond to noxious stimuli. The cell bodies of these primary sensory neurons are located in the dorsal root ganglia (DRG) and in the trigeminal ganglia. Dorsal root and trigeminal ganglion neurons are pseudo-unipolar cells. This means that these neurons have one axon that splits into two processes, with each process functioning as an axon. One of these processes conveys information from the periphery to the soma while the other one transmits information from the soma to neurons in the dorsal horn of the spinal cord or in the trigeminal nucleus caudalis of the brainstem. Two major classes of nociceptors have been described: Aδ- and C-nociceptors (Raja et al., 1999). Aδ-nociceptors are small-diameter, thinly myelinated fibers that conduct at 5–30 m/s and respond to either heat (thermal nociceptors) or intensive pressure (mechanical nociceptors), whereas C-nociceptors are small-diameter unmyelinated fibers that conduct slowly (<1 m/s) and respond to high-intensity mechanical, thermal, or chemical stimuli.

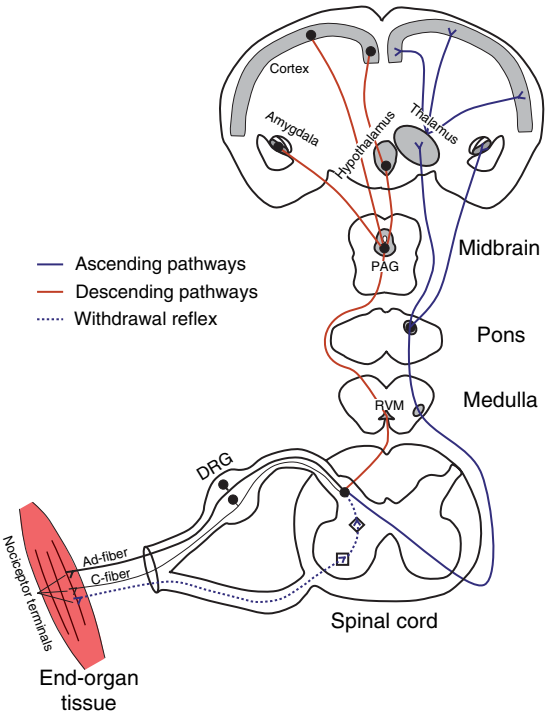


Figure 2 Illustration of anatomical pain pathways. Nociceptive information is transduced in nociceptor terminals located in the skin, muscle, and other end-organ tissues. Small-diameter unmyelinated C-fibers transmit the nociceptive information to neurons in the dorsal horn of the spinal cord, which in turn project to various brain regions via ascending pathways (solid blue line) that relay in the medulla, pons, and thalamus. Activation of motor neurons (□) by excitatory interneurons (◇) mediates the pain withdrawal reflex (dotted blue line). Descending pathways from the brain (red line), that relay in the periaqueductal gray matter (PAG) and rostroventromedial medulla (RVM), modulate nociceptive transmission in the dorsal horn. DRG, dorsal root ganglia.

The exact mechanism by which noxious stimuli are translated to neurophysiological signals in the peripheral nerve endings is not completely understood, but it is thought to involve the activation of proteins designed to convert noxious stimuli to depolarizing electric potentials. A family of receptors that has received increasing attention in recent years as major sensory transducers is the transient receptor potential (TRP) ion channel family. TRP channels are expressed in nociceptor terminals and transduce a wide range of thermal, mechanical, and chemical stimuli (Wang and Woolf, 2005; Story and Gereau, 2006). The TRPV1 receptor is activated by noxious heat ($\geq$43 °C), protons, or capsaicin (the pungent compound found in hot chili peppers); TRPV2 by noxious heat ($\geq$52 °C); TRPV4 by mechanical stimuli or protons; TRPM8 by cold ($\leq$23–28 °C), menthol, and icilin (a super cooling agent); and TRPA1 by noxious cold ($\leq$18 °C), icilin, mustard, cinnamon, or mechanical stimuli. Since TRP channels are activated within a range of noxious temperatures, and also by protons, chemicals, and intensive pressure, it has been hypothesized that these receptors have a prominent role in thermal, chemical, and mechanical nociception. Other receptors and ion channels such as the metabotropic glutamate receptors, the TREK-1 potassium channel, and the acid-sensing ion channels (ASIC) and dorsal root acid-sensing ion channels (DRASIC) have also been identified as potential sensory transducers in nociceptor terminals. The ultimate function of these channels is to provide a transduction current that depolarizes the nociceptor terminals and thereby initiate action potential firing in the nociceptor.

4.05.3.2 Pain Transmission

Nerve impulses generated in the periphery by noxious stimuli propagate via axons of DRG neurons to the dorsal horn of the spinal cord (**Figure 2**). The dorsal horn of the spinal cord is subdivided into six layers or laminae based on cytoarchitectural characteristics. C nociceptive afferents terminate mainly in laminae I and II, whereas Aδ afferents terminate mainly in laminae I and V (Basbaum and Jessell, 2000). Following DRG activation by peripheral noxious stimuli, neurotransmitters and peptides are released from DRG afferent terminals into the dorsal horn of the spinal cord. In the dorsal horn, these neurotransmitters and peptides act on postsynaptic receptors expressed by three types of dorsal horn neurons: excitatory interneurons, inhibitory interneurons, and projection neurons. Excitatory and inhibitory interneurons are part of the dorsal horn local circuitry; these neurons synapse on and modulate the firing of projection neurons, motor neurons, and other interneurons (**Figure 2**). Activation of motor neurons by excitatory interneurons mediates the classic pain withdrawal reflexes. Inhibitory interneurons can also exert presynaptic inhibition of nociceptive primary afferents, indirectly regulating the excitability of dorsal horn neurons by decreasing primary afferent input. Projection neurons, on the other hand, are the dorsal horn output neurons. These second-order neurons convey nociceptive information from the dorsal horn of the spinal cord to the thalamus, hypothalamus, and different brainstem structures via five major ascending pain pathways: the spinothalamic, spinohypothalamic, spinoparabrachial, spinoreticular, and spinomesencephalic tracts (Basbaum and Jessell, 2000; Almeida et al., 2004). These ascending pathways in turn project to various cortical and limbic structures such as the somatosensory cortex, cingulate cortex, insular cortex, prefrontal cortex, and amygdala (**Figure 2**). Sensory discriminative information about the localization, quality, and intensity of the noxious stimulus as well as the attentional, emotional, and cognitive components of pain are processed in these cortical and limbic structures (Almeida et al., 2004).

4.05.3.3 Pain Modulation

As discussed in the previous section, nociceptive transmission and synaptic efficacy in both primary afferent endings and dorsal horn neurons can be modulated by local circuits in the spinal cord. But this is not the only modulatory mechanism; the excitability of dorsal horn projection neurons and primary afferent endings can also be modulated by descending pathways from the brain that originate in the periaqueductal gray matter (PAG), project to the rostroventromedial medulla (RVM), and then terminate in the dorsal horn of the spinal cord (**Figure 2**).

Earlier studies focused on descending inhibition of spinal nociceptive processing, but now we know that descending modulatory pathways can be both inhibitory and facilitatory (Fields, 2000). Early in the 1990s, pioneering work by Howard Fields and colleagues identified three types of neurons in the RVM: on-cells, which facilitate nociceptive transmission; off-cells, which have a net inhibitory effect on nociceptive transmission; and neutral cells that show no nociception-related change in activity (Fields et al., 1983, 1991). *In vivo* electrophysiological recordings in the RVM revealed that on-cells discharge just prior to

withdrawal from a noxious stimulus, whereas off-cells pause during withdrawal from a noxious stimulus (Fields et al., 1983). Both types of cells are activated by electrical stimulation of the PAG and they both project directly to laminae I, II, and V of the dorsal horn, where nociceptive-specific neurons are located (Fields et al., 1995; Fields, 2000). Descending modulation of dorsal horn projection neurons and primary afferent endings seems to be mediated via activation or inhibition of excitatory and inhibitory local circuit interneurons in the dorsal horn. Physiologically, activation of descending inhibitory control upon peripheral noxious stimuli seems to provide a negative feedback mechanism that decreases nociceptive transmission at the level of the spinal cord, whereas activation of descending facilitatory pathways has been proposed to contribute to the development and maintenance of hyperalgesia and allodynia (Helmstetter and Tershner, 1994; Fields, 2000; Gebhart, 2004).

4.05.3.4 Pain Perception

The mechanisms of transduction, transmission, and modulation of noxious sensory stimuli described in the previous sections offer an explanation for how noxious sensory stimuli are translated to neurophysiological signals and how these signals are then propagated to and modulated by the CNS. However, these neural processes do not explain how certain stimuli are ultimately perceived as painful. The perception of pain is more than just direct processing of sensory stimuli. Clinical studies have demonstrated that the processes underlying pain perception comprise complex behavioral, psychological, and emotional factors (Turk, 2003; Lewandowski, 2004). Social and environmental factors influence the perception of pain, as do past experience and culture. Consequently, an apparently similar stimulus can generate enormous individual differences in pain perception. Several questions thus arise. How does pain perception occur? What are the neural mechanisms underlying how we perceive pain?

Projections from ascending nociceptive pathways to various cortical and limbic structures such as the cingulate cortex, insular cortex, prefrontal cortex, and amygdala have been described (Almeida et al., 2004) (**Figure 2**). Because of the long-standing role of these cortical and limbic structures in cognitive, emotional, and motivational processes, it has been proposed that their activation upon noxious stimuli is involved in mediating the negative emotional reactions to pain that can result in activity avoidance and escape behaviors (Coleman-Mesches and McGaugh, 1995; Johansen et al., 2001; Gao et al., 2004; Johansen and Fields, 2004; Lei et al., 2004; Tang et al., 2005). Much progress has been made in recent years in identifying brain regions and potential neural mechanisms underlying the cognitive and affective components of pain. This progress has been limited, however, by the methodological and conceptual difficulties associated with studying a subjective process such as pain perception, which becomes even more technically challenging when using animal models. The emergence of novel brain imaging techniques with better image quality and resolution has allowed researchers to perform studies that examine the neuroanatomical basis of different cognitive and emotional components of pain in humans.

4.05.4 Pain Sensitization

Under normal physiological conditions, pain acts as an early warning system to prevent injury. Many acute pain responses are simple reflexes (**Figure 2**). A classic example is the reflexive withdrawal experienced by individuals when they accidentally touch a hot surface. That immediate reflexive withdrawal from the heat source protects the body from burning. This physiological experience encompasses all of the pain components described in the previous section. First, the heat stimulus is translated to a neurophysiological signal in peripheral nerve endings, probably via the activation of one of the TRP ion channels. The nociceptive information is then transmitted to the dorsal horn of the spinal cord, where several processes take place:

1. Interneurons in the dorsal horn activate motor neurons in the ventral horn that mediate the withdrawal reflex.
2. Projection neurons send the nociceptive information to the somatosensory cortex, where information about the location, intensity, and quality of the stimulus is processed.
3. Projection neurons in the dorsal horn convey the nociceptive information to different cortical and limbic structures such as the anterior cingulate cortex, the insular cortex, and the amygdala, where pain awareness and associative learning occur.

The neural processes described in this example occur under normal physiological conditions, in uninjured tissue, when pain acts as a warning system to prevent

injury. When injury does occur, however, pain serves a slightly different but still physiologically adaptive role. After injury, hypersensitivity to both noxious and previously innocuous stimuli develops. Hypersensitivity after injury can be seen as a memory of the injury that engages a heightened protective system developed to prevent further injury, which could interfere with the healing process. A good example of this type of pain is the hypersensitivity experienced when a bone is broken. In this case, normally innocuous movements or stimuli such as light touch become painful. Movements that would otherwise be normal would interfere with the healing process of a broken bone; thus pain, in this case, serves a protective role to support the healing process.

After an injury, the responses of the sensory system to a given stimulus also increase. These increases can be seen at both the peripheral and central levels and are thought to underlie pain hypersensitivity. Heightened neuronal responses of the sensory system are commonly referred to as sensitization. Pain sensitization is characterized by a decreased threshold for action potential firing, increased responsiveness to a given stimulus, and receptive field enlargement. Different types of sensitization have been described in the peripheral and central nervous systems. Sensitization can occur as a consequence of an injury such as in the broken bone example; in this case, sensitization is an adaptive process and should subside as the injury heals. However, there are many instances in which sensitization can be maladaptive, outlasting the initial injury or with an unknown pathology. This pathological sensitization is thought to underlie chronic pain conditions. The properties, characteristics, and potential mechanisms of these various kinds of sensitization will be discussed in the following sections.

4.05.4.1 Peripheral Sensitization

Following tissue injury, multiple inflammatory mediators, such as substance P, prostaglandin E_2, bradykinin, nerve growth factor, tumor necrosis factor, and glutamate, are released by mast cells, macrophages, immune cells, and injured cells at the site of injury (Bhave and Gereau, 2004). Release of these inflammatory mediators initiates a cascade of events that leads to increased responsiveness of nociceptors, a phenomenon called peripheral sensitization (**Figure 3**).

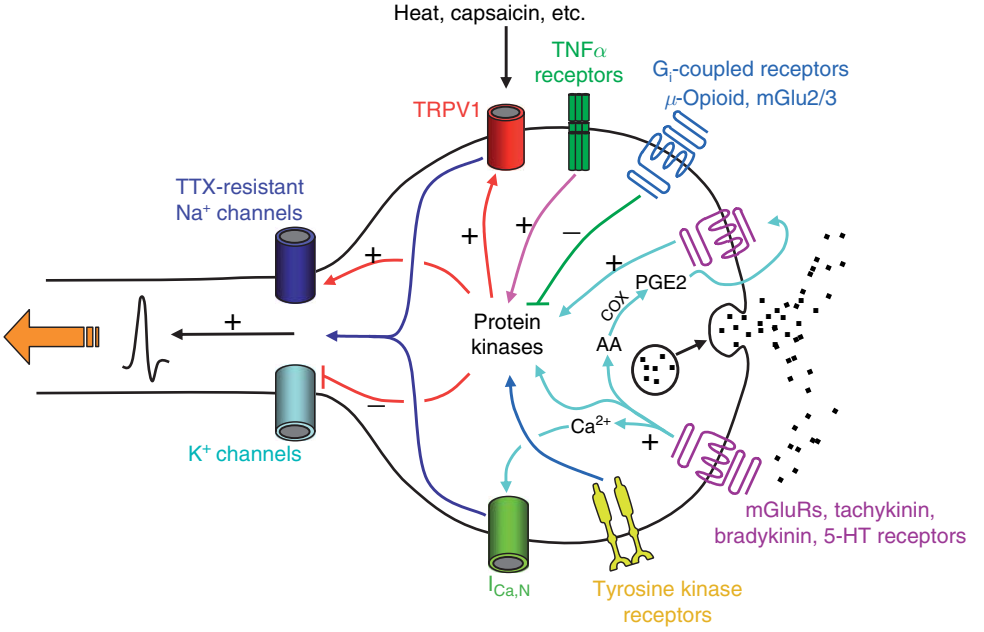


Figure 3 Potential mechanisms underlying peripheral sensitization. Following tissue injury, multiple inflammatory mediators are released at the site of injury and activate several membrane receptors. Subsequent activation of protein kinases and the phosphorylation of ion channels increase the excitability of dorsal root ganglia (DRG) neurons and thus contribute to peripheral sensitization. Sensitization of DRG responses is thought to underlie the reduced threshold that is commonly observed after tissue injury. 5-HT, serotonin; AA, arachidonic acid; Ca^{2+}, calcium; COX, cyclooxygenase; $I_{Ca,N}$, calcium-activated nonselective cation currents; K^+, potassium; mGluR, metabotropic glutamate receptor; PGE2, prostaglandin E2; TNFα, tumor necrosis factor alpha; TRPV1, transient receptor potential V1; TTX, tetrodotoxin.

Peripheral sensitization could result from a change in the cell-surface expression of ion channels that contribute to sensory transduction or action potential generation or from posttranslational modifications of ion channels that modulate the channel properties without necessarily affecting their cell-surface expression. Inflammatory mediators can directly activate ion channel proteins or indirectly activate intracellular pathways via the activation of G-protein-coupled receptors or receptor tyrosine kinases. Activation of intracellular pathways can result in acute modification of ion channels or in the activation of transcription factors, with a resultant increase in transcription and translation of new proteins. Transcriptional, translational, and posttranslational changes can therefore underlie the phenomenon of peripheral sensitization.

4.05.4.1.1 Acute modification of primary sensory neurons

Increased responsiveness of nociceptors to a given stimulus can be observed fairly quickly after tissue injury. Given that transcriptional and translational processes take longer to occur than the development of peripheral sensitization, it has been proposed that the initial stages of peripheral sensitization involve posttranslational modifications of ion channels or transducer molecules and not transcriptional or translational processes. On the other hand, maintenance of peripheral sensitization is thought to involve all three of these processes; that is, gene transcription, protein translation, and posttranslational modifications.

Posttranslational modifications such as phosphorylation can alter the biophysical properties of ion channels that are already inserted in the membrane at the time of injury. Many posttranslational modifications of this type are known to occur and to modulate the excitability of DRG neurons (Bhave and Gereau, 2004). An example of a posttranslational modification that occurs in DRG neurons and that sensitizes the responses of the neuron to a given stimulus is the phosphorylation of the sensory transducer ion channel TRPV1. Phosphorylation of TRPV1 by protein kinase A (PKA), protein kinase C (PKC), and calcium/calmodulin-dependent protein kinase II (CaMKII) results in sensitization of the channel's responses to capsaicin, heat, or pH and also in blockade of desensitization. These alterations in the biophysical properties of the channel could therefore contribute to the reduced threshold for noxious stimuli commonly observed after tissue injury *in vivo*. A caveat of most of the studies that have examined posttranslational modifications in DRG neurons is that the experiments have been conducted in *in vitro* preparations. It is still unknown whether these posttranslational modifications occur after tissue injury and if they contribute to pain hypersensitivity. The development of antibodies specific for modified proteins (i.e., phosphorylated TRPV1) would help answer this type of question.

As in many neurons in the CNS, it is also possible that after injury, posttranslational modifications of ion channels in DRG neurons promote the insertion of ion channels into the membrane. Increased availability of ion channels for activation would increase neuronal depolarizing responses to a given stimulus without necessarily altering the biophysical properties of the individual channels. Although not studied in DRG neurons thus far, trafficking of ion channels is a phenomenon that has received a lot of attention in recent years as one of the major mechanisms used by neurons to control synaptic responses. It is therefore very likely that ion channel trafficking in DRG neurons also underlies a component of peripheral sensitization.

In order for posttranslational modifications to occur, intracellular signaling molecules such as protein kinases need to be activated in DRG neurons in response to tissue injury (**Figure 3**). Activation of receptor tyrosine kinases and G-protein-coupled receptors by inflammatory mediators, such as nerve growth factor (NGF) or glutamate, trigger the activation of intracellular biochemical cascades that are initiated by the production or release of second messengers (Crawford et al., 1997; Bhave and Gereau, 2004). Examples of these second messengers are cyclic adenosine monophosphate (cAMP), diacylglycerol (DAG), inositol 1,4,5-triphosphate (IP_3), calcium (Ca^{2+}), and arachidonic acid (AA). Each of these second messengers either activates protein kinases that directly phosphorylate target proteins such as ion channels or produce other second messengers that can also directly bind to and modulate target proteins. AA is a second messenger system that has been extensively studied in DRG neurons (**Figure 3**). AA is released inside the cell in response to the activation of phospholipase A_2 or phospholipase C, which are enzymes that are activated by G-protein-coupled receptors. AA is oxidized by cyclooxygenases to produce prostaglandins and thromboxanes. Prostaglandins diffuse out of the cell and act on nearby prostanoid receptors. Activation of prostanoid receptors triggers biochemical cascades that mediate peripheral sensitization. The mechanism of action of nonsteroidal

anti-inflammatory drugs commonly used to treat inflammatory pain is to inhibit cyclooxygenase and subsequently reduce prostaglandin synthesis. It has been demonstrated that administration of these anti-inflammatory drugs prior to injury (i.e., before a surgical procedure) is more effective at decreasing inflammatory pain than administration after injury. Administration of these anti-inflammatory drugs prior to injury presumably prevents the development of peripheral sensitization.

4.05.4.1.2 Long-term modifications of primary sensory neurons

In addition to acutely modulating the biophysical properties of ion channels via posttranslational modifications, activation of intracellular signaling cascades after injury can also result in the activation of transcription factors that regulate gene expression and can subsequently result in the synthesis of new proteins such as ion channels, sensory transducers, and/or neuromodulators.

Changes in protein expression after injury can last for days, weeks, or even longer. For this reason, increases in protein expression have been proposed as one of the main mechanisms involved in the maintenance of persistent pain. For example, following persistent inflammation, there is an increase in mRNA levels of the tetrodotoxin (TTX)-resistant sodium channel Nav1.8 in small-diameter DRG neurons (Tanaka et al., 1998). This increase in mRNA levels is accompanied by an increase in the amplitude and density of TTX-resistant sodium currents, suggesting that Nav1.8 protein levels are also increased. Increased sodium currents could in turn increase the excitability of DRG neurons by decreasing the action potential threshold, thus producing the increased response to a fixed stimulus that typifies peripheral sensitization.

Translational changes independent of changes in transcription have also been observed in DRG neurons after inflammation and are thought to contribute to hypersensitivity (Ji et al., 2002b). Increased levels of the sensory transducer TRPV1 are observed at the protein level, but not at the mRNA level, in DRG neurons after persistent inflammation. These inflammation-induced increases in TRPV1 are dependent on NGF-induced activation of p38 mitogen-activated protein kinase (MAPK) and are thought to be mediated via downstream activation of a translational regulator such as eIF4E. In general, transcription and translational processes in DRG neurons after injury are thought to be initiated by the activation of the same receptors and second messenger pathways that mediate posttranslational modifications, but it is thought to require repeated stimulation and/or prolonged action of these second messenger systems.

As discussed in an earlier section of this chapter, nociceptive information is conveyed to the dorsal horn of the spinal cord via Aδ- and C-nociceptive fibers, whereas nonnoxious tactile information is transmitted to the dorsal horn by large-diameter Aβ-sensory fibers. Pain sensitization can be mediated by cellular and molecular changes that occur in peripheral nociceptive fibers that lead to increased responsiveness of nociceptors. Enhanced nociceptor responses in turn lead to increased release of neurotransmitters and neuromodulators from central DRG terminals, subsequently increasing dorsal horn excitability. In addition to these plastic phenomena, a phenotypic switch in Aβ-sensory fibers has also been observed after injury (Neumann et al., 1996). Increased excitability of dorsal horn neurons after injury is thought to be partly mediated by an elevation in the release of substance P by primary nociceptive afferents in the dorsal horn. Under normal conditions, substance P is exclusively expressed in nociceptive afferents; however, part of the phenotypic switch of Aβ-sensory fibers includes that they start synthesizing substance P, which is a peptide not normally expressed by these neurons. Thus, both *de novo* substance P protein synthesis in Aβ-sensory fibers and increases in the translation of this peptide in nociceptive fibers are thought to contribute to the maintenance of pain sensitization by increasing the excitability of dorsal horn neurons. Recruitment of Aβ-sensory fibers is also thought to mediate mechanical allodynia, which is pain to previously innocuous tactile stimulation (Neumann et al., 1996).

4.05.4.2 Sensitization in the Dorsal Horn of the Spinal Cord

Activity-dependent increases in excitability and synaptic transmission occur in the dorsal horn of the spinal cord in response to a short burst of repeated nociceptive input. This spinal sensitization is traditionally referred to as central sensitization to distinguish it from peripheral sensitization. Windup, spinal long-term potentiation (LTP), and classic central sensitization are three different forms of pain-induced sensitization that have been described in the dorsal horn and that are thought to underlie persistent pain. Although these three types of sensitization are considered three different phenomena, their

cellular and molecular mechanisms share many common properties. All these forms of sensitization are blocked or decreased by ablation of neurokinin 1 (NK1) receptor-expressing projection neurons in the dorsal horn, and they all depend on glutamatergic synaptic transmission. Some of these forms of sensitization are restricted to the activated synapse (homosynaptic), whereas others spread to adjacent synapses (heterosynaptic). The properties, characteristics, and potential mechanisms of windup, spinal LTP, and classic central sensitization will be discussed in the following sections.

4.05.4.2.1 Windup: Short-term sensitization of dorsal horn neurons

Normal healthy individuals report higher pain intensity ratings to electrical, mechanical, or thermal stimuli of constant intensity as the frequency of the stimulus delivered increases (Herrero et al., 2000). This change in reported pain is considered a behavioral correlate of windup, which is a progressive and frequency-dependent increase in the excitability of dorsal horn neurons that is evoked by a short train of repeated stimulation of nociceptive afferents. Windup can therefore be considered a cellular memory of nociceptor input and one form of sensitization of dorsal horn neurons. Experimentally, windup can be induced in the dorsal horn by stimulating nociceptive afferents at frequencies between 0.2 and 20 Hz, with the greatest degree of windup generated by 1- to 2-Hz stimulations (Herrero et al., 2000). Stimulations lower than 0.2 Hz fail to induce windup, whereas stimulations higher than 20 Hz induce habituation of the response, or winddown. A characteristic property of windup is that, unlike the other forms of central sensitization, it is only manifested during the course of the stimulus (Ji et al., 2003).

C-fiber stimulation generates slow excitatory synaptic potentials in the dorsal horn. Windup is thought to result from the temporal summation of these slow excitatory synaptic potentials (Herrero et al., 2000; Ji et al., 2003). The current view is that windup is mediated by cumulative depolarization of the neuron induced by the activation of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors after repeated presynaptic stimulation. AMPA-mediated depolarization triggers the removal of the voltage-dependent Mg^{2+} block of *N*-methyl-D-aspartate (NMDA) receptors, resulting in further increase of temporal summation. In addition, activation of voltage-gated Ca^{2+} channels by neuronal depolarization has been proposed to generate plateau potentials that also contribute to the expression of windup (Herrero et al., 2000; Ji et al., 2003).

As discussed in previous sections, nociceptor stimulation results in presynaptic release of neurotransmitters and peptides in the dorsal horn of the spinal cord. Glutamate is the primary excitatory neurotransmitter that mediates synaptic transmission between peripheral afferents and the dorsal horn, and its release has been shown to be necessary for the development of windup. Presynaptic release of neuropeptides such as substance P and calcitonin gene-related peptide (CGRP) are also crucial for the expression of windup (Herrero et al., 2000). At the postsynaptic level, windup seems to depend on the activation of G-protein-coupled receptors and NMDA receptor activation, the latter being important for both the induction and the maintenance of windup (Herrero et al., 2000; Ji et al., 2003). Windup can be recorded in acute spinal cord slice preparations, demonstrating that spinal intersegmental and supraspinal modulation are not necessary for its development. At the same time, infusion of opioids or serotonin into the spinal cord inhibits the generation of windup, suggesting that inputs from supraspinal structures could potentially modulate windup (Herrero et al., 2000).

Under normal physiological conditions, windup is expressed as a homosynaptic form of sensitization; thus it can be induced by stimulation of C-fibers but not by A-fiber stimulation (Thompson et al., 1994; Ji et al., 2003). After inflammation, several plastic changes modulate the induction of windup (Thompson et al., 1994; Herrero et al., 2000) (**Figure 4**). Thus, stimulation of C-fibers that innervate the inflamed area produces enhanced windup, stimulation of afferent fibers that innervate uninflamed areas adjacent to the inflamed area or of Aβ-fibers that normally fails to induce windup can now induce windup, and a general reduction in the threshold for windup is observed. The physiological significance of windup is a matter of debate. The high-frequency synchronized stimulation used to induce windup does not naturally occur in nociceptor afferents in response to noxious stimulation; therefore, the physiological relevance of windup has been questioned. At the same time, because a windup-like phenomenon has been observed clinically, it is still possible that some form of windup occurs in response to repeated noxious stimulation of constant intensity, contributing to hypersensitivity.

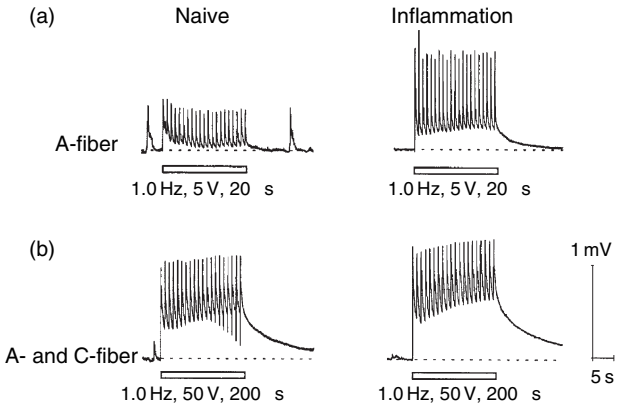


Figure 4 Effects of tissue injury on windup. Summated ventral root potentials (VRP) following low-frequency (1-Hz) repetitive stimulation of A-fibers or both A- and C-fibers recorded in spinal cords from naive or ultraviolet (UV)-injured animals. The horizontal bar indicates the stimulation period. (a) Stimulation of A-fibers (5 V, 20 μs) in naive animals failed to sustain a summated depolarization (left panel). An identical stimulation in spinal cords from UV-injured animals induced a robust and sustained depolarization (right panel) indicating that, after injury, windup can be induced by stimulation of A-fibers that fail to induce windup under normal conditions. (b) Stimulation of both A- and C-fibers (50 V, 200 μs) induced a robust and sustained depolarization in spinal cords from naive animals (left panel). An identical stimulation in spinal cords from UV-injured animals increased the amplitude of the sustained depolarization (right panel) indicating that, after injury, windup is enhanced. Adapted from Thompson SW, Dray A, and Urban L (1994) Injury-induced plasticity of spinal reflex activity: NK1 neurokinin receptor activation and enhanced A- and C-fiber mediated responses in the rat spinal cord in vitro. *J. Neurosci.* 14: 3672–3687.

4.05.4.2.2 Spinal long-term potentiation

Activity-dependent synaptic LTP and long-term depression (LTD) of excitatory postsynaptic potentials (EPSPs) can both be evoked in dorsal horn neurons following brief high-frequency repeated stimulation of presynaptic central DRG afferents (Randic et al., 1993). The development of LTP or LTD seems to depend on the postsynaptic membrane potential, with hyperpolarized potentials (−85 mV) favoring the development of LTD and more depolarized potentials (−70 mV) favoring the development of LTP. Both LTP and LTD are homosynaptic forms of sensitization; thus, potentiation or depression of EPSPs is normally restricted to the activated synapse and not other adjacent synapses. LTP or an LTP-like phenomenon can be induced in animals and in humans and has been strongly correlated with pain behavior. Synchronized high-frequency stimulation of peripheral afferents in intact animals or in acute spinal cord slice preparations can result in long-lasting increases in evoked postsynaptic responses of dorsal horn neurons (Randic et al., 1993; Sandkuhler and Liu, 1998). Similarly, in humans, high-frequency electrical stimulation of cutaneous afferents, using stimulation protocols that resemble the ones used to induce experimental LTP in animals, has been shown to induce long-term increases in reported pain sensitivity (Klein et al., 2004). The cellular and intracellular signaling mechanisms that underlie dorsal horn LTP and those that underlie injury-induced pain hypersensitivity are strikingly similar. For this reason, dorsal horn LTP has been commonly used as a cellular model of afferent-induced hyperalgesia.

LTP induction in the dorsal horn is dependent on a rise in free intracellular calcium in the postsynaptic cell as well as on postsynaptic activation of AMPA, NMDA, metabotropic glutamate receptors (mGluRs), and low-threshold T-type voltage-gated calcium channels. Activation of various intracellular signaling molecules such as PKA, PKC, extracellular signal-regulated protein kinase (ERK), Src tyrosine kinase (Src), and CaMKII in dorsal horn neurons is also necessary for the induction of LTP (Sandkuhler, 2000; Sandkuhler et al., 2000; Woolf and Salter, 2000; Ikeda et al., 2003). The activation of these intracellular molecules leads to posttranslational changes of target proteins such as ion channels, membrane receptors, and/or accessory subunits. Posttranslational modifications modulate the cell-surface expression and function of these proteins, ultimately increasing synaptic efficacy in the dorsal horn. Activation and posttranslational modifications of neuropeptide receptors such as NK1 and tachykinin receptors in the dorsal horn are also necessary for the induction of LTP (Sandkuhler et al., 2000). An interesting feature of dorsal horn LTP is that it can only be induced in a subpopulation of dorsal horn neurons.

LTP is preferentially induced in dorsal horn projection neurons that express the NK1 receptor but not in dorsal horn neurons that do not express this receptor (Ikeda et al., 2003). These findings, together with behavioral studies that show that ablation of NK1-expressing neurons in the dorsal horn attenuates the development of inflammation-induced hypersensitivity (Mantyh et al., 1997), suggest that NK1-expressing projection neurons are an important site of nociceptive plasticity in the dorsal horn.

Despite the cellular and molecular similarities between dorsal horn LTP and injury-induced pain hypersensitivity, the biological significance of dorsal horn LTP has long been questioned. High-frequency synchronized stimulation like the one used to induce experimental LTP does not resemble the low-frequency unsynchronized afferent barrage induced physiologically by natural noxious stimuli. In addition, although LTP has been shown to occur in the dorsal horn *in vivo* in response to natural noxious stimulation, this naturally occurring LTP can be induced in spinalized animals but not in intact animals. A recent study by Ikeda and colleagues put an end to this debate (Ikeda et al., 2006) (**Figure 5**). In their elegant study, Ikeda and colleagues showed that low-frequency stimulation (LFS) of C-fibers, at a frequency that resembles the afferent barrage that occurs during inflammation, induces a robust synaptic LTP of evoked EPSCs in NK1-expressing dorsal horn neurons that project to the periaqueductal grey (PAG). Ikeda et al. further demonstrated that LTP can be induced *in vivo*, in intact animals, by a natural low-frequency afferent barrage that is induced by peripheral inflammation. The mechanisms underlying the induction of LFS-induced LTP in the dorsal horn resemble those of injury-induced hyperalgesia (Ikeda et al., 2006), further supporting the idea that LTP of synaptic strength in the dorsal horn underlies inflammation-induced pain hypersensitivity.

4.05.4.2.3 Classic central sensitization

Following tissue injury and under pathological pain conditions, second-order nociceptive-specific neurons in the dorsal horn of the spinal cord show a decreased threshold for action potential firing, increased responsiveness to a given stimulus, and receptive field enlargement (Woolf, 1983; Woolf and Wall, 1986; Cook et al., 1987; Ji et al., 2003) (**Figure 1**). These electrophysiological changes are collectively referred to as central sensitization and, as peripheral sensitization, are thought to underlie the development of chronic pain. Unlike windup and dorsal horn LTP, central sensitization is considered a heterosynaptic form of sensitization, as these electrophysiological changes occur in both the activated synapse and also in adjacent synapses. Sensitization and recruitment of synapses adjacent to the activated synapses are thought to contribute to the receptive field enlargement that is characteristic of central sensitization and are thought to underlie the pain hypersensitivity that is experienced in noninjured areas following an associated tissue injury (secondary allodynia).

The molecular mechanisms underlying central sensitization and dorsal horn LTP are very similar. As is the case for dorsal horn LTP, central sensitization selectively occurs in NK1-expressing neurons in the dorsal horn and depends on the activation of AMPA, NMDA, and mGluRs. Multiple intracellular signaling pathways, such as ERK, PKA, PKC, CaMKII, and Src, are activated in dorsal horn neurons downstream of the activation of these receptors (Ji et al., 2003). Activation of an intracellular signaling pathway can exert both short-term and long-term neuronal modifications. Acute posttranslational modifications of ion channels are known to occur fairly quickly after the activation of intracellular molecules and are thought to mediate the early component of central sensitization, whereas sustained activation of intracellular molecules commonly results in their translocation to the nucleus, where they can activate different transcription factors. Transcription and translation of new proteins, in combination with sustained posttranslational modifications of ion channels, are thought to contribute to the maintenance of central sensitization.

The ERK signaling cascade is a good example of an intracellular signaling pathway that exerts both short-term and long-term neuronal modifications that contribute to central sensitization (**Figure 6**). ERK is activated in dorsal horn neurons shortly after tissue injury, downstream of the activation of a diversity of membrane receptors (Ji et al., 1999; Karim et al., 2001; Pezet et al., 2002; Adwanikar et al., 2004; Kawasaki et al., 2004; Slack et al., 2004; Kawasaki et al., 2006; Svensson et al., 2006). ERK directly phosphorylates the potassium channel Kv4.2, decreasing Kv4.2-mediated potassium currents, thereby increasing neuronal excitability (Hu and Gereau, 2003; Hu et al., 2003, 2006). ERK also regulates phosphorylation and the activity of the NR1 NMDA receptor subunit (Slack et al., 2004). In addition to these acute modifications of ion channels, ERK can also translocate to the nucleus, where it phosphorylates and activates the cAMP response element binding protein (CREB), which in turn promotes the transcription of immediate early genes such as c-fos

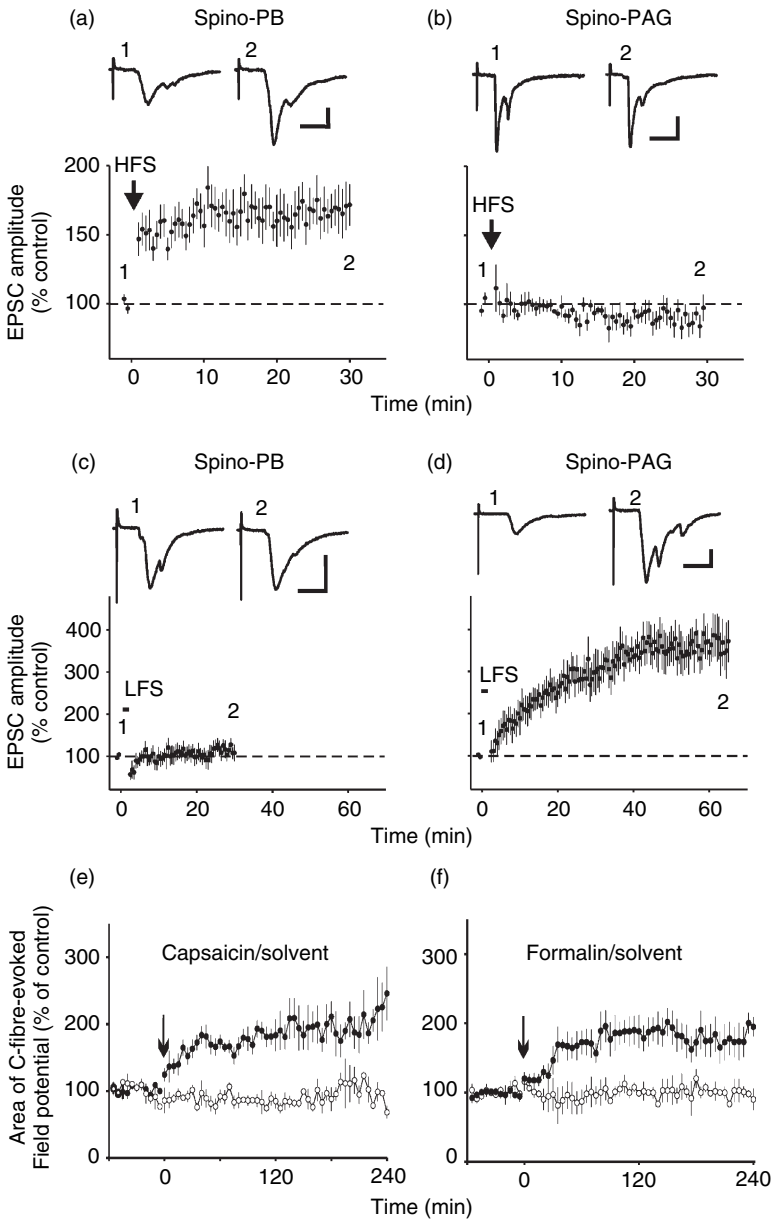


Figure 5 Spinal LTP can be induced by low-frequency afferent barrage evoked by electrical stimulation in vitro or inflammation of peripheral tissue in vivo. (a, b) High-frequency stimulation (HFS) of C-fibers selectively induced LTP in parabrachial (PB)-projecting but not in periaqueductal gray (PAG)-projecting lamina I neurons. (c, d) Low-frequency stimulation of C-fibers selectively induced LTP in PAG-projecting but not in PB-projecting lamina I neurons. Insets: Representative C-fiber-evoked excitatory postsynaptic currents (EPSCs) before and after C-fiber high frequency (a, b) or low-frequency (c, d) stimulation. Calibration bars: 20 ms/200 pA. (e, f) Inflammation induces LTP in the dorsal horn of the spinal cord *in vivo*. Extracellular recordings in the superficial dorsal horn following electrical stimulation of the sciatic nerve in vivo in anesthetized animals. Intraplantar injection (indicated by the arrow) of capsaicin (e) or formalin (f) induced spinal LTP *in vivo* (solid circles). Intraplantar injection of vehicle (open circles) failed to induce spinal LTP. Adapted from Ikeda H, Stark J, Fischer H, et al. (2006) Synaptic amplifier of inflammatory pain in the spinal dorsal horn. *Science* 312: 1659–1662.

and of other late-expressing genes such as prodynorphin and NK1 (Ji et al., 2002a; Kominato et al., 2003; Kawasaki et al., 2004; Song et al., 2005). Blockade of ERK activation in the dorsal horn decreases injury-induced hypersensitivity, NR1 phosphorylation, mGluR5-mediated increases in excitability, and CREB-mediated transcriptional regulation of c-fos, NK1, and prodynorphin.

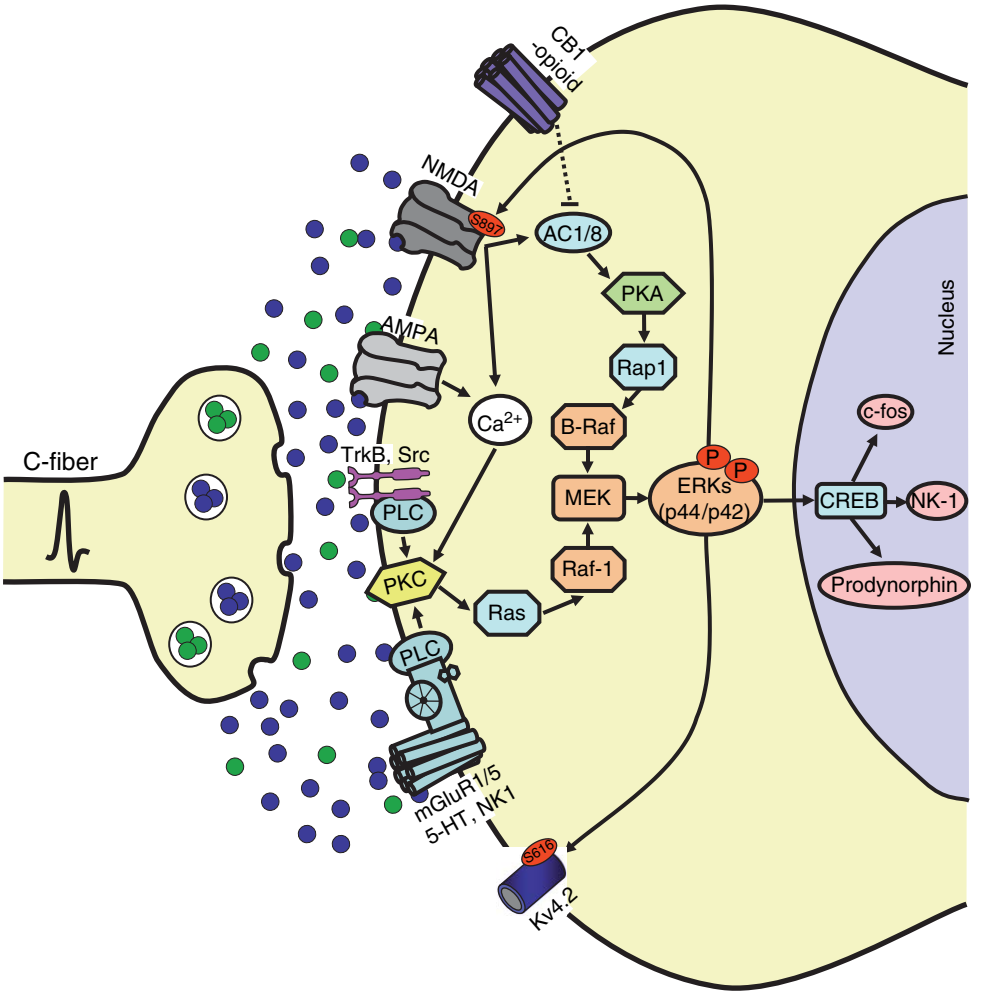


Figure 6 The extracellular signal-regulated protein kinase (ERK) signaling cascade in the dorsal horn contributes to central sensitization. Numerous cell-surface signals can activate the ERK signaling cascade in dorsal horn neurons in response to tissue injury. ERK directly phosphorylates the potassium channel Kv4.2 at Serine-616 (S616) and regulates phosphorylation of the NMDA receptor subunit NR1 at Serine-897 (S897). ERK can also translocate to the nucleus and activate CREB-dependent transcription of c-fos, NK1, and prodynorphin. 5-HT, 5-hydroxytryptamine (serotonin receptor); AC1/8, adenylyl cyclases 1 and 8; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptor; B-Raf, B-Raf kinase; Ca^{2+}, calcium; CB1, cannabinoid receptor 1; CREB, cAMP-response element binding protein; MEK, mitogen-activated protein kinase/ERK; mGluR1/5, metabotropic glutamate receptors 1 and 5; NK1, neurokinin 1 receptor; NMDA, *N*-methyl-D-aspartate receptor; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; Raf1, Raf-1 kinase; Rap1, repressor activated protein 1; Ras, Ras GTPase; Src, Src tyrosine kinase; TrkB, tyrosine kinase receptor B.

In addition to the increased excitatory transmission observed in the dorsal horn during central sensitization, a decrease in inhibitory tone also contributes to the development and maintenance of central sensitization. Decreased synaptic efficacy in local inhibitory circuits seems to result from decreased gamma-aminobutyric acid (GABA) release, decreased receptor expression and function, and, although a controversial hypothesis, by a loss of inhibitory interneurons. Decreases in depolarization-induced GABA release are observed in the dorsal horn in a nerve injury model (Lever et al., 2003). Reduction in primary afferent-evoked inhibitory transmission and a decrease in the expression of GABA receptors and the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD) are also observed after tissue or nerve injury (Castro-Lopes et al., 1995; Moore et al., 2002). In addition, intrathecal administration of GABA receptor antagonists has been shown to mimic central sensitization, further supporting

the hypothesis that decreased GABAergic tone contributes to the development of central sensitization (Sivilotti and Woolf, 1994). The dramatic loss of GABA and GAD immunostaining that is observed in the dorsal horn after nerve injury has led to the hypothesis that excitotoxic death of GABAergic neurons might contribute to decreased inhibitory tone after tissue injury. This is a controversial hypothesis, however, because stereological analysis of neuronal packing density and staining with apoptotic markers have failed to demonstrate a significant loss of neurons after tissue or nerve injury (Polgar et al., 2003, 2004, 2005). At the same time, the lack of changes in stereological studies is also debatable (Scholz et al., 2005). Despite this debate, it is well accepted that a decrease in GABAergic tone contributes to central sensitization; therefore, increasing GABAergic transmission in the dorsal horn could offer an alternative to alleviate pain. Preclinical studies in rodents have indeed demonstrated that transplanting neuronal cells bioengineered to synthesize GABA into the lumbar subarachnoid space decreases hypersensitivity in animal models of chronic neuropathic pain (Eaton et al., 2007).

4.05.4.3 Sensitization in Supraspinal Structures

Plastic changes in neuronal responses to noxious stimuli after tissue injury have also been observed and studied in supraspinal structures. Descending modulatory pathways are activated after tissue injury and can inhibit or facilitate the excitability of dorsal horn neurons. Descending inhibition or facilitation of pain transmission seems to depend on the nature of the stimulus, the environment in which the stimulus is applied, and the behavioral state of the animal. Supraspinal structures thus provide yet another level of modulation of pain processing. Pain-induced sensitization of the rostroventral medulla (RVM), the anterior cingulate cortex (ACC), and the amygdala has been described. The mechanisms of sensitization in these three supraspinal structures will be discussed in this section.

4.05.4.3.1 Rostroventral medulla

The RVM is a major source of descending modulation to the spinal dorsal horn. Realization that stimulation of the RVM could produce hyperalgesia led to the hypothesis that descending facilitation of nociceptive transmission could underlie injury-induced hypersensitivity (Zhuo and Gebhart, 1990). Many studies have followed up this observation and there is now substantial evidence that supports that sensitization of the RVM indeed contributes to the maintenance of injury-induced hypersensitivity (Porreca et al., 2002). Three types of neurons have been identified in the RVM. On-cells discharge just prior to withdrawal from a noxious stimulus, off-cells pause during withdrawal from a noxious stimulus, and neutral-cells show no nociception-related change in activity (Fields et al., 1983). Activation of on-cells has been proposed to facilitate nociceptive transmission in the dorsal horn. Consistent with this hypothesis, inflammation-induced persistent nociceptive input that is associated with hypersensitivity has been shown to increase the activity of on-cells in the RVM in an NMDA-dependent manner (Xu et al., 2007). Furthermore, chemical inactivation of the RVM or infusion of NMDA receptor antagonist into the RVM reduces injury-induced hypersensitivity (Urban et al., 1999; Wei and Pertovaara, 1999; Porreca et al., 2002). Interestingly, after inflammation, neutral-cells in the RVM that were initially unresponsive to noxious stimuli undergo a phenotypic switch, exhibiting response profiles that are characteristic of either on- or off-cells (Miki et al., 2002). Recruitment of neutral-cells might therefore contribute to RVM sensitization after inflammation. RVM sensitization has also been linked to the hypersensitivity that is commonly observed following opioid withdrawal. Opioid withdrawal-induced hypersensitivity is correlated with increased activity of on-cells in the RVM, and chemical inactivation of the RVM decreases opioid withdrawal-induced hypersensitivity (Bederson et al., 1990; Kaplan and Fields, 1991). Release of various neurotransmitters and neuropeptides such as glutamate, neurotensin, and cholecystokinin in the RVM have been shown to contribute to pain-induced RVM sensitization (Urban et al., 1996).

RVM modulation of injury-induced hypersensitivity seems to result, at least in part, from the activation of a spino-bulbo-spinal positive feedback loop (Suzuki et al., 2002). Following tissue injury, NK1-expressing projection neurons in the dorsal horn activate serotonergic neurons in the RVM, which in turn, via the activation of serotonergic descending facilitatory pathways, increase the excitability of dorsal horn neurons. Ablation of NK1-expressing dorsal horn neurons, which decreases injury-induced hypersensitivity, also decreases injury-induced activation of serotonergic RVM neurons. Furthermore, blockade of serotonin receptors in the dorsal horn reduces injury-induced

hypersensitivity, reproducing the effects of ablation of NK1-expressing dorsal horn neurons. *In vitro*, in an acute spinal cord slice preparation, serotonin application transforms silent synapses in the dorsal horn into functional ones, thus providing another potential mechanism for the RVM-mediated facilitation of nociceptive transmission in the dorsal horn (Li and Zhuo, 1998). In summary, activation of the spino-bulbo-spinal circuit in response to sustained nociceptive input generates a positive feedback loop that seems to perpetuate central sensitization, contributing in this way to the maintenance of injury-induced hypersensitivity.

4.05.4.3.2 Anterior cingulate cortex

Imaging studies in humans have repeatedly demonstrated increased neural activity in the ACC of healthy individuals as well as of chronic pain patients in response to noxious stimulation (Apkarian et al., 2005). Noxious stimulus-induced neuronal activity in the ACC positively correlates with the reported unpleasantness of the noxious stimuli (Rainville et al., 1997). For this reason, the ACC has been proposed to be a neural center that mediates the negative emotional component of pain. Consistent with this hypothesis, frontal lobotomies or cingulotomies in chronic pain patients have been shown to decrease the reported unpleasantness of pain without affecting nociception, or the sensory component of pain (Romanelli et al., 2004). Similarly, electrolytic lesions of the ACC have been shown to decrease injury-induced hypersensitivity in animal models of inflammatory and neuropathic pain (Donahue et al., 2001).

Zhuo and colleagues have extensively studied the cellular and molecular mechanisms that underlie ACC modulation of pain. They have shown that under normal physiological conditions, ACC neurons respond to peripheral noxious stimuli and can exhibit both LTP and LTD of synaptic transmission (Zhuo, 2005). Under chronic pain conditions, or after amputation of a digit, ACC neurons exhibit increased excitability, a long-lasting potentiation of noxious stimulus-induced synaptic responses, and lose the capability to undergo synaptic LTD (Wei et al., 1999; Wei and Zhuo, 2001; Gao et al., 2006) (**Figure 7**). Potentiation of synaptic responses to noxious stimuli and loss of LTD have therefore been proposed to mediate ACC modulation of nociception. Both ACC modulation of nociception and LTP are dependent on the activation of adenylyl cyclases 1 and 8 (AC1 and AC8) and on the expression levels of the NMDA receptor subunit NR2B (Wei et al., 2001, 2002; Wu et al., 2005). Thus, genetic elimination of AC1 and AC8 decreases injury-induced hypersensitivity and local activation of adenylyl cyclases in the ACC of these animals rescues the injury-induced hypersensitivity phenotype (Wei et al., 2002). On the other hand, genetic overexpression of NR2B in forebrain regions, including the ACC, increases inflammation-induced nociceptive behavior. Moreover, NR2B expression has been shown to be upregulated in the ACC after injury, and pharmacological blockade of these receptors in the ACC decreases injury-induced hypersensitivity. After tissue injury, activation of NMDA, AC1, and AC8 in the ACC results in long-lasting activation of the transcription factor CREB and in a subsequent increase in the transcription of immediate early genes such as c-fos (Wei et al., 2001, 2002). Transcriptional changes in the ACC are therefore thought to mediate pain-induced long-lasting sensitization of the ACC.

As mentioned, the ACC has been proposed to mediate an emotional component of pain. Multiple studies in animals support this hypothesis. ACC neurons have been shown to be activated during pain avoidance behaviors (Koyama et al., 2001). In addition, lesions of the ACC decrease both inflammation-induced conditioned place avoidance and noxious stimulus-induced escape-avoidance behavior after nerve injury (Johansen et al., 2001; Gao et al., 2004; LaGraize et al., 2004) (**Figure 8**). Furthermore, glutamatergic activation of the ACC during conditioning produces avoidance learning in the absence of peripheral noxious stimulation, suggesting that activation of the ACC is sufficient to induce pain unpleasantness (Johansen and Fields, 2004) (**Figure 8**). In support of this hypothesis, human neuroimaging studies have shown a reduction in ACC activity when the unpleasantness of pain is removed from the pain experience by performing hypnotic suggestions that affect pain unpleasantness without altering the perceived intensity of pain (Rainville et al., 1997).

4.05.4.3.3 Amygdala

The amygdala is part of the spino-ponto-amygdaloid pathway, a major ascending pronociceptive pathway that originates in lamina I of the spinal cord and medullary dorsal horn and relays in the parabrachial (PB) area to terminate in the central nucleus of the amygdala (CeA) (Bernard et al., 1989; Bernard and Besson, 1990; Jasmin et al., 1997). The amygdala also

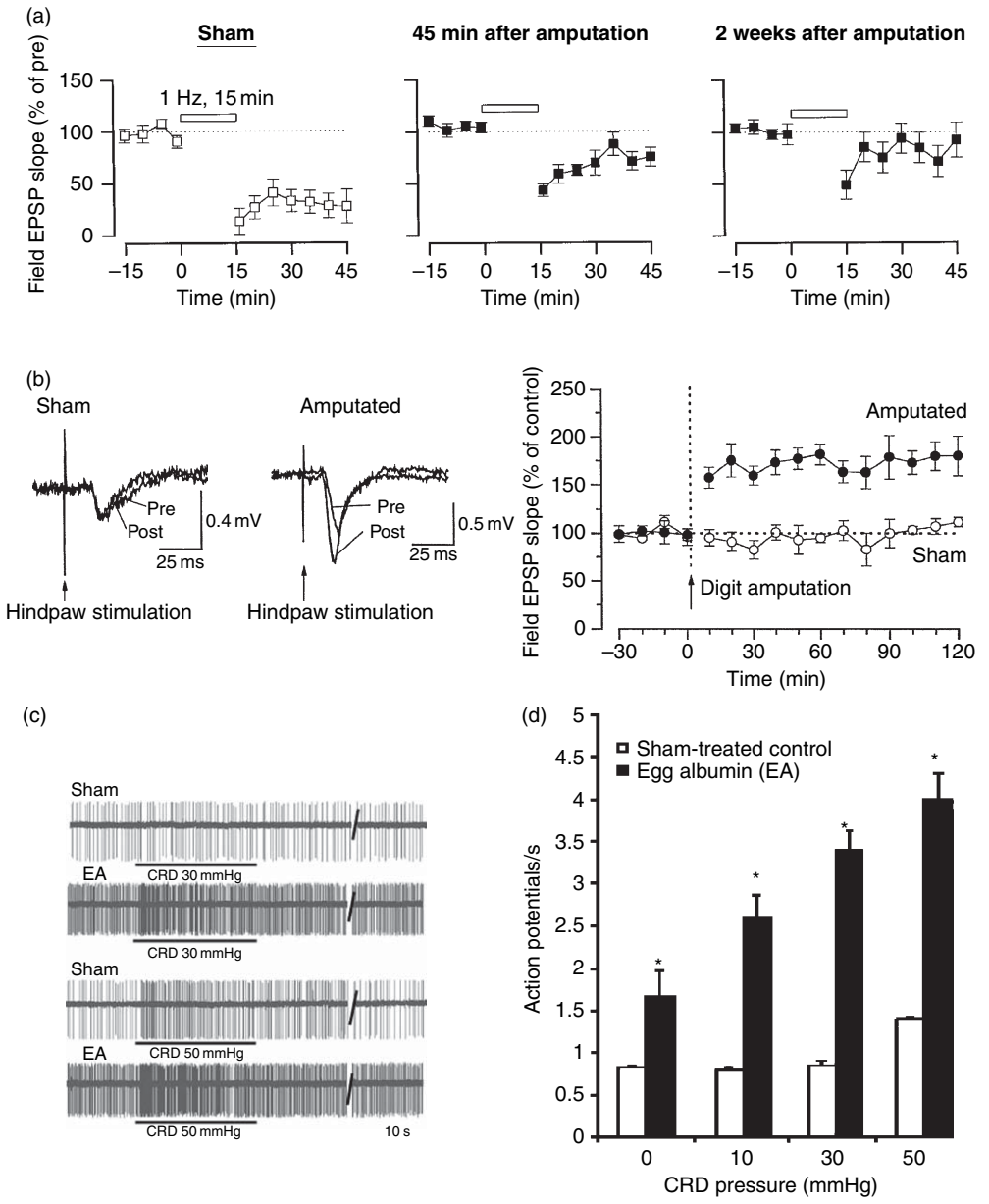


Figure 7 Injury induces loss of long-term depression (LTD), potentiation of synaptic transmission, and increased excitability in the anterior cingulate cortex (ACC). (a) Long-term potentiation (LTP) was recorded in ACC slices from sham animals or animals that had a single hindpaw digit amputated. Low-frequency stimulation, indicated by the horizontal bar, induced a robust LTD in the ACC of sham animals (left panel). An identical stimulation failed to induce LTD in the ACC of amputated animals 45 min (middle panel) and 2 weeks (right panel) after the amputation. (b) Amputation of a single hindpaw digit potentiates synaptic transmission in the ACC. Left panel shows representative traces of hindpaw-stimulation-evoked EPSPs in the ACC before (Pre) and after (Post) sham treatment or amputation. Right panel shows the time-course of hindpaw-stimulation-evoked EPSPs in the ACC. Amputation, but not sham treatment, induced a long-lasting potentiation of ACC responses to hindpaw stimulation. (c) Increased excitability in the ACC of viscerally hypersensitive rats. Representative single-unit recordings in the ACC in response to colorectal distention (CRD) in sham-treated and viscerally hypersensitive (EA) animals. Viscerally hypersensitive animals show increased background activity and CRD-evoked responses in the ACC when compared to sham-treated animals. (d) Frequency of firing in the ACC in response to CRD increases in a stimulus-intensity-dependent manner in viscerally hypersensitive animals (EA) when compared to sham-treated animals. Adapted from (a) Wei F, Li P, and Zhuo M (1999) Loss of synaptic depression in mammalian anterior cingulate cortex after amputation. *J. Neurosci.* 19: 9346–9354; (b) Wei F and Zhuo M (2001) Potentiation of sensory responses in the anterior cingulate cortex following digit amputation in the anaesthetised rat. *J. Physiol.* 532: 823–833; and (c, d) Gao J, Wu X, Owyang C, and Li Y (2006) Enhanced responses of the anterior cingulate cortex neurones to colonic distension in viscerally hypersensitive rats. *J. Physiol.* 570: 169–183.

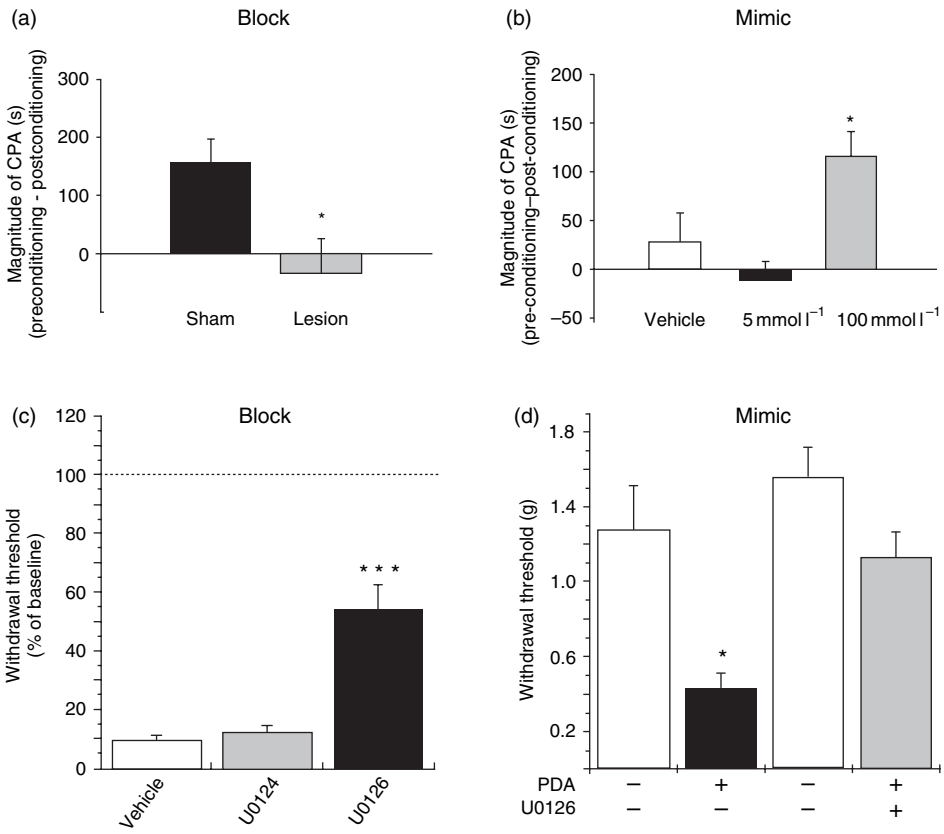


Figure 8 The anterior cingulate cortex (ACC) and the amygdala are important sites of modulation of inflammation-induced behavioral sensitization. (a) Inflammation-induced conditioned place avoidance (CPA) is blocked by lesions of the ACC. Animals with sham treatment show a higher magnitude of CPA scores than animals with ACC lesions. (b) Glutamatergic stimulation of the ACC mimics inflammation-induced conditioned place avoidance in the absence of inflammation. Animals were intra-ACC infused with vehicle, 5 mmol l^{-1} or 100 mmol l^{-1} of the glutamate agonist homocysteic acid (HCA). Infusion of HCA into the ACC induced a robust CPA in a dose-dependent manner. (c) Pharmacological blockade of ERK activation in the amygdala decreases inflammation-induced mechanical hypersensitivity. Animals were injected with 5% formalin into the hindpaw to induce inflammation. The MEK inhibitor U0126, the structural analog control compound U0124, or vehicle was infused into the amygdala and the effects of these treatments on mechanical thresholds were analyzed. Infusion of U0126 into the amygdala decreased inflammation-induced hypersensitivity when compared to vehicle- or U0124-treated animals. (d) Pharmacological activation of ERK in the amygdala mimics inflammation-induced mechanical hypersensitivity in the absence of inflammation. Mice received an intra-amygdala infusion of the phorbol ester PDA (120 pmol), which results in robust ERK activation, or a co-infusion of PDA (120 pmol) plus U0126 (1.5 nmol), and mechanical withdrawal thresholds were measured. Infusion of PDA into the amygdala decreased paw-withdrawal thresholds in response to mechanical stimulation when compared to baseline thresholds. PDA effects were blocked by co-infusion with U0126. Adapted from (a) Johansen JP, Fields HL, and Manning BH (2001) The affective component of pain in rodents: Direct evidence for a contribution of the anterior cingulate cortex. *Proc. Natl. Acad. Sci. USA* 98: 8077–8082; (b) Johansen JP and Fields HL (2004) Glutamatergic activation of anterior cingulate cortex produces an aversive teaching signal. *Nat. Neurosci.* 7: 398–403; and (c, d) Carrasquillo Y and Gereau RW IV (2007) Activation of the extracellular signal-regulated kinase in the amygdala modulates pain perception. *J. Neurosci.* 27: 1543–1551.

receives a direct projection from the spinal cord, with most of this tract originating in lamina V of the dorsal horn where nociceptive-specific neurons are located (Burstein and Potrebic, 1993). In addition to being part of ascending pain pathways, the amygdala has also been proposed to be part of a descending modulatory pain pathway (Hopkins and Holstege, 1978; Helmstetter et al., 1998; McGaraughty and Heinricher, 2002; Shane et al., 2003). Projections from the amygdala to the PAG have been identified using anatomical retrograde tracers (Hopkins and Holstege, 1978); these projections are thought to feed into the PAG-RVM-dorsal horn descending modulatory pain pathway (**Figure 2**).

In vivo electrophysiological recordings have mapped amygdala neurons that respond to peripheral

noxious stimuli (Bernard et al., 1992; Neugebauer and Li, 2002, 2003) (**Figure 9**). The majority of these neurons are located in the CeA, which is the target of the spino-ponto-amygdaloid ascending pain pathway (Bernard et al., 1989; Bernard and Besson, 1990; Jasmin et al., 1997). Under normal physiological conditions, CeA neurons have been shown to be preferentially excited or inhibited by cutaneous noxious stimuli or by noxious stimulation of deep tissue in a stimulus intensity-dependent manner, with receptive fields that included most areas of the body (four limbs, tail, trunk, face, cornea, tongue, and intraoral region). Moreover, in chronic pain conditions, CeA neurons have been shown to undergo neuroplastic changes, suggesting that plasticity in the amygdala might underlie pain hypersensitivity (Neugebauer and Li, 2003) (**Figure 9**). For example, induction of arthritis has been shown to result in increased responsiveness of CeA neurons to nociceptive afferent input, reduction of response threshold, and receptive field enlargement (Neugebauer and Li, 2003) (**Figure 9**). This arthritis-induced amygdala sensitization is dependent on the activation of mGluR1, both NMDA and non-NMDA ionotropic glutamate receptors, and the CGRP1 receptor (Li and Neugebauer, 2004a, 2004b; Han et al., 2005b).

Sensitization of amygdala neurons is preserved in slices obtained from arthritic animals (**Figure 9**). This has allowed for further examination of the mechanisms of amygdala sensitization at a cellular level. Whole-cell voltage-clamp recordings of CeA neurons from brain slices revealed enhanced synaptic transmission and increased excitability in CeA neurons after the induction of arthritis, visceral pain, or neuropathic pain (Neugebauer et al., 2003; Han et al., 2004, 2005b; Ikeda et al., 2007). Interestingly, in the neuropathic pain model, the degree of amygdala potentiation positively correlates with the degree of behavioral hypersensitivity (Ikeda et al., 2007). Consistent with the *in vivo* recording, the changes in synaptic transmission in slices from arthritic and visceral pain animals are dependent on the activation of mGluR1, NMDA receptors, and the CGRP1 receptor. Surprisingly, amygdala potentiation after visceral pain is not dependent on NMDA receptor activation, suggesting that different types of pain activate different molecular pathways in the amygdala. Persistent pain-induced amygdala sensitization correlates with upregulation of mGluR1 protein expression levels and with increased phosphorylation of the NR1 subunit of the NMDA receptor in the amygdala (Neugebauer et al., 2003; Bird et al., 2005). Finally, enhancement of group III mGluR-mediated inhibition of CeA synaptic transmission (Han et al., 2004) and increases in PKA-mediated NMDA receptor function (Bird et al., 2005) have also been observed after the induction of arthritis.

Despite all the progress made in characterizing the responses of amygdala neurons to noxious stimuli under conditions of persistent pain, very little is known about the contribution of the amygdala to the behavioral expression of injury-induced persistent hypersensitivity. Numerous studies have repeatedly demonstrated that lesions or chemical inactivation of the amygdala do not affect baseline nociception. Surprisingly, at present, the effect of lesions or chemical inactivation of the amygdala on injury-induced persistent hypersensitivity has not been evaluated. However, ERK activation is observed in noxious-responsive amygdala neurons during inflammation, and pharmacological blockade of ERK activation in the amygdala reduces inflammation-induced hypersensitivity (Carrasquillo and Gereau, 2007) (**Figures 8 and 9**). In addition, blockade of mGluR1 or the CGRP1 receptor in the amygdala has been shown to reduce arthritis-induced hypersensitivity, demonstrating that neuroplastic changes in the amygdala indeed contribute to behavioral hypersensitivity (Han et al., 2005b; Han and Neugebauer, 2005). Moreover, pharmacological activation of ERK in the amygdala has been shown to induce behavioral hypersensitivity that mimics inflammation-induced hypersensitivity but occurs in the absence of peripheral tissue injury, suggesting that ERK activation in the amygdala is sufficient to induce pain hypersensitivity (Carrasquillo and Gereau, 2007).

Similar to the ACC, the amygdala has also been proposed to mediate an emotional component of pain. Consistent with this, lesions of the amygdala significantly reduce conditioned place avoidance induced by paw inflammation, electric foot shock, or visceral pain in rats (Tanimoto et al., 2003; Gao et al., 2004). In addition, blockade of mGluR1, mGluR5, or the CGRP1 receptor in the amygdala decreases vocalization after noxious stimulation of an arthritic knee, a behavioral response that has been proposed to represent an affective component of pain (Han et al., 2005a, 2005b; Han and Neugebauer, 2005).

4.05.5 Cognitive Component of Pain

Many psychobehavioral studies have demonstrated that pain-related neural activity, and concurrently,

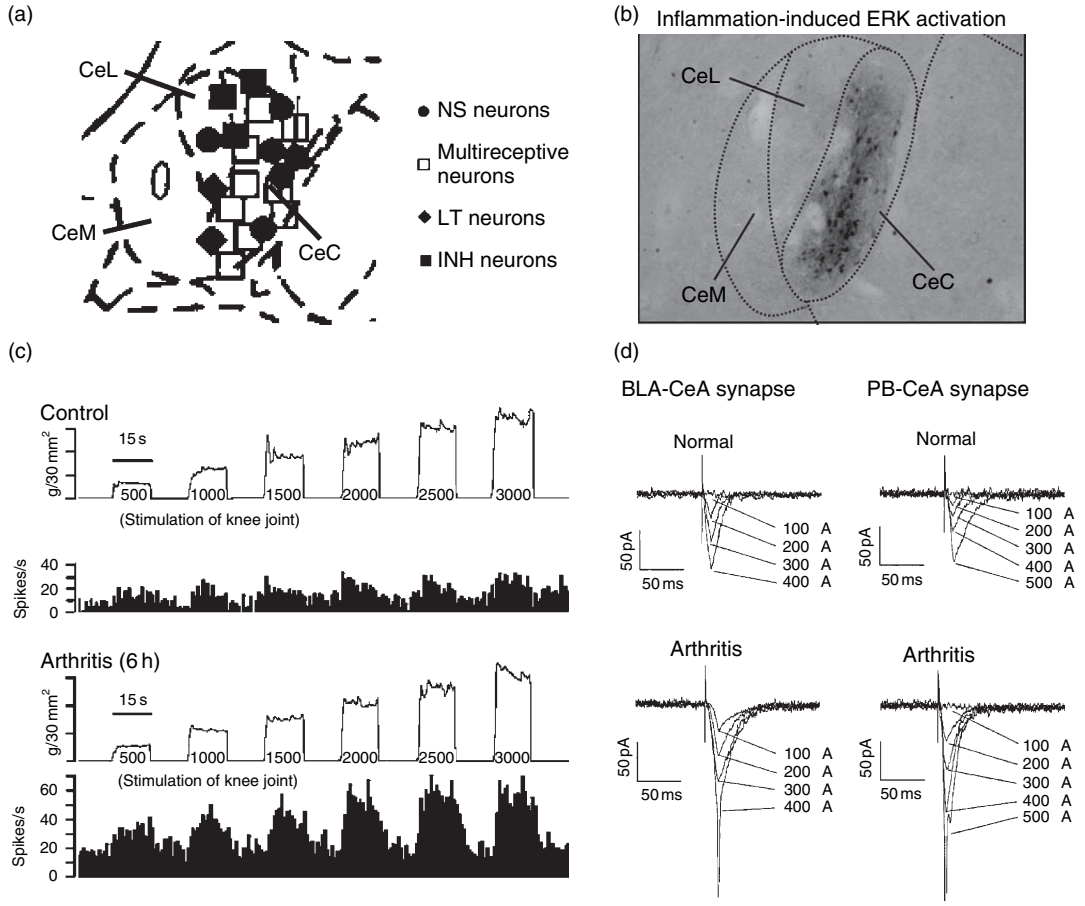


Figure 9 Neuroplastic changes in the amygdala occur after tissue injury. (a, b) Diagrams depicting the subdivisions of the central nucleus of the amygdala (CeA) in a rodent coronal brain section. CeM, medial subdivision; CeL, lateral subdivision; CeC, lateral capsular subdivision. (a) Amygdala neurons with knee-joint input are localized to the lateral capsular subdivision of the CeA. NS, nociceptive specific; LT, low threshold; INH, inhibited. (b) Immunohistochemistry for phospho-ERK in the amygdala after formalin-induced inflammation of one hindpaw. Inflammation-induced ERK activation is localized to the lateral capsular subdivision of the CeA. (c) Responses of amygdala neurons to deep tissue stimulation increase after the induction of arthritis. Representative single-unit recordings in the amygdala in response to graded stimulation of the knee joint in control animals and after the induction of arthritis. Arthritic animals show increased background activity and deep tissue stimulation-evoked responses in the amygdala when compared to control animals. (d) Synaptic transmission in the amygdala is enhanced after the induction of arthritis. Lower thresholds for evoked EPSCs and orthodromic spike generation were observed in slices from arthritic animals after stimulation of either the basolateral amygdala-central amygdala (BLA-CeA) or the parabrachial area-central amygdala (PB-CeA) when compared to slices from control animals. Adapted from (a) Neugebauer V and Li W (2002) Processing of nociceptive mechanical and thermal information in central amygdala neurons with knee-joint input. *J. Neurophysiol.* 87: 103–112; (b) Carrasquillo Y and Gereau RW IV (2007) Activation of the extracellular signal-regulated kinase in the amygdala modulates pain perception. *J. Neurosci.* 27: 1543–1551; (c) Neugebauer V and Li W (2003) Differential sensitization of amygdala neurons to afferent inputs in a model of arthritic pain. *J. Neurophysiol.* 89: 716–727; and (d) Neugebauer V, Li W, Bird GC, Bhave G, and Gereau RW IV (2003) Synaptic plasticity in the amygdala in a model of arthritic pain: Differential roles of metabotropic glutamate receptors 1 and 5. *J. Neurosci.* 23: 52–63.

the sensation of pain, can be strongly modulated by emotions and cognitive awareness (Turk, 2003; Lewandowski, 2004). For many years, anecdotal evidence has supported the idea that attention and distraction can influence pain perception. Distraction has thus been commonly used by parents and pediatricians to perform procedures that produce minor discomfort and pain in children such as injections, cleaning of wounds, and tooth extraction. Scientific studies in both humans and nonhuman primates also support the hypothesis that attention and distraction influence pain perception (Villemure

and Bushnell, 2002). These studies show that increased attention to a painful stimulus increases both pain intensity ratings and neuronal activity in certain brain regions such as the ACC, thalamus, and the prefrontal cortex and that, conversely, distraction or diverted attention from the pain stimulus decreases pain intensity ratings, reported unpleasantness of pain, and neuronal activity (Villemure and Bushnell, 2002). Although much progress has been made in elucidating the neural mechanisms of modulation of pain perception by attention and distraction, the results of these studies are hard to interpret because of the difficulty in controlling and measuring the degree of attention between subjects and because other factors such as vigilance, mood, and anxiety can also contribute to the reported pain and the unpleasantness ratings.

Anticipation or expectation can also strongly influence the perception of pain (Bushnell et al., 2004; Vase et al., 2004). This works in both directions; thus, anticipation or expectation of a painful stimulus increases the intensity of perceived pain, and expectation of pain relief decreases the intensity of perceived pain. The latter is classically exemplified by the phenomenon of placebo analgesia. Placebo analgesia is accompanied by decreases in neuronal activity of pain-related brain regions such as the ACC, insular cortex, and thalamus (Vase et al., 2004). Conversely, anticipation and expectation of a painful stimulus activates brain regions associated with pain processing such as the ACC, prefrontal cortex, insular cortex, and PAG (Bushnell et al., 2004). Interestingly, ACC activity in response to painful stimulation is very similar to the activation induced by anticipation of a painful stimulus even when the anticipation is not followed by painful stimulation. These results suggest that cognitive processes such as expectation can strongly influence the brain's responses to certain stimuli and ultimately affect the perception of pain. The power of cognitive awareness in pain perception was very well exemplified in a recent study by deCharms and colleagues at Stanford University, which showed that both healthy volunteers and chronic pain patients can learn to control their pain-induced neural activity in the ACC by using various cognitive strategy guidelines and real-time neuroimaging feedback (deCharms et al., 2005). This study specifically showed that modulation of ACC activity is accompanied by an associated change in reported pain perception.

4.05.5.1 Implications for Pain Management

An interesting yet counterintuitive fact is that injury is neither necessary nor sufficient to induce pain (Fields, 2000). In many instances, pain is not necessarily the manifestation of an injury but rather the manifestation of pathophysiological neural changes in the pain neuraxis. In support of this hypothesis, studies in both animals and humans have shown that electrical stimulation of certain brain regions can elicit pain in the absence of injury (Fields, 2000). Furthermore, as mentioned in previous sections of this chapter, pharmacological manipulations in the amygdala or the ACC can mimic inflammation-induced pain hypersensitivity in the absence of tissue injury or can result in avoidance learning in the absence of peripheral noxious stimulation, respectively (Johansen and Fields, 2004; Carrasquillo and Gereau, 2007) (**Figure 8**). A classic clinical example of pain without injury is seen in the phantom limb syndrome, when patients with amputated limbs report feelings of pain in the missing limb.

The limbically augmented pain syndrome (LAPS) theory could offer an explanation for situations in which pain is not accompanied by any obvious injury. The LAPS theory states that stress and exposure to emotionally traumatic events lead to a sensitized corticolimbic state that is analogous to central sensitization in the spinal cord and brainstem, but occurs in supraspinal structures that subserve both nociceptive processing and affective regulation (Rome and Rome, 2000). This sensitized corticolimbic state can in turn increase the perception of pain.

The scientific evidence demonstrating that emotions and cognitive factors strongly influence pain perception and the data suggesting that neuronal modifications in different regions of the pain neuraxis could explain these psychological modulations changed the clinical view of pain and consequently the approaches that are typically used for pain management. In addition to the more traditional pharmacological, physical, and surgical treatments for chronic pain, now a cognitive-behavioral approach is also commonly included in standard pain management practices. Cognitive-behavioral therapy (CBT) is designed to offer patients strategies to cope with their pain by identifying beliefs and fears that interfere with their functioning and well-being (Osborne et al., 2006). CBT has been shown to improve not only the reported pain intensity but also other non-pain-related variables such as mood, depression, pain interference, disability, return to work, and health care utilization (Kerns et al., 2006).

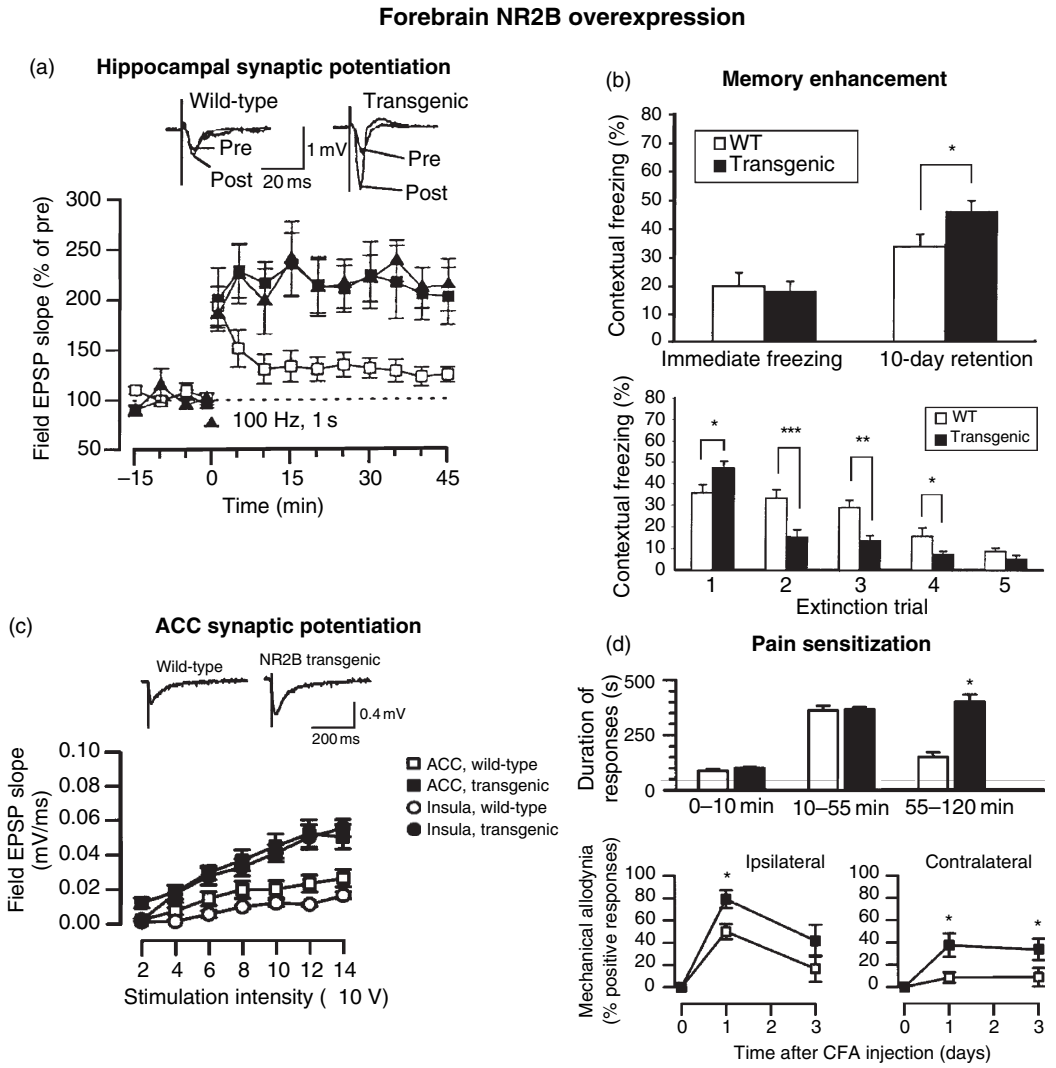


Figure 10 Overexpression of *N*-methyl-D-aspartate-type glutamate receptor subunit (NR2B) in the forebrain facilitates synaptic potentiation in the hippocampus and the ACC and enhances learning and memory and pain hypersensitivity in mice. (a) Synaptic potentiation in the hippocampus of NR2B-overexpressing mice. Time course of evoked excitatory postsynaptic potentials (EPSCs) in slices from wild-type (WT) and transgenic animals. Tg-1, filled squares; Tg-2, filled triangles; WT, open squares. Tetanic stimulation-evoked EPSPs are potentiated in NR2B overexpressing mice when compared to WT. Inset: Representative evoked EPSP before (pre-) and after (post-) tetanic stimulation in WT (left) and transgenic (right) slices. (b) Enhancement of contextual fear memory (top panel) and faster fear extinction (bottom panel) in NR2B-overexpressing mice. Top panel: Animals received a single conditioned stimulus/unconditioned stimulus (CS/US) pairing training. Retention test was performed 10 days after training. Percentage of freezing rate was increased in NR2B-overexpressing mice when compared to WT. Bottom panel: Animals received a single CS/US pairing training and were then subjected to five extinction trials. Percentage of freezing decreased faster in NR2B-overexpressing mice than in WT mice. (c) NR2B overexpression enhances NMDA receptor-mediated synaptic responses in the ACC. The slope of the field EPSP in response to graded stimulation intensities is higher in slices from NR2B-overexpressing mice than in slices from WT animals. Inset: Representative field EPSPs recorded from ACC slices of WT and NR2B-overexpressing animals. (d) Behavioral responses to intraplantar injection of formalin (top panel) or complete Freund's adjuvant (CFA) (bottom panel) are increased in NR2B-overexpressing mice. Top panel: Total time that WT (□) and NR2B-overexpressing (■) mice spent engaging nociceptive behavior in each phase of the formalin test. NR2B-overexpressing mice spent more time engaging in nociceptive behavior in the third phase of the formalin test when compared to WT mice. Bottom panel: The percentage of positive responses to mechanical stimulation was measured before (time 0) and 1 and 3 days after intraplantar injection of CFA. Increased percentage of responses after CFA was observed in NR2B-overexpressing mice (■) when compared to wild-type mice (□). Adapted from (a, b) Tang YP, Shimizu E, Dube GR, et al. (1999) Genetic enhancement of learning and memory in mice. *Nature* 401: 63–69, with permission from Nature Publishing Group; (c, d) Wei F, Wang GD, Kerchner GA, et al. (2001) Genetic enhancement of inflammatory pain by forebrain NR2B overexpression. *Nat. Neurosci.* 4: 164–169, with permission from Nature Publishing Group.

4.05.6 Learning and Memory Versus Chronic Pain

As we have discussed in this chapter, neural changes occur at different sites in the pain neuraxis in response to injury. This plasticity is thought to underlie the development and maintenance of chronic pain. Similar changes in excitability and synaptic strength occur in the hippocampus, cortex, and amygdala and are thought to underlie learning and memory. The similarities in the cellular mechanisms underlying pain and learning and memory are striking; many cellular processes and signaling molecules that are known to be important mediators of pain are also important regulators of learning and memory (Ji et al., 2003). The overlapping function of these cellular processes in pain and learning and memory was evident in genetically modified mice that overexpress NR2B in the forebrain (Tang et al., 1999; Wei et al., 2001) (**Figure 10**). Overexpression of NR2B in the forebrain resulted in facilitation of synaptic potentiation in the hippocampus and in superior performance in learning and memory tasks (Tang et al., 1999). A separate study showed that, unfortunately, these NR2B-overexpressing mice are smarter at the expense of also being more susceptible to pain (Wei et al., 2001). As in the hippocampus, enhanced synaptic responses were also observed in NR2B-overexpressing mice in the ACC and insular cortex, two key structures for pain processing. Accordingly, these mice also exhibited enhanced nociceptive responses after inflammation. The similarities in the neural mechanisms underlying pain and learning and memory have made the development of novel analgesic therapies even more challenging. At the same time, as discussed in the previous section of this chapter, the tight relationship that exists between pain and cognition has led to the development of a cognitive-behavioral approach to managing chronic pain that has been shown to be highly effective in helping patients cope with their pain.

Acknowledgments

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4.06 Molecular Mechanism of Associative Learning in the Bee

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4.06.1 Associative Olfactory Learning in Bees

To cope with the highly variable environment, as well as individual and social tasks, honeybees perform a variety of behaviors including orientation, foraging, and social communication. When searching bees discover flowers providing food, they learn to associate the visual and olfactory signals with the reward (nectar and pollen) and quickly optimize their foraging strategy based on nectar flow and sucrose concentration. For optimal learning, stimuli of food sources like odor and color (conditioned stimuli, CS) that predict reward have to be perceived before the bees actually experience the sucrose reward (unconditioned stimuli, US) (reviewed in Menzel and Müller, 1996). This associative learning under natural conditions reveals many characteristics of associative learning in mammals. Studying the honeybee's learning behavior in the laboratory was a major breakthrough and provided the opportunity to study associative conditioning of the proboscis extension reflex (PER) at the behavioral, the cellular, and the molecular level under controlled conditions (**Figure 1**). The PER that is elicited by an appetitive stimulus (sucrose) is conditioned by pairing an odor stimulus (CS, conditioned stimulus) with a sucrose reward (US, unconditioned stimulus). A single pairing that lasts only a few seconds induces an associative memory that is initially at a high level but decays over the following days (Menzel and Müller, 1996; Menzel, 1999). As in other model systems, repetitions of conditioning trials induce a stable memory (**Figure 2(a)**). In the honeybee, three successive conditioning trials, given within a time window of a few minutes, induce a long-lasting memory that is stable for many days (>7 days) and which shows all properties of a long-term memory (LTM) (Menzel and Müller, 1996). Memories induced by a single or by repeated conditioning trials differ in their sensitivity to amnestic treatments like cooling. While cooling immediately after a single-trial conditioning impairs memory formation, the same treatment after multiple-trial conditioning does not (Menzel et al., 1974; Erber et al., 1980; Müller, 1996). Thus, repetition of conditioning trials accelerates the transfer into amnesia-resistant memory.

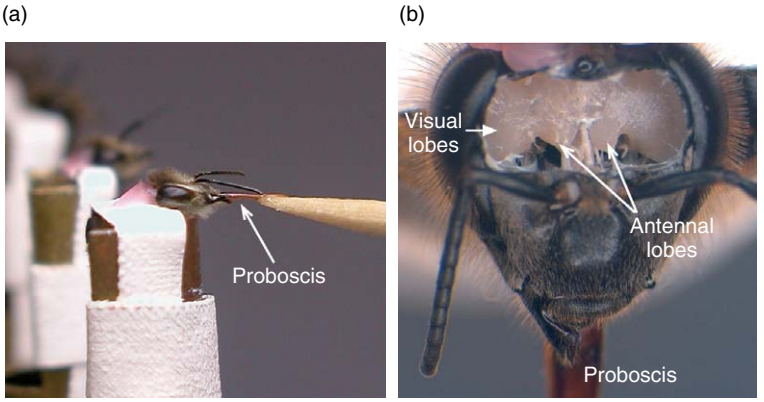


Figure 1 Honeybees fixed in metal tubes for olfactory conditioning of the proboscis extension reflex (PER). (a) Bees mounted in the metal tubes can freely move their proboscis and antennae. Stimulation of the antennae or the proboscis with sucrose solution (toothpick) elicits the PER. (b) Dorsal view of a honeybee head with open head capsule. The direct access to neuronal networks like the antennal lobes allows monitoring of neuronal activity and manipulation of signaling cascades during learning *in vivo*.

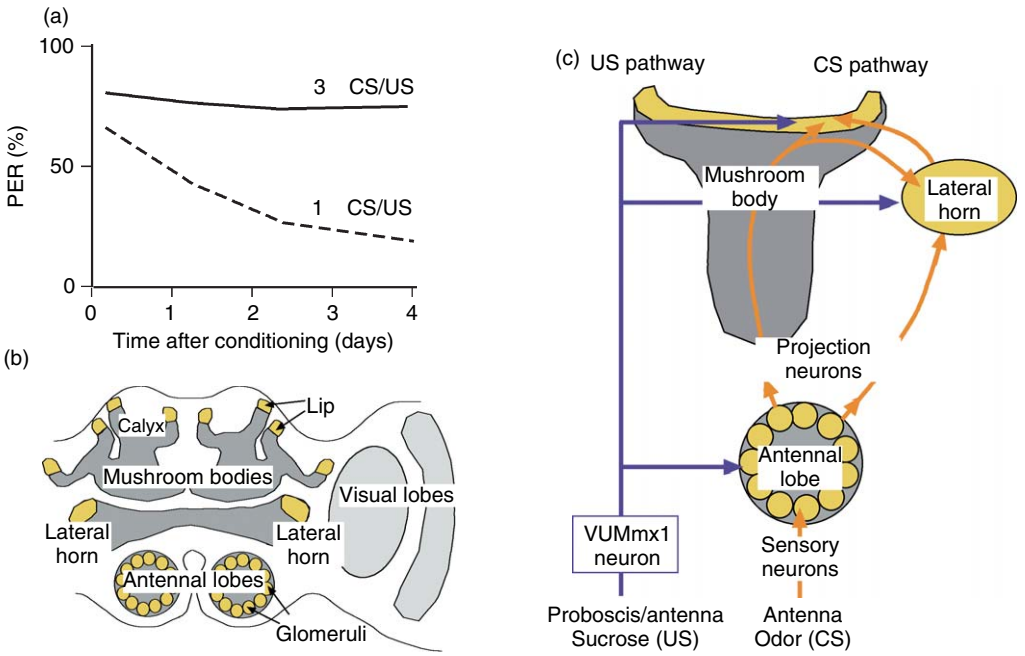


Figure 2 Associative olfactory learning in honeybee and the underlying neuronal circuits. (a) Associative conditioning of the PER consists of the pairing of an odor, the conditioned stimulus (CS), with a following sucrose reward, the unconditioned stimulus (US). A single pairing (1×CS/US) that lasts a few seconds leads to formation of a memory that decays over days. Multiple conditioning trials (3×CS/US with an intertrial interval of 2 min) induce a robust long-term memory that provides the basis for the analysis of the molecular signaling cascades underlying learning and memory formation. (b) The schematic section through the honeybee brain shows the major neuronal circuits implicated in processing olfactory and visual information. While the antennal lobes and the visual lobes are primary sensory centers, the mushroom bodies are sites that process different sensory modalities. The antennal lobes with their glomerular structure, the lip of the mushroom body calyces, and the lateral horn are areas implicated in olfactory information processing. (c) The neuronal circuits that mediate CS and the US information are well characterized in honeybees. CS pathway: the antennal lobes are the primary processing site of odor information and receive their input from sensory neuron in the antenna. CS information leaves the antennal lobes via projection neurons that transmit the information to the calyces of the mushroom bodies and the lateral horn. The antennal lobes, the calyces of the mushroom bodies, and the lateral horn are convergence sites of the CS and the US pathways. These brain areas are innervated by the ventral unpaired median (VUM) mx1 neuron that can substitute the US function in associative learning. The neuronal circuits connecting the output sites of these brain areas to the motor circuitry mediating the PER are unknown.

4.06.2 Neural Circuits Mediating Associative Olfactory Learning

The brain areas and neuronal circuits mediating odor information (CS pathway) and reward information (US pathway) in associative learning in the honeybee have been analyzed using a variety of techniques and approaches. While histological and immunohistological techniques provided the basic information concerning morphological features of the neuronal pathways, studies using electrophysiological and optical recording approaches in intact animals added important functional aspects to our knowledge of the neuronal circuits involved in CS and US processing (**Figures 2(b)** and **2(c)**).

4.06.2.1 CS Pathway

Olfactory information from the chemosensory receptors on the antennae is relayed via the antennal lobes (AL) to the calyces of the mushroom bodies (MB) and the lateral horn (LH) (**Figure (2c)**). In each AL, the 160 glomeruli comprise sites of dense synaptic connections between sensory neurons (≈60 000), local interneurons (4000) and projection neurons (PNs) (≈800) (Galizia and Menzel, 2000). A considerable fraction of the local interneurons connecting the glomeruli within an AL exhibit gamma-aminobutyric acid (GABA) immunoreactivity (Schäfer and Bicker, 1986). This GABAergic network modulates the overall activity in the ALs and, together with a second glomerular-specific inhibitory network, contributes to the sharpening of the odor representation at the level of output neurons that transmit the information to higher-order neuropils (Sachse and Galizia, 2002). Blocking of the inhibitory network in the ALs impairs odor discrimination but not learning (Stopfer et al., 1997).

Different types of PNs transmit odor information from the AL to the lip region of the MB calyces and the LH in the lateral protocerebrum (**Figure 2(c)**). While PNs in the median antennocerebral tract show odor-specific activity profiles for different odors and conduct the information with a delayed code, the PNs in the lateral antennocerebral tract have rather unspecific broadband activity profiles for different odors and transmit the information without delay (Müller et al., 2002). This points to a dual coding of odor information by extracting and transmitting different features in the time domain by different sets of PNs. Immunohistochemical studies demonstrate the presence of acetylcholinesterase and acetylcholine receptors in the PNs and their target area, the lip region of the MB. Thus, the major CS input into the MBs seems to be cholinergic (Kreissl and Bicker, 1989).

The MBs, which consist of densely packed Kenyon cells (KC) are prominent, well-characterized brain structures and play a central role in learning and memory formation in several species (*See* Chapters 1.28, 4.07). The olfactory input by the PNs is confined to the lip region of the calyces. Optical recording techniques demonstrate that odors evoke combinatorial activity patterns in both the glomeruli of the ALs and the lip region of the MBs. While these patterns are prominent at the level of the ALs, odors generate only brief and sparse responses at stimulus onset in the KC of the MBs (Szyszka et al., 2005). The inhibitory recurrent neurons connecting output areas of MBs with input in the lip region of the MBs are probably involved in this process (Ganeshina and Menzel, 2001).

4.06.2.2 US Pathway

Appetitive chemosensory pathways from the antennae and the proboscis project to the suboesophageal ganglion and terminate near motor neurons involved in proboscis extension. Ventral unpaired median (VUM) neurons receive chemosensory input, and the single identified VUMmx1 neuron can substitute for the US function in associative learning, as demonstrated by depolarization of VUMmx1 shortly after a CS presentation (Hammer, 1993). The VUMmx1 innervates the ALs, the MBs, and both LHs and thus converges with brain areas that process odor information (**Figure 2(c)**). Injections of octopamine, the putative transmitter of VUMmx1 (Kreissl et al., 1994), either into the ALs or the MBs substitute for the US function (Hammer and Menzel, 1995, 1998), supporting the role of VUMmx1 in reward processing in these two brain areas. Pairing of CS stimulation with octopamine injection into either the AL or the MB leads to a conditioned response as tested after 20 min, supporting independent contributions of the AL and MB in memory formation. This function in memory formation, however, differs between the AL and the MB, since only CS presentation followed by octopamine injections into the AL leads to a normal detectable acquisition. Such different roles of the ALs and the MBs have initially been proposed by experiments using local cooling of AL and MB as amnestic treatment to interfere with associative learning (Menzel et al., 1974; Erber et al., 1980).

4.06.3 The Molecular Cascades Mediating CS and US Pathways in Olfactory Learning

The robust learning paradigm in combination with the accessibility and the size of the honeybee brain provides the unique opportunity to apply biochemical techniques to monitor *in vivo* induced activities of signaling cascades (Hildebrandt and Müller, 1995a,b). Rapid termination (<0.5 s) of biochemical reactions by liquid nitrogen freezing of the whole animal, followed by freeze-drying, tissue dissection, and optimized biochemical assays, allows the determination of learning-induced activation of signaling cascades in brain areas like the ALs and the MBs at defined times after *in vivo* stimulation.

The search for the signaling cascade contributing to US processing revealed a specific role of the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA). Stimulation of the antenna with sucrose, the US, induces a fast transient activation of PKA in the ALs (Hildebrandt and Müller, 1995b) that is back to baseline within a few seconds. US-induced PKA activation in the ALs is stimulus specific, since odor stimulation (CS) or mechanical stimulation of the antennae does not affect PKA activity (Hildebrandt and Müller, 1995b). The exclusively US-induced PKA activation in the ALs is sensitive to the monoamine reuptake blocker reserpine and thus depends on the monoamines present in the AL. One of the latter is octopamine, the putative transmitter of the VUMmx1 neuron that densely innervates the ALs. Thus, octopamine is responsible for linking US stimulation to transient PKA activation (Hildebrandt and Müller, 1995a).

The PKA immunostaining is to its largest extent detected in the local interneurons that connect the glomeruli within the AL (Müller, 1997a). This, together with the biochemical measurements and the dense innervation of the ALs by the VUMmx1 neuron, suggests a global modulatory function of US-induced processes mediated via the octopamine–cAMP/PKA cascade in the AL. The observation that silencing expression of the octopamine receptor in the honeybee specifically impairs olfactory learning but not odor discrimination (Farooqui et al., 2003), together with the specific requirement of octopamine in *Drosophila* appetitive, but not aversive, olfactory learning (Schwaerzel et al., 2003), points to a conserved role of octopamine in insect appetitive learning.

In addition to the specific activation of the cAMP/PKA pathway, US stimulation also triggers Ca^{2+}-regulated signaling cascades like the Ca^{2+}-phospholipid-dependent protein kinase C (PKC) in the AL (Grünbaum and Müller, 1998). In contrast to the US-specific activation of the cAMP/PKA pathway, PKC is activated by both US and CS stimulation (Grünbaum and Müller, 1998). Inhibition of the transient CS- or US-induced PKC activation does not affect associative learning, suggesting that PKC-mediated processes may contribute to chemosensory information processing rather than to learning and memory formation.

In addition to the ALs, the VUMmx1 neuron innervates the lip region of the MB calyces that receives olfactory input from the ALs (**Figure 2(c)**). In contrast to the ALs, however, US stimulation does not activate the cAMP/PKA cascade in the MBs *in vivo*. This, together with the observation that octopamine stimulates the cAMP/PKA cascade in cultured KC from the MBs (Müller, 1997a), points to a different coupling of the octopamine receptors in the MBs: most likely, US stimulation activates octopamine receptors that trigger Ca^{2+}-regulated signaling cascades in the MBs (Balfanz et al., 2005). The observed differences in the expression of voltage-sensitive and Ca^{2+}-dependent currents in neurons of the ALs and the MBs (Grunewald, 2003) support the idea that information in olfactory learning is processed differently in ALs and MBs.

4.06.4 Associative Conditioning: Induction of Molecular Processes Underlying Memory Formation

During associative conditioning, parameters of the CS and US stimulus (strength, duration) and the temporal relations between stimuli are deciding factors for the process of memory formation. Depending on these parameters, different molecular processes are triggered during the short training phase, and depending on the triggered molecular processes, distinct forms of memory are developed. At least three memory phases can be identified in the various model systems: a short-term memory (STM) in the range of minutes, a mid-term memory (MTM) in the range of hours, and a stable LTM which lasts for days and weeks. In honeybees, as in many other systems, the LTM can be pharmacologically divided into a translation-dependent early phase (eLTM, 1–2 days)

and a transcription-dependent late phase (lLTM, ≥3 days) (Menzel, 1999; Müller, 2002). The defined period of associative conditioning (seconds to minutes) and the experimental accessibility of the convergence sites of CS and US processing in honeybees provided the ideal situation to determine the learning-induced dynamic activation of molecular signaling cascades and their contribution to formation of distinct memory phases *in vivo*.

4.06.5 Induction of LTM: The Critical Role of the cAMP/PKA Cascade

The requirement of cAMP/PKA-dependent processes in LTM formation and processes of long-lasting neural plasticity is observed in all systems investigated so far and seems to be conserved throughout the animal kingdom (*See* Chapters 4.07, 4.10, 4.21). Blocking of PKA during and immediately after the training phase leads to a selective loss of LTM, while STM and MTM are unaffected (Müller, 2000). Direct measurements show that the temporal dynamics of US-triggered PKA activation in the ALs is modified by the temporal sequence of CS and US stimuli and by the number of successive conditioning trials (**Figure 3**). A single CS/US forward-pairing, which induces a weak olfactory memory, leads to a transient increase in PKA activity that returns to basal levels 60 s after the conditioning trial. Repetition of CS/US forward-pairings (3× CS/US with an intertrial interval of 2 min) that induce LTM prolongs PKA activity in the ALs up to more than 3 min. US stimulation alone, as well as single or repeated US/CS backward-pairings, which do not induce appetitive memory, lead to a significantly shorter but similar pattern of PKA activation. This first *in vivo* evidence for a link between the sequence

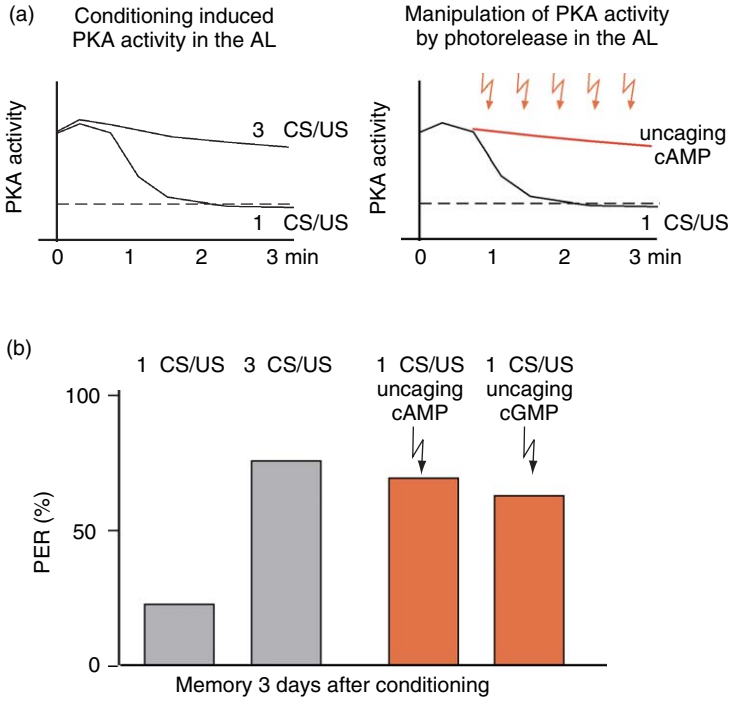


Figure 3 Induction of LTM requires a prolonged PKA activation in the antennal lobes. (a) The activation pattern of PKA in the antennal lobes depends on the sequence and the number of successive conditioning trials. While a single CS/US pairing induces a transient activation of about 1 min, repeated CS/US pairings (2-min intertrial interval) that induce an LTM lead to a prolongation of PKA activity up to more than 3 min. Local uncaging of cAMP in the antennal lobes enables the imitation of the prolonged PKA activation *in vivo,* while the animal receives a single CS/US pairing. (b) Honeybees are trained under conditions allowing access to the brain to photorelease cAMP or cyclic guanosine monophosphate (cGMP) during conditioning. Under these conditions honeybees form an LTM as tested 3 days after 3×CS/US pairings, but not after a single CS/US pairing. A single CS/US pairing together with the imitation of the prolonged PKA activation by uncaging cAMP in the AL induces LTM. Since the prolonged PKA activation is mediated via the nitric oxide/cGMP cascade, single CS/US pairing combined with uncaging of cGMP in the AL also induce LTM.

and number of CS and US stimulation and temporal dynamics of PKA activation in the ALs suggested that prolonged PKA activation in the AL is involved in the formation of associative LTM.

This hypothesis was tested by photolytic release of caged cAMP in the ALs (**Figure 1(b)**) to artificially prolong PKA activation during olfactory conditioning. A single conditioning trial combined with the local replay of the prolonged PKA activation in the ALs *in vivo* is sufficient to induce long-lasting memory (**Figures 3** and **4**). These experiments provided direct functional evidence for a link between conditioning parameters, temporal patterns of PKA activation in the ALs, and formation of LTM in intact animals.

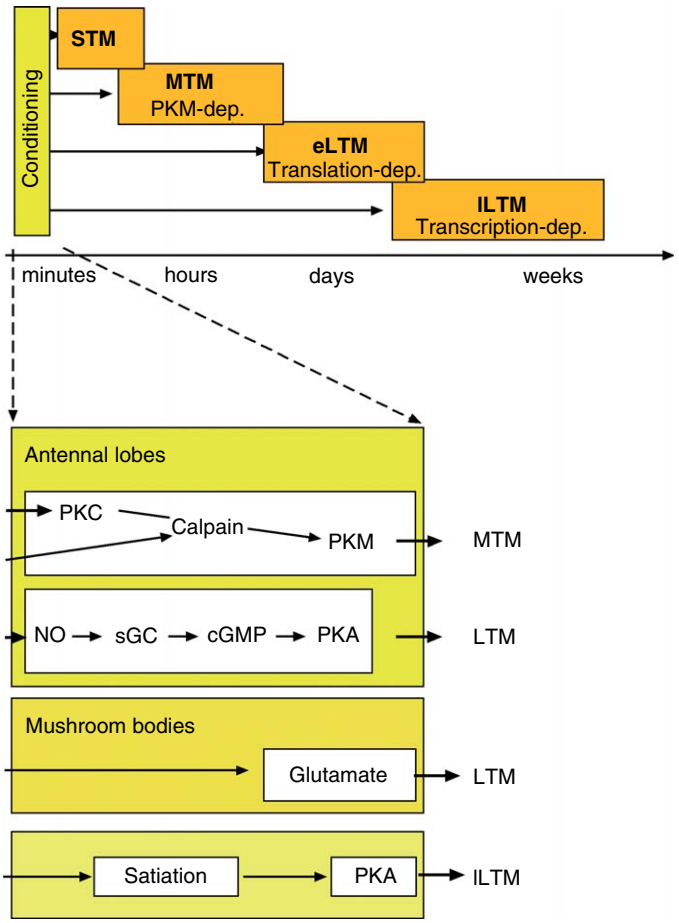


Figure 4 Scheme of the molecular mechanisms underlying the induction and maintenance of different memory phases in honeybees. As in other species, different memory phases are mechanistically distinguishable in the honeybee. While short-term memory (STM) and mid-term memory (MTM) can be formed by existing proteins, long-term memory (LTM) requires protein and RNA synthesis. LTM can be pharmacologically dissected into an early phase (eLTM) that requires protein synthesis and a late phase (lLTM) that, in addition, requires RNA synthesis. STM, MTM, and LTM are induced independently and by parallel processes. Two independent processes involved in maintenance of MTM and induction of LTM are localized in the antennal lobes. MTM requires the formation of a constitutive protein kinase M (PKM) for its maintenance. PKM is formed in the antennal lobes by cleaving protein kinase C (PKC) by the protease calpain. Also located in the antennal lobes is the molecular process underlying the prolonged activation of the protein kinase A (PKA) that is required for LTM induction. This prolonged PKA activation is mediated by activation of the soluble guanylate cyclase (sGC) by nitric oxide (NO) and induces subsequent processes of LTM formation. In addition to the antennal lobes, processes located in the mushroom bodies also contribute to LTM formation. Photolytic release of glutamate in the mushroom bodies immediately after conditioning facilitates LTM formation. The satiation status during conditioning modulates learning and memory formation. The formation of the late LTM can be specifically rescued via a PKA-dependent process, indicating that eLTM and lLTM are induced by two parallel PKA-dependent mechanisms. The molecular targets triggered by these early events in the ALs and the MBs are presently unknown.

However, the molecular processes triggered by the prolonged PKA activation in the ALs that finally lead to LTM are unknown.

Artificial prolongation of PKA activation in the ALs, in conjunction with single-trial conditioning, does not reach the level of conditioned responses that is achieved after multiple-trial conditioning. Thus, it is possible that other molecular processes or neural circuits (e.g., in the MBs) also contribute in parallel to LTM induction.

4.06.6 Induction of LTM: The Nitric Oxide/cGMP System Acts as a Signal Integrator during Associative Conditioning

In the nervous system, nitric oxide (NO) has been implicated in various physiological functions including chemosensory information processing and learning (Müller and Hildebrandt 1995; Müller, 1996, 1997b). Inhibition of NO synthase (NOS) in honeybees that is mainly localized in brain areas like the ALs and the lip of the MB calyces impair both LTM formation (Müller, 1996) and prolonged PKA activation (Müller, 2000). The finding that blocking of the soluble guanylate cyclase, the major target of NO in neuronal tissues, also impairs multiple-trial induced prolonged PKA activation and LTM formation provided first evidence for a crosstalk between cyclic guanosine monophosphate (cGMP) and cAMP signaling in this system. Theoretically, cGMP can interact with the cAMP cascade via cGMP-dependent protein kinase, cGMP-regulated phosphodiesterases, and cyclic nucleotide-gated channels. Although the target of cGMP has not yet been identified *in vivo*, the synergistic activation of honeybee PKA by cAMP and cGMP (Leboulle and Müller, 2004) points to a direct function of cGMP in the prolonged increase in PKA activity in the AL.

This systemic analysis does not allow for localizing the contributing brain areas. Again the uncaging technique served as valuable approach to verify that the prolonged PKA activation in the ALs is mediated via NO and cGMP *in vivo* (**Figures 3** and **4**). As demonstrated with uncaging cAMP, a single conditioning trial followed by photorelease of cGMP leads to formation of a long-lasting memory (**Figure 3**). A single conditioning trial in combination with uncaging NO in the ALs impairs memory formation. Although the reason for this is unknown, photolyzing NO in the ALs may interfere with other functions of NO in signal processing in the ALs. It has been demonstrated that blocking NO in the AL impairs odor discrimination (Hosler et al., 2000). Due to the localization and Ca^{2+}-dependence of NOS, the odor-specific changes in Ca^{2+}-concentrations in subsets of glomeruli during odor processing (Joerges et al., 1997) may induce an odor-specific release of NO required for odor discrimination. The artificial release of NO within the whole ALs would interfere with such a process. Moreover, the NO/cGMP system in the ALs is also implicated in integrative processing of appetitive signals during habituation (Müller and Hildebrandt, 1995). Characterizing the temporal aspects of NO and cGMP function using the uncaging technique *in vivo* revealed clear differences between NO/cGMP function in associative and nonassociative learning. Both the cellular network mediating the NO/cGMP function and the temporal parameters of the stimulus-induced activation of the NO/cGMP system differ between associative learning and habituation (Müller and Hildebrandt, 2002). Irrespective of their differences, however, both NO/cGMP-mediated processes contribute to integrative signal processing during application of stimuli. In case of associative learning, the function of the NO/cGMP system in a very narrow time window during conditioning is critical for induction of LTM and thus for processes that become evident days later (**Figure 4**).

4.06.7 Glutamate-Mediated Signaling Cascades in the Mushroom Bodies Are Involved in Memory Formation

Glutamate is the major excitatory transmitter in the mammalian brain and plays a central role in neuronal plasticity in vertebrates (*See* Chapters 4.16, 4.20). Although the function of glutamate as main transmitter at insect neuromuscular junctions is well studied, the role of glutamate in the insect brain is poorly understood. The existence of glutamate-like immunoreactivity (Bicker et al., 1988), glutamate-induced ion currents (Barbara et al., 2005), glutamate transporters, and glutamate receptors (Kucharski et al., 2000; Funada et al., 2004; Zannat et al., 2006) provides considerable evidence for a potential role of glutamate in the honeybee brain.

Systemic application of drugs affecting reuptake of glutamate before repeated training trials impairs memory tested after 24 h but does not affect acquisition or memory tested at 1 h (Maleszka et al., 2000).

A similar effect is observed by injection of MK-801, a noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist (Si et al., 2004). Although interpretation of the function of glutamate transmission based on pharmacological studies is difficult, a molecular approach used in *Drosophila* (Xia et al., 2005) supports the idea of an implication of glutamate in insect learning. Stable and transient knockdown of NMDA receptors in the whole *Drosophila* brain disrupts aversive olfactory learning and impairs long-term memory. However, as in the honeybee, the particular brain site at which glutamate influences memory remains unclear. Photolytic uncaging of glutamate in the honeybee brain revealed direct evidence for a defined spatial and temporal contribution of glutamate in LTM formation (Locatelli et al., 2005) and, in addition, overcame problems caused by the insufficient pharmacological characterization of the drugs used on insect glutamate receptors and reuptake systems.

Uncaging glutamate in the MBs immediately after a weak training protocol (single-trial training) improves the formation of a long-lasting memory (2 days) and thus mimics the effect of a strong training protocol (**Figure 4**). The effect of glutamate seems to be confined to a time window after associative training, since release of glutamate immediately before training has no effect. This post-conditioning effect of glutamate release on late memory formation allows two interpretations: Either it mimics a situation that is usually induced by repeated conditioning trials and thus facilitates learning, or post-conditioning release of glutamate directly contributes to early processes of memory formation. The function of glutamate is restricted to the MBs circuitry, since glutamate release in the ALs does not affect processes of learning or memory formation. The demonstrated role of glutamate in memory formation strengthens the notion that glutamatergic neurotransmission is a conserved key element in neural plasticity across species.

4.06.8 Induction and Maintenance of MTM: The Ca^{2+}-Dependent Cleavage of PKC by Calpain

Calcium-mediated signaling cascades play a fundamental role in the regulation of cellular processes. The signaling cascade that involves PKC is implicated in synaptic plasticity in various systems (*See* Chapter 4.22). Initiated by the finding that both US and CS stimulation induce a transient PKC activation in the ALs, the function of the signaling cascade that involves PKC has been analyzed in detail (Grünbaum and Müller, 1998). The temporal dynamics of PKC activation in the ALs are independent of the sequence of CS and US stimulation and of the number of successive conditioning trials. Inhibition experiments reveal evidence that these transient PKC modulations during the conditioning phase do not directly contribute to learning or memory formation. In maintenance of a specific memory phase, however, a function of the signaling cascade involving PKC has been demonstrated.

Repeated conditioning trials that induce LTM lead to an increase in PKC activity in the ALs beginning 1 h after conditioning and lasting up to 3 days (Grünbaum and Müller, 1998). This long-lasting elevation of PKC activity is not induced by a single conditioning trial. Two independent and mechanistically distinct mechanisms contribute to this training-induced increase in PKC activity. In the early phase, ranging from 1 to 16 h, the increased PKC activity can be attributed to a constitutively active PKC, a form of PKC known as PKM. PKM is formed by cleavage of the activated PKC by the Ca^{2+}-dependent protease calpain. Inhibition of calpain activity during conditioning eliminates the early phase of elevated PKC activity from 1–16 h after training. This supports the idea that training activates PKC and calpain. Only activated PKC is susceptible to calpain action resulting in the production of PKM. The inhibition of calpain during conditioning impairs memory in a time window between 1 and 16 h after learning, suggesting that learning-triggered PKM production is required for maintaining the MTM (**Figure 4**). This is supported by the observation that blocking calpain does not affect acquisition, the early memory phase up to 30 min, and memory after 1 day.

The late phase of training-induced elevation of PKC activity observed 1–3 days after learning is eliminated by injection of blockers of protein and RNA synthesis in a narrow time window during and after conditioning. Thus, induction of the two mechanistically different processes expressing the early and late phase of elevated PKC activity occur in parallel during and/or shortly after conditioning. The mechanism underlying the elevated PKC activity in the late phase, as well as its function in memory formation, is unclear. It is probably one of several parallel mechanisms occurring in different neuronal circuits required for the formation of the late phase of long-term memory.

4.06.9 Influence of Satiation Reveals a Parallel Function of the cAMP Cascade in the Process of Memory Formation

In addition to parameters concerning the relation and number of US and CS stimulation during associative learning, factors like stress, satiation, etc., influence learning and memory formation. Although the latter parameters are usually strictly controlled in behavioral experiments, the manipulation of satiation level during associative learning in honeybee uncovered new features of the molecular machinery leading to LTM formation (Friedrich et al., 2004).

As in other paradigms using appetitive associative conditioning, a reliable induction of LTM in the honeybee requires training of hungry animals. Only multiple-trial conditioning of animals starved for about 18 h leads to a robust acquisition and induction of MTM and the two LTM phases, eLTM and lLTM. An additional feeding 4 h before multiple-trial conditioning is sufficient to impair acquisition and all subsequent memory phases. Such an additional feeding also impairs memory induced by a single-trial conditioning. This is of particular importance, since presently only manipulations of satiation during learning or amnestic treatment by cooling immediately after learning have been shown to interfere with memory induced by a single conditioning trial. The restriction of the different satiation levels to the time window of conditioning and the impairment of both LTM phases that require the cAMP/PKA cascade during conditioning (Müller, 2000; Friedrich et al., 2004) pointed to a contribution of the cAMP/PKA cascade in satiation-dependent impairment of memory formation. Determination of the basal PKA levels in honeybee brains at different satiation levels revealed a characteristic difference: hungry bees starved for 18 h show higher basal PKA activity in the brain than bees fed 4 h before. Elevating the low basal PKA activity levels in animals fed 4 h before conditioning specifically rescues the transcription-dependent lLTM (**Figure 4**). This is remarkable, since acquisition, MTM, and eLTM are still impaired. Since in all model systems, both eLTM and lLTM require PKA for their induction, the findings in honeybees support the existence of two parallel cAMP/PKA pathways implicated in LTM formation: one triggers molecular cascades leading to translation-dependent eLTM, and the other is involved in triggering cascades required for transcription-dependent lLTM. The specific rescue of lLTM by elevating basal PKA activity in fed animals suggests that other molecular pathways involved in learning and memory formation also contribute to the network of molecular interactions between satiation and learning processes.

4.06.10 Summary

The accessibility of the honeybee brain provides the basis for the analysis of learning-induced changes of signaling cascades in defined brain areas. The conditioning-induced transient activation of different signaling cascades occurs in a narrow time window of a few minutes during and after conditioning and is highly dependent on the training parameters, which are critical for induction of distinct memories. The temporal action of signaling cascades can be imitated by local uncaging of substances activating these cascades. Thus, combination of behavioral and biochemical techniques reveals important insights into the organization of the molecular network underlying memory formation *in vivo*.

The temporal analysis of the conditioning-induced activation of PKA, which plays a conserved role in LTM formation in all model systems, demonstrated a critical function for a prolonged PKA activation in the ALs in LTM formation. This prolonged PKA activation is mediated by the NO/cGMP system and is necessary to induce molecular processes underlying eLTM and lLTM. An independent, PKA-mediated process specifically implicated in lLTM formation could be identified by altering the bee's satiation level during conditioning. The network of molecular processes implicated in LTM formation also includes processes localized in the MBs. Here, signaling cascades activated by the neurotransmitter glutamate are involved in processes that facilitate LTM formation, an observation that supports the idea that the function of glutamate in synaptic plasticity is conserved. The molecular processes involved in induction and maintenance of MTM are independent of those underlying induction of LTM. In the ALs, conditioning triggers the cleavage of PKC by calpain that results in production of PKM. The autonomously active PKM is specifically required to maintain MTM in the range of a few hours.

Taken together, the temporal and spatial analysis of signaling cascades triggered by associative learning in honeybees reveals a parallel organization of the molecular network contributing to memory formation. Such a parallel-organized network is perfect for the fine-tuning of the multiphasic process of memory formation by training parameters and other factors influencing learning like satiation, stress, and circadian rhythm.

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4.07 Molecular and System Analysis of Olfactory Memory in *Drosophila*

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4.07.1 Introduction: Three Types of Olfactory Memories and a Memory Center

Drosophila melanogaster has been used as a model system in investigating many cellular and developmental processes. The *Drosophila* central nervous system is composed of neurons and glia that operate on the same fundamental principles as their mammalian counterparts. Even though the *Drosophila* brain has only 100 000 cells (Ito et al., 2003), it produces complex behaviors and sustains various forms of learning and memory, which are highly amenable to analysis by current genetic methods. Moreover, *Drosophila* studies make it possible to investigate different concepts of memory that may eventually be generalized to other species, because roughly 50% of human genes have a *Drosophila* ortholog (Rubin et al., 2000).

4.07.1.1 Olfactory Aversive Conditioning

We will focus on the most commonly used paradigm to assess *Drosophila* memory, olfactory aversive conditioning (*See* also Chapter 1.28). This paradigm was instrumental in the discovery of most learning and memory mutants, and still contributes to our increasing comprehension of molecular and cellular processes that underlie associative memory. Importantly, the characterization of specific anatomical and memory mutants has allowed us to partially decipher the dynamics of memory phases in this system.

The principle, originally developed by Quinn et al. (1974) and improved by Tully and Quinn (1985), is as follows: for training, a single associative-learning trial consisting of an odor (the conditioned stimulus, CS^+) is accompanied by electric shocks (the unconditioned stimulus, US); and after a short rest period, a second odor (the conditioned stimulus, CS^-) is presented in

absence of shock. To test memory, flies are placed in an apparatus where they can choose between two compartments that are presented simultaneously: one containing the CS^+ odor, the other one the odor CS^-. After 2 min of test, flies from each compartment are isolated and counted to calculate a memory performance index (PI) that typically ranges from 0 (no memory) to 1 (perfect score). This protocol generates immediate memory scores of around 0.85, indicating that the majority of flies are able to learn and remember. To increase the efficiency of this process, our laboratory has developed an automated conditioning setup that allows parallel training of six groups of flies by means of a barrel (for schematic representation, see Pascual and Preat, 2001).

If flies are submitted to one training cycle (i.e., odor 1 paired with electric shock reinforcement for 1 min, hereafter called the short protocol), 1-day memory retention is weak. Multiple massed repetitions (hereafter called the massed protocol) of this single cycle generate memory that can be measured for a few days. Multiple spaced repetitions with a rest period of 15 min between each cycle (hereafter called the long protocol) generate a stabilized memory, called long-term memory (LTM), which can be measured for 1 week (Tully et al., 1994).

In addition to these quantitative variations, an important question is whether the different protocols induce distinct types of memory. Several general treatments have been used to disrupt specific memory components in wild-type flies. After the short protocol, 3-h memory retention is disrupted by submitting the flies to a cold shock immediately after training, but becomes progressively more resistant when this treatment is administrated at later times (Quinn and Dudai, 1976; Tully et al., 1994). This observation characterizes two memory phases, one anesthesia-sensitive memory (ASM, or labile memory) and one anesthesia-resistant memory (ARM, or consolidated memory). ASM and ARM are also generated after the massed protocol. Flies fed with an inhibitor of protein synthesis, cycloheximide, show a disrupted LTM but normal ARM (Tully et al., 1994) and ASM (Dudai, 1977), suggesting that unlike ARM and ASM, LTM depends on *de novo* protein synthesis (cf **Table 1**). These results suggest that flies can form three different types of olfactory memory, and the search for mutants that affect these phases differently is a major goal of *Drosophila* memory research.

4.07.1.2 The Mushroom Bodies Are the Center of Olfactory Memory

Olfactory receptor neurons, located in the antennae and maxillary palps, project their axons into the antennal lobes (ALs). From the ALs, projection neurons convey the olfactory information via the antennoglomerular tract in part to mushroom bodies (MBs) (**Figure 1**), a pair of prominent and characteristically shaped neural centers. In the 1970s and 1980s, the function of the MBs was assessed in different insect species with classical interventionism approaches, cooling or ablation (Menzel et al., 1974; Erber et al., 1980). In the cockroach, MBs are a specialized neuropile involved in processing and storing multimodal sensory information (Li and Strausfeld, 1997). Over the years, MBs have been implicated in olfactory learning and memory (Davis, 2005), and in a variety of complex functions including courtship learning (Mehren et al., 2004), context generalization in visual learning (Liu et al., 1998), walking activity (Martin et al., 1998), and sleep (Joiner et al., 2006; Pitman

Table 1 Synopsis of different memory phases, the protocols that generate them, and their properties

	Anesthesia-sensitive memory	*Anesthesia-resistant memory*		*Long-term memory*
Duration	Short-lasting memories: Short-term memory (STM) lasting a few minutes and middle-term memory (MTM) lasting a few hours	Semi-stabilized memory lasting a few days		Stabilized memory lasting more than 1 week
Protocol	All protocols	Short protocol	Massed protocol	Long protocol
Anesthesia treatment	Sensitive	Resistant	Resistant ?	Resistant ?
Protein-dependent synthesis	No	No		Yes

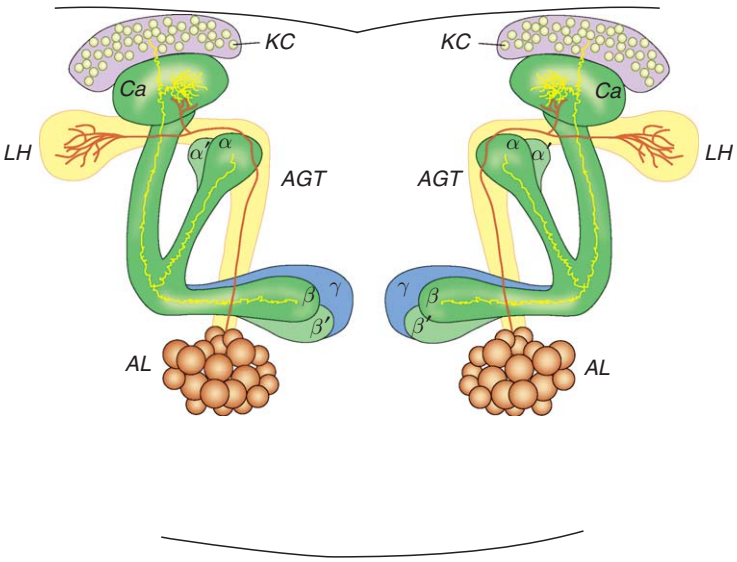


Figure 1 *Drosophila* olfactory system. Olfactory sensory neurons project to the antennal lobes (AL). From there, projection neurons project through the antennoglomerular tract (AGT) and connect mushroom body (MB) dendrites localized in the calyx (Ca), as well as the lateral horn (LH). Each MB is composed of about 2500 neurons, the Kenyon cells (KC). Three types of KC project in five lobes: α/β, α'/β', and γ.

et al., 2006). In *Drosophila*, MBs are composed of three main classes of neurons whose axons divide to form two vertical lobes (α and α') and three median ones (β, β', and γ) (Crittenden et al., 1998) (**Figure 1**).

Brain mutants with MB structural defects were first isolated in the 1980s. Unlimited numbers of animals with the same anatomical defect could be assessed using mutants, without interventionism. This helped to highlight the role of MBs in olfactory learning and memory (Heisenberg et al., 1985). However, one caveat of this approach is that the anatomical defects are generally not specific to the MBs (de Belle and Heisenberg, 1994). An alternative approach was used to generate flies without MBs (de Belle and Heisenberg, 1994). Hydroxyurea, an anti-mitotic agent, was fed to newly hatched wild-type larvae. At this early developmental stage, only five neuroblasts – the neurons' precursor cells – are mitotically active within each brain hemisphere: the four that generate the MBs, and one in the antennal lobe. Thus, hydroxyurea treatment leads to viable adults flies with almost no MBs. These flies turned out to show no olfactory memory (de Belle and Heisenberg, 1994), although abilities to mediate olfactory memory (shock reactivity, olfaction, and locomotor behavior) were intact. Together, these data strongly implicate the MBs in associative olfactory memory.

The essential role of MBs in olfactory memory is further outlined by the high expression in the MBs of many proteins involved in learning and memory, as will be seen.

4.07.2 Short-Lasting Memories

4.07.2.1 The cAMP Pathway Plays a Key Role in Associative Memory

We do not intend to review here in detail all *Drosophila* learning and memory genes, or the various conditioning protocols (for review see Waddell and Quinn, 2001; Dubnau et al., 2003b; Heisenberg, 2003; Davis, 2005; McGuire et al., 2005; Skoulakis and Grammenoudi, 2006). Rather, we will describe the different approaches that have allowed the analysis of *Drosophila* associative memory at the molecular, cellular, network, and systemic levels.

The strength of *Drosophila* molecular genetic tools underlies the discovery and the dissection of new molecular pathways involved in memory. The use of mutants, and in particular the recently designed conditional mutants, is a powerful means to link the molecular, cellular, network, and system levels. The global experimental plan is to generate random single-gene mutations and to select mutants that show a specific learning or memory defect. In the second

step, the aim is to identify the mutated gene and to understand its function in memory (Benzer, 1973). The first screens for *Drosophila* learning and memory mutants were produced by Seymour Benzer and colleagues more than 30 years ago. Four thousand fly stocks carrying ethyl methane sulfonate-induced mutations were tested for their ability to learn in an olfactory conditioning assay (Quinn et al., 1974; Dudai et al., 1976; Quinn et al., 1979). The first *Drosophila* learning and/or memory mutants characterized were *dunce* (*dnc*) and *rutabaga* (*rut*). Biochemical approaches showed that *dnc* flies were deficient in one form of cyclic adenosine monophosphate (cAMP) phosphodiesterase activity (Byers et al., 1981; Davis and Kiger, 1981), and that *rut* flies were deficient in the activity of a Ca^{2+}/calmodulin-sensitive adenylyl cyclase (Livingstone et al., 1984). The current view is that Rut adenylyl cyclase could act during learning as a coincidence detector for the US and CS, because of the ability of adenylyl cyclase to respond to two different types of signals, namely extracellular monoamines and intracellular calcium levels (Livingstone et al., 1984; McGuire et al., 2003).

However, EMS-induced mutations are often difficult to localize in genome, and therefore the main problem with these first learning and/or memory mutants was to identify the mutated gene. The first step consists of genetically mapping the mutation by complementation tests, using *Drosophila*'s deletion collection. This, however, only defines a broad area covering many other genes (Dudai et al., 1976; Livingstone et al., 1984). Thus, the cloning of the responsible gene took years and was facilitated by the use of new mutant alleles induced by the insertion of a transposable P-element. Description of P-element insertions within 200 nucleotides of the *rut* transcription start site identified *rut* as the gene for the Ca^{2+}/calmodulin-responsive adenylyl cyclase (Han et al., 1992; Levin et al., 1992). On the other hand, new EMS-induced *dnc* mutants came from a screen for female sterility mutants (Mohler, 1977). The *dnc* gene was identified by recombinational mapping of *dnc* mutations with restriction site polymorphisms as genetic markers (Davis and Davidson, 1984) and by identification of homologous cDNA clones to known phosphodiesterases (Chen et al., 1986). *dnc* encodes a cAMP-specific-phosphodiesterase II. Another mutant, *amnesiac* (*amn*) mutant, was identified in a screen for flies with affected memory (Quinn et al., 1979). The *amn* gene was later identified thanks to a P-element-induced allele, which acts as a second site suppressor of the *dnc* female sterility phenotype, and has been repeatedly isolated since (Feany and Quinn, 1995; Moore et al., 1998; Toba et al., 1999). The Amn peptide shows some similarity to a putative adenylyl cyclase activating peptide (PACAP). The biochemical activity of Amn is unknown, but both the genetic and molecular evidence suggests that Amn acts through adenylyl cyclase to increase the concentration of cAMP (Feany and Quinn, 1995).

Discoveries of these proteins displaying major roles in memory processes suggested important roles for intracellular cAMP and cell signaling cascade in olfactory learning. Protein kinase A (PKA) being a major effector for cAMP, Drain et al. tested the hypothesis that PKA is involved in memory processes (Drain et al., 1991). PKA is a tetramer composed of two catalytic (C) and two regulatory (R) subunits. In the absence of cAMP, regulatory subunits bind to the catalytic subunits and inhibit their activity, and conversely, at elevated levels, cAMP binds to the regulatory subunits, which then release the catalytic subunits to phosphorylate target proteins. Protein kinase inhibitor (PKI) is a PKA inhibitory peptide that binds the PKA catalytic subunit and prevents its release in response to cAMP. Ubiquitous PKI overexpression disrupts olfactory memory (Drain et al., 1991).

Another approach identified a new mutant of the catalytic subunit gene (DCO) of PKA. Enhancer detection screening is a combination of histological screening and P-element mutagenesis. The modified P-element carries a reporter gene and is randomly inserted in genome by classical techniques of mutagenesis. Screening is based upon the fact that expression of the reporter gene mimics the expression of the endogenous gene where this reporter gene was inserted (**Figure 2**). The aim is to first find by histology lines whose reporter expression pattern includes the MBs. The integrated P-element may disrupt expression of the MB-expressed gene; therefore testing memory performance of the mutant lines identifies new proteins involved in this process. Mutants in the catalytic subunit gene of PKA were isolated by this approach (Skoulakis et al., 1993). Reduction of 80% PKA activity in heteroallelic mutants of DCO generated a memory disruption. The R-type I subunit of PKA is also crucial for associative learning (Goodwin et al., 1997).

An additional study outlining the important role of cAMP signaling in learning and memory came from a study of mutations in neurofibromatosis type 1 (*Nf1*) *Drosophila* homolog. Mutations in the human *NF1* gene cause benign and malignant tumors of the nervous system and generate learning deficits in humans (Ferner et al., 1996; North et al., 1997; Gutmann, 1999). Mice heterozygous for *Nf1*

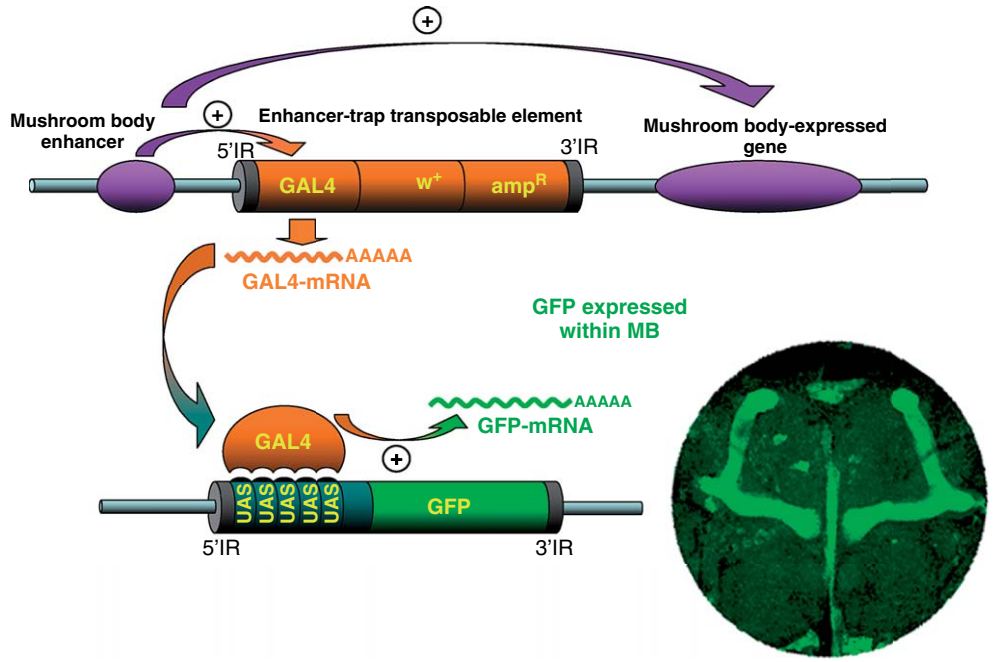


Figure 2 The GAL4/upstream activating sequence (UAS) system. When an enhancer-trap transposable element is inserted near transcription enhancers that control expression in a given structure, the GAL4 gene that is contained in the P-element is expressed in the same structure. If this fly also contains a reporter gene downstream of the UAS sequences, GAL4 will drive transcription of the reporter. GFP, green fluorescent protein; MB, mushroom body.

mutations have an increased predisposition to learning impairment (Silva et al., 1997). The *Drosophila* NF1 protein shows a high degree of conservation with the human protein (60%). Flies lacking NF1 at the adult stage show an olfactory memory defect, suggesting that the human homolog is involved in learning and memory, and memory impairment is not due to developmental defect (Guo et al., 2000). Genetic and biochemical analysis have revealed that NF1 inhibits Ras activity (Cichowski and Jacks, 2001; Zhu and Parada, 2002). Interestingly, NF1 also participates in the activation of adenylyl cyclase, increasing cAMP levels via at least two distinct pathways: by an NF1/Ras-dependent mechanism stimulated by growth factors and by an NF1/Gαs-dependent pathway, acting through Rut (Hannan et al., 2006). By overexpressing a constitutively active PKA catalytic subunit in an *NF1* mutant background, Guo et al. rescued the NF1 memory defect, suggesting the biochemical deficiency in the *NF1* mutants must reside upstream of PKA induction in the cAMP pathway (Guo et al., 2000).

In conclusion, the cAMP is particularly important to support olfactory associative memory. Both a lack (in *rut, DCO, amn, NF1*) or an excess (in *dunce*) of cAMP pathway activity can cause memory impairment, and the learning score of double mutant *dnc*, *rut* is reduced when compared with either single mutants (Tully and Quinn, 1985), which suggests that cAMP levels have to be finely regulated during memory formation to control behavioral plasticity. Alternatively, Dnc and Rut may act in different subsets of neurons.

4.07.2.2 Mushroom Bodies and the cAMP Pathway

Drosophila MBs are required for olfactory learning and memory, and insights from mutants have indicated that the cAMP pathway plays a key role in memory establishment. Nevertheless, until recently it remained to be proved that cAMP metabolism is strictly required in the MBs for learning and memory. A powerful tool for spatial control of gene expression is the GAL4/UAS enhancer-trap technique (Bellen et al., 1989; Wilson et al., 1989; Brand and Perrimon, 1993) (**Figure 2**), which enables selective activation of any cloned gene in a wide variety of tissues and cells. With this system (Brand and Perrimon, 1993), Connolly and colleagues (1996) ectopically stimulated MB cAMP signaling by expressing a constitutively activated Gα_s-protein (Gα_s^*). Permanent adenylyl cyclase activation

specifically in the MBs leads to an impaired associative memory.

That memory is impaired after ablation or functional disruption of a brain structure does not necessarily imply that the memory trace itself is localized within this structure. To localize short-term memory (STM), it was necessary to rescue the memory abilities of a mutant by expressing the corresponding protein in a specific brain structure. As seen earlier (**Figure 1**), MBs are composed of three main classes of neurons. One class of neurons bifurcates to generate one vertical branch in the α lobe and one horizontal branch in the β lobe; another generates one branch in another vertical lobe, the α' lobe, and one in the horizontal β' lobe; and a third class of neurons generates a single projection into the horizontal γ lobe (Crittenden et al., 1998). Zars and colleagues showed that expression of Rut Ca^{2+}/calmodulin adenylyl cyclase (Rut-AC) in γ lobes of MBs, but not in α/β lobes, partially restores a normal learning capacity in *rut* flies (Zars et al., 2000), whereas McGuire et al. reported that Rut-AC has to be expressed simultaneously in γ and α/β lobes to rescue the memory defect due to *rut*. A new recent study indicates that marginal *rut* rescue is observed when cyclase is expressed only in some α/β neurons (depending on the odor pair used for conditioning), an intermediate rescue is observed with γ expression, while a full rescue is obtained with α/β and γ expression (Akalal et al., 2006).

However, in these experiments, Rut was expressed not only in the MBs at the adult stage, but also during development. Thus, the behavioral rescue might have been due to the correction of an MB developmental defect, an indirect cause of the learning defect. To circumvent this hypothesis, techniques have been developed to control spatial and temporal expression of a protein. Using the TARGET and SWITCH methods (**Figure 3**), Ron Davis and his team showed that the presence of Rut in adult $\alpha/\beta/\gamma$ lobes of MBs alone was sufficient to rescue *rut* memory defect (McGuire et al., 2003; Mao et al., 2004).

These discoveries raise a new set of questions. For example, at which step of information processing are the MBs involved: acquisition, consolidation, or retrieval? Do the MBs carry a memory trace or are they a relay? These challenging problems are being solved with new genetic and imaging techniques.

4.07.2.3 Mushroom Bodies Anatomic-Functional Maps

A sophisticated approach to disturb neuronal circuits, based on a rapid and reversible blockage of synaptic

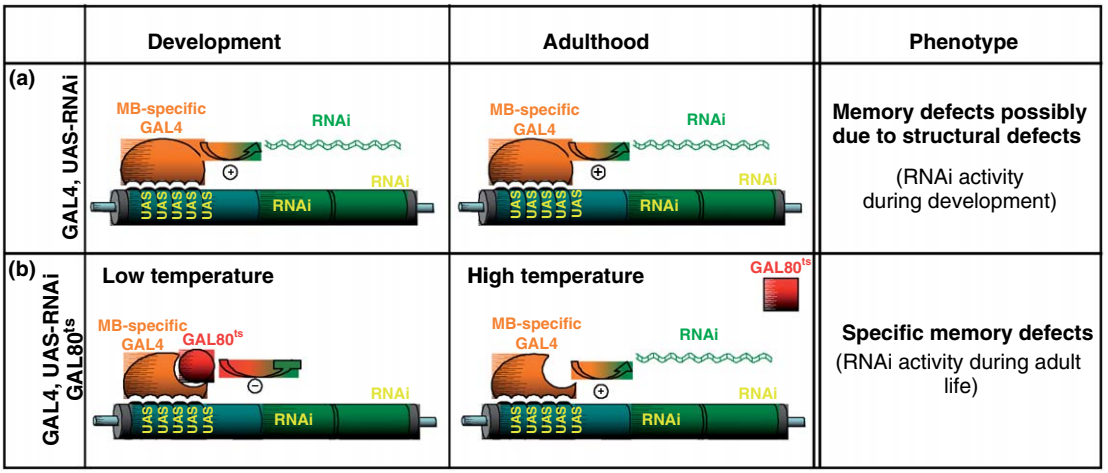


Figure 3 The TARGET technique helps us to discriminate between developmental and adult physiological effects. A sequence encoding an inverted repeat RNA (RNAi) is placed under UAS control. (a) GAL4 activates RNAi expression during development and adulthood. A memory defect could be caused indirectly by structural defects during development. (b) The TARGET system solves this problem. Flies are maintained at low temperature during their developmental stages. At this temperature, $GAL80^{ts}$ inhibits the transcription activity of GAL4, so that no RNAi is expressed. When adults emerge, they are transferred at permissive temperature (30 °C); the $GAL80^{ts}$ protein becomes ineffective and GAL4 activates the transcription of the RNAi in adult MBs. The putative memory defects are due to the decreased concentration of the mRNA targeted by the RNAi. In another use of the TARGET system, Rut expression in adult *rut* mutant background was sufficient to rescue *rut* memory defect (McGuire et al., 2003).

transmission, was developed by Kitamoto (2001). The *shibire* (*shi*) gene encodes a microtubule-associated guanosine triphosphatase (GTPase), dynamin, which is involved in endocytosis and is essential for synaptic vesicle recycling and maintenance of the readily releasable pool of synaptic vesicles. The temperature-sensitive allele, shi^{ts1}, is defective in vesicle recycling at the restrictive temperature (>29 °C), resulting in a rapid blockade of synaptic transmission. The shi^{ts1} mutation has a dominant effect, blocking chemical synapses even in the presence of a normal shi^{+} allele. Expression of the shi^{ts1} tool can therefore be used within the GAL4/UAS system. The GAL4/UAS-shi^{ts1} approach is very powerful, as it allows us to inhibit particular brain circuits at precise times. To determine whether the MBs are required during the acquisition, consolidation or retrieval phases, the temperature-sensitive Shi^{ts1} protein was specifically expressed in MB neurons in order to transiently disrupt synaptic neurotransmission (Dubnau et al., 2001; McGuire et al., 2001; Schwaerzel et al., 2002). It was shown that the synaptic outputs of MB neurons are required during retrieval of the STM but not during acquisition or consolidation. Which of the MB lobe outputs are required for STM retrieval? Two groups implicated mainly α/β and γ neurons (Dubnau et al., 2001; Schwaerzel et al., 2003), while another study suggested that only the α/β neurons were required (McGuire et al., 2001). Using a γ-specific Gal4 driver, we showed that γ neuron output is indeed required for STM and middle-term memory (MTM) retrieval (Isabel et al., 2004). All together, these initial studies indicate that a STM/MTM trace is localized at α/β and γ output synapses or upstream of these synapses. Interestingly, it was shown recently that α'/β' lobes are involved in acquisition and consolidation but not in memory retrieval (Krashes et al., 2007), showing that the MB lobes play very distinct roles.

Taken together, these experiments suggest that associative memory requires the sequential involvement of different subsets of MB neurons: α'/β' lobes for acquisition and consolidation, and $\alpha/\beta/\gamma$ for memory retrieval. However, as seen above, Rut-AC is thought to act as the major temporal integrator of associative learning (Livingstone et al., 1984), and the learning defect of *rut* flies can be totally rescued by expression of Rut-AC only in the $\alpha/\beta/\gamma$ lobes (McGuire et al., 2003; Akalal et al., 2006). This observation is paradoxical with the shi^{ts1}approach, which shows that α'/β' lobes (Krashes et al., 2007), but not $\alpha/\beta/\gamma$ lobes, are required for acquisition. It is possible that α'/β' neurons stimulate α/β or γ neurons. Perhaps the coincidence detection model is too simple, and further studies will be necessary to elucidate this discrepancy.

4.07.2.4 Imaging Mushroom Bodies and Antennal Lobes

One major caveat of *Drosophila* central brain studies is that direct electrophysiological analysis is scarce (Wilson et al., 2004), due to the small size of neuron cell bodies (less than 5 μm in diameter). To circumvent this difficulty, two partially alternative approaches have been followed: analysis of learning and memory mutants at the neuromuscular junction (Zhong and Wu, 1991; Renger et al., 2000), which avoids the complexity of brain physiology, as well as the analysis of isolated MB neurons in culture (Wright and Zhong, 1995). These experimental systems can provide interesting molecular and cellular information, but they are inadequate for assessing neuronal function at the level necessary for a global understanding of memory systems.

Odor processing occurs in a complex tissue environment, and the identification of the repertoire of brain cell assemblies involved in olfactory memory requires visualization of their network activity at high spatial and temporal resolution, in relatively intact preparations. Optical neural activity recordings allow the study of brain activity with micrometer spatial resolution, and activity-sensitive fluorescent probes have been recently used in *Drosophila*. Those sensors are proteins, and their expression can therefore be restricted to specific subsets of neurons with the GAL4/UAS system. Several sensors have been successfully brought to *Drosophila*, including those that monitor the local change of pH that accompanies neurotransmitter release (Yu et al., 2004) or changes in the intracellular calcium concentration that provide a valuable indicator of electrical activity (Aequorin (Rosay et al., 2001); Cameleon (Fiala et al., 2002); Camgaroo (Yu et al., 2003); and G-CaMP (Wang et al., 2003, 2004b)). Using the G-CaMP reporter and two-photon microscopy, stereotyped odor-evoked patterns have been observed in the antennal lobe glomeruli (Wang et al., 2003) and in the MBs (Wang et al., 2004b).

The ultimate goal of future imaging studies is to build a functional map of cell assemblies encoding memory in different regions of *Drosophila* brain, by comparing the activity of trained and naive animals, in normal flies or memory mutants. A first step, achieved as a transient change in the spatial code, was

observed in the antennal lobe of wild-type flies 3 min after olfactory associative conditioning (Yu et al., 2004). What happens to MB calcium concentrations during memory processes? The G-CaMP reporter was driven in α/β lobes to assess whether these lobes, which are functionally active during memory retrieval (McGuire et al., 2001; Isabel et al., 2004; Akalal et al., 2006), present an odor-evoked calcium signal dependent on associative short-lasting memory (Yu et al., 2006). Surprisingly, although calcium responses to odor and electric shock can readily be detected in the α/β neurons, one pairing of odor and electric shock did not alter odor-evoked calcium concentration in these neurons. Yu et al. suggest two alternative explanations. Firstly, short-lasting memory traces are not formed in α/β neurons but in other types of MB neurons such as α'/β' lobes, a hypothesis which fits with *shi*^{ts1}experiments driven in α'/β' neurons (Krashes et al., 2007), and/or γ lobes (Yu et al., 2006). Secondly, short-lasting memory traces might be produced by calcium-independent cellular mechanisms (Yu et al., 2006).

4.07.2.5 Around the Mushroom Bodies

MBs are innervated by two groups of well-characterized amnesiac and dopaminergic neurons that are involved in olfactory associative memory: dopaminergic neurons (DNs) and the dorsal paired median (DPM) neurons that express the Amn peptide.

4.07.2.5.1 Dopaminergic neurons are required for aversive conditioning

Dopamine (DA) is a neuromodulator that is involved in appetitive reinforcement in mammals (Mirenowicz and Schultz, 1996) and in *Aplysia* (Brembs et al., 2002). It has been known for a while that *Drosophila* synthesizes DA (Hirsh and Davidson, 1981; Livingstone and Tempel, 1983; Wright, 1987; Neckameyer and Quinn, 1989). The recent genetic and imaging tools developed in *Drosophila* allow us to assess the roles of DA in learning and memory. Based on the fact that tyrosine-hydroxylase (TH) is the rate-limiting step in DA biosynthesis, a Gal4-driver whose expression mimics that of TH was tested (Friggi-Grelin et al., 2003). This tool has allowed the identification of six neuronal dopaminergic clusters that project in particular to MBs and to the central complex (Friggi-Grelin et al., 2003), confirming earlier TH and DA immunolabeling studies (Nassel and Elekes, 1992). By combining the TH-GAL4 driver and UAS-Shi^{ts1}, Schwaerzel et al. showed that blocking DNs only during acquisition disrupts STM (Schwaerzel et al., 2003). Thus DNs are required for aversive conditioning, and could convey US (in this case, electric shock) information (Schwaerzel et al., 2003). Another study has shown that in naive flies, electric shock generates a strong activity in the DNs, whereas the odor generates a weak signal. However, after several pairings between the odor and the shock, odor-evoked activity is significantly prolonged. In agreement with the behavioral approach (Schwaerzel et al., 2003), *in vivo* imaging therefore suggests that DNs play a role in aversive conditioning in *Drosophila* (Riemensperger et al., 2005). To study if the activation of DNs is sufficient to mediate the aversive cue, Schroll et al. have developed a technique to remotely stimulate DNs. By expressing a light-activated cation channel (channelrhodopsin-2), in larvae, DNs can be stimulated specifically by illuminating the larvae with blue light. The authors used a discriminatory learning paradigm: one odor (CS^+) is associated with a reinforcing salt stimulus as the aversive US, while another odor (CS^-) is presented without salt (Gerber and Hendel, 2006). Interestingly, the reinforcing salt stimulus could be replaced functionally by DN activation with blue light. DNs are therefore sufficient to convey the US for aversive conditioning (Schroll et al., 2006).

4.07.2.5.2 Dorsal paired median neurons are required for consolidation

Although *amn* is an MTM mutant (Quinn et al., 1979) putatively involved in cAMP metabolism (Feany and Quinn, 1995), Amn is not expressed in the MB but in the DPM neurons which project onto all the MB lobes (Waddell et al., 2000). In the *amn* mutant, expression of wild-type Amn in the DPM neurons re-establishes normal MTM (Waddell et al., 2000). Conversely, when Shi^{ts1} is expressed at restrictive temperature into DPM, MTM is affected (Waddell et al., 2000). Blocking the DPM during the consolidation phase only (Keene et al., 2004), and more precisely, 30 min after conditioning (Keene et al., 2006), phenocopies the *amn* mutant memory defect (Waddell et al., 2000). Scott Waddell's group has used an elegant approach to determine the site of action of DPM neurons projecting onto the MBs. By driving Dscam expression, a protein involved in axonal guidance (Wang et al., 2002), in the DPM neurons during development, DPM innervation of the $\alpha/\beta/\gamma$ lobes is disrupted. However, those flies show normal memory, indicating that MTM is stabilized in α'/β' lobes in response to DPM. Co-expression of Dscam

and Shi^{ts1} abolishes memory (Keene et al., 2006) and confirms the role of the α'/β' lobes during memory consolidation. Also, as mentioned above, Amn might activate an adenylyl cyclase. Thus, Amn could activate Rut via a G-protein-coupled receptor, in order to extend MTM. However, *rut* MTM is rescued when Rut is specifically expressed in the $\alpha/\beta/\gamma$ lobes of the MBs, even though Amn stimulates the α'/β' lobes. It is therefore difficult to imagine that Amn is directly involved in Rut activation, unless Amn diffuses into the $\alpha/\beta/\gamma$ lobes. Since it is likely that DPM neurons also synthesize acetylcholine (Keene et al., 2004), it is possible that this transmitter is directly involved in the stimulating MBs.

Yu et al. took an imaging approach to study the function of the Amn-expressing DPM neurons in memory. DPM neurons respond to both the shock (the US) and to the odor (the CS), and pairing the CS and the US increases odor-evoked calcium signals in the same time window during which DPM neuron synaptic transmission is required for normal memory (Yu et al., 2005). Interestingly, the delayed olfactory memory trace of DPM neuron processes that innervate the vertical lobes is branch-specific (Yu et al., 2005). Conclusions from the behavioral experiments using Shi^{ts1} (Waddell et al., 2000; Keene et al., 2004, 2006) are therefore strengthened by imaging. One hypothesis is that the α'/β' neurons and the DPM neurons form a mutually reinforcing loop that is necessary for this consolidation (Krashes et al., 2007).

4.07.3 Anatomical, Molecular, and Systemic Analysis of Consolidated Memories: Anesthesia-Resistant Memory and Long-Term Memory

4.07.3.1 Anesthesia-Resistant Memory

As described earlier, ARM is a semi-stabilized memory that can be generated either by a single training cycle or massed training (ARM is a subset of total memory, whose proportion increases with time after training, conversely to ASM).

4.07.3.1.1 Anesthesia-resistant memory is localized in mushroom bodies

MBs are involved in acquisition, consolidation, and retrieval of short-lasting memory, but does ARM depend on MBs? To assess the anatomical location of ARM, flies whose different MB neurons were blocked by Shi^{ts} were trained for one cycle, submitted 1 h afterward to a cold shock to disrupt ASM, and tested 1 h after that to measure ARM. When α/β neurons are blocked, ARM is almost totally abolished, whereas γ neuron blockade does not disrupt ARM. Thus we showed that ARM generated after one training cycle relies mainly on α/β neurons MBs (Isabel et al., 2004).

4.07.3.1.2 Molecular pathways

4.07.3.1.2.(i) Radish *Radish* (*rsh*) is a mutant whose residual memory is erased by cold-shock anesthesia (Folkers et al., 1993). Thus *rsh* is specifically deficient in ARM, and it is the only currently known mutant that presents this characteristic. The *rsh* gene was localized within a 180-kb interval in the 11D-E region of the X chromosome, and several candidate genes were identified (Folkers et al., 1993). Recently, Josh Dubnau's group reported that the gene responsible for the *rsh* phenotype was a phospholipase A2 (PLA2), whose expression could rescue the mutant defect (Chiang et al., 2004). However, this rescue experiment could not be reproduced, and it turned out that PLA2 maps 95 kb outside the behaviorally determined deletion interval (Folkers et al., 2006). Instead a second team has reported that *rsh* encodes a novel protein, corresponding to the predicted gene CG15720, and containing possible nuclear localization motifs and 23 predicted PKA and 14 predicted PKC phosphorylation sequences (Folkers et al., 2006). Expression of this gene under the control of an inducible heat-shock promotor in a *rsh* background restores normal ARM (Folkers et al., 2006). The Rsh protein is highly expressed in the MBs (Folkers et al., 2006), which corroborates the behavioral localization of ARM (Isabel et al., 2004).

4.07.3.1.2.(ii) Atypical protein kinase M The atypical protein kinase M (aPKM) is a persistently active truncated isoform of atypical protein kinase C (aPKC). Overexpression of either a mouse or *Drosophila* aPKM transgene enhances memory after massed conditioning, but not after spaced training (Drier et al., 2002). It is therefore conceivable that aPKC is a molecular component of ARM. Because this effect is not blocked in a *rsh* background, the authors proposed that PKM acts downstream of *rsh*. In support of this, inhibition of aPKM disrupts consolidated memory after massed conditioning (Drier et al., 2002).

4.07.3.2 Long-Term Memory

4.07.3.2.1 Long-term memory is localized in vertical lobes of mushroom bodies

MB lobes are known to be required to form short-lasting memory: α'/β' lobes for acquisition and consolidation and $\alpha/\beta/\gamma$ for retrieval. Are MBs involved in LTM? We have identified in our lab the *alpha-lobes absent* (*ala*) mutant, which shows abnormal MB anatomy (Boquet et al., 2000). This mutant shows a particularly unusual MB phenotype: 10% of *ala* individuals possess all five MB lobes, 36% lack the horizontal β and β' lobes, and 4.5% lack vertical α and α' lobes (the remaining subpopulations present different MB phenotypes in the left and the right hemispheres) (Pascual and Preat, 2001). The *ala* mutant flies were trained according to the three following procedures: the short protocol, massed protocol, and long protocol. We analyzed separately the brains of flies that had made the correct and the wrong choice during the memory test, to calculate the memory score of each class of *ala* mutants. The *ala* flies lacking α/α' lobes display a normal STM and ARM, but no LTM at 24 h (Pascual and Preat, 2001) or 5 h (Isabel et al., 2004) after spaced conditioning. Thus, MBs, and more precisely, the α/α' vertical lobes, are necessary to form LTM (Pascual and Preat, 2001). By expressing Shi^{ts1} in α/β lobes, it was further shown that α lobe outputs are required during LTM retrieval (Isabel et al., 2004). Is there an LTM trace in the α vertical lobes?

The G-CaMP reporter was driven in α/β neurons to analyze whether these lobes present an odor-evoked calcium level dependent on associative long-lasting memory (Yu et al., 2006). Whereas α/β neurons presented no increase in calcium during STM, α branches, but not β branches (which belong to the same group of neurons), present an increase in response to conditioned odor after spaced conditioning. This result corroborates the behavioral data (Pascual and Preat, 2001; Isabel et al., 2004). Moreover, Ron Davis's group has shown that this increased calcium signal is blocked after inhibition of protein synthesis, and also by the expression of the inhibitory form of the transcription factor Creb (see next section for details), which blocks LTM (Yu et al., 2006). The authors confirmed that *amn* is required to form LTM, as the mutant fails to increase calcium activity after LTM conditioning (Yu et al., 2006). The hypothesis that DPM neurons participate in LTM consolidation is supported by the observation of a delayed memory trace in the DPM neurons that innervate vertical lobes (Yu et al., 2005, 2006).

4.07.3.2.2 Molecular pathways

4.07.3.2.2.(i) Transcriptional regulation

4.07.3.2.2.(i).a Creb

PKA, in response to increased cAMP, is believed to activate a subset of Creb family proteins in the nucleus (Bacskai et al., 1993) that could in turn activate gene expression required for LTM. The *Drosophila* Creb family gene produces seven alternatively spliced isoforms, among which is a PKA-responsive transcriptional factor (dCreb-a) and another that is an antagonist of the PKA-responsive transcription (dCreb2-b) (Yin et al., 1995). LTM is disrupted by dCreb2-b repressor overexpression (Yin et al., 1994; Perazzona et al., 2004), likely due to inhibition of the gene expression required to establish LTM. Yin and colleagues reported that flies overexpressing the dCreb2-a activator generated LTM after a single training cycle (Yin et al., 1995). However, this result could not be replicated, and it was shown that the original dCreb2-a transgenic flies carried an accidental mutation that produced a truncated protein with no DNA binding domain (Perazzona et al., 2004). Moreover, ubiquitous adult induction of the correct Creb2-a isoform led to lethality (Perazzona et al., 2004), likely because of ectopic expression of downstream proteins. The exact role of Creb in LTM formation therefore remains to be elucidated.

4.07.3.2.2.(i).b Notch

Notch is a signaling receptor controlling cell fate determination and pattern formation in development (Artavanis-Tsakonas et al., 1999). This transmembrane protein is cleaved in response to ligands such as Delta. Its cytoplasmic part can enter the nucleus to promote regulation of gene expression (Kidd et al., 1998; Schroeter et al., 1998; Struhl and Adachi, 1998). Besides its involvement in development, Notch is required for the regulation of neurite outgrowth in the adult mammalian brain (Sestan et al., 1999). Because of this role in neural ultrastructure regulation, and thus potentially in neuronal plasticity (Wang et al., 2004a) and memory (Costa et al., 2003) in mammals (for review, see Costa et al., 2005), its role in associative memory in *Drosophila* was assessed independently by two teams (Ge et al., 2004; Presente et al., 2004). To circumvent developmental roles of Notch, inducible Notch

manipulations were performed at the adult stage. A temperature-sensitive Notch mutant allele and a dominant-negative Notch mutant displayed intact STM and ARM and impaired LTM when adult flies were submitted to the nonpermissive temperature for 2 days (Ge et al., 2004; Presente et al., 2004). Because these experiments do not demonstrate where Notch is required in the brain, the authors used RNAi-mediated Notch repression, restricted to the MBs by means of an MB-specific driver. MB Notch impairment led to an LTM defect (Presente et al., 2004). Finally, overexpression of a wild-type copy of Notch generates a protein synthesis-dependent memory resembling LTM after only one or two spaced training cycles, a protocol that normally induces only short-lasting memories (Ge et al., 2004). The identity of the Notch ligand(s), and the downstream gene(s) regulated by Notch signaling during LTM, remain to be discovered.

4.07.3.2.2.(ii) Translational regulation

4.07.3.2.2.(ii).a Staufen/pumilio pathways

Dubnau and colleagues (Dubnau et al., 2003a) tested a behavioral screen for LTM mutants, parallel to microarray experiments aimed to select genes with altered expression after LTM training. This work led to the identification of several proteins involved in mRNA processing as well as in translation (Dubnau et al., 2003a): (1) Pumilio is a protein known to act as a transcript-specific translational repressor, regulating localized mRNA translation in oocytes; (2) Staufen and Oskar mediate the translocation of several proteins to posterior poles of oocytes; and (3) eIF-5C is a translation initiation factor. In the adult brain, these genes are preferentially expressed in the MB. Disruption of either of these four genes impairs LTM. Thus LTM requires local mRNA translation regulation, possibly postsynaptically in the MB calyces or presynaptically in the vertical lobes.

4.07.3.2.2.(ii).b RNA-induced silencing complex

In an elegant study, Ashraf et al. (2006) showed that protein synthesis at the synapse is required for LTM, and that LTM formation depends on calcium/calmodulin-dependent kinase II (CaMKII) signaling, a pathway also implicated in synaptic plasticity in mammals (Kelleher et al., 2004). By driving the expression of a tagged CaMKII in projection neurons that link olfactory sensory neurons to MBs, they showed that the recruitment of CaMKII to postsynaptic sites in the antennal lobe glomeruli is required to induce LTM (Ashraf et al., 2006). By performing this expression in different mutant backgrounds, they further showed that synaptic protein synthesis is regulated by the RNA interference silencing complex (RISC).

4.07.3.2.2.(iii) Posttranslational regulation of long-term memory formation

4.07.3.2.2.(iii).a Crammer

We have described *crammer* (*cer*), a gene involved specifically in the formation of LTM (Comas et al., 2004). The *cer* mutant has reduced LTM but normal STM, MTM, and ARM. Interestingly, in the wild-type strain, *cer* expression is transiently reduced 3 hours after LTM training. As the Cer peptide is an inhibitor of cysteine proteinases, the decrease in its expression shortly after intensive training must lead to a transient activation of its cysteine proteinase(s) target(s) (Comas et al., 2004). The overexpression of *cer* in glial cells but not in MB neurons induces an LTM decrease, indicating that glial cells expressing *cer* might be involved in LTM formation. However, in this experiment Cer is overexpressed during development, and further experiments should be carried out to disrupt or overexpress Cer specifically during the adult stage in glia cells. Whether Cer is secreted to act on MB neurons or whether Cer is involved only within glial cells also remains to be resolved. In conclusion, this work suggests that regulation of cysteine proteases is required to perform LTM, possibly in glia cells surrounding the MBs (Comas et al., 2004).

4.07.3.2.2.(iii).b Tequila

Mutations in the human neurotrypsin gene are associated with nonsyndromic mental retardation (MR) (Molinari et al., 2002). An important question is whether the MR generated by this mutation is a consequence of a brain development defect and/or a consequence of a physiological plasticity defect. To address this question, we took advantage of the high degree of homology between the human and the fly genomes, and the genetic tools offered by the fly model. Tequila (teq), a serine protease, is the *Drosophila* ortholog of the human neurotrypsin. First, we showed that a constitutive *teq* mutant has normal STM and ARM, but presents an LTM defect. To study whether Teq regulation for LTM processing is required in the MBs, RNAi-mediated Teq inhibition was induced in these structures. LTM in these flies was specifically impaired. To further study

whether Teq is required only in the adult stage or during development, the inducible Gene-Switch system was used to disrupt Teq specifically in adult stage. LTM was disrupted, but not STM or ARM, which conclusively demonstrates that Teq is required for the plasticity process and not for development. Is Teq transcription finely regulated during LTM formation? Levels of Teq mRNA in adult heads were measured at different times after spaced conditioning and showed an approximately 30-fold increase 4 h after training. This increase took place in MBs, especially in the MB peduncle (a dense structure where axons project before giving rise to lobes), as shown by immunostaining 5 h after training. Interestingly, transient Teq silencing in the adult stage, before but not during training, had no impact on LTM, showing that LTM impairment is reversible if Teq is naturally expressed *de novo* after artificial disruption. We conclude that this serine protease is required for information processing and functional plasticity in *Drosophila* and could have a preponderant role in postnatal cognition processes in children (Didelot et al., 2006).

4.07.3.3 Dynamics of Memory Phases

The short protocol induces two labile phases: STM, which is disrupted in mutants affected for cAMP metabolism and lasts about 30 min, and MTM, which is disrupted in *amn* flies and lasts for a few hours. STM and MTM are both anesthesia-sensitive, as they are erased if flies are cooled down to 4 °C after conditioning. This property suggests that STM and MTM are sustained by brain electrical activity.

LTM is induced by the long protocol and can be measured for at least 1 week. What are the dynamics of memory phase interaction in *Drosophila*? In a previous model, information acquired during learning is processed into consolidated memories (ARM and LTM) by passing sequentially through two earlier memory phases (STM and MTM) (Tully et al., 1994). In contrast, we recently proposed a model that involves two parallel memory pathways, one with cAMP-dependent STM/MTM, and the other with ARM (**Figure 4**). Indeed, *dnc* and *rut* retain a significant level of early memory (Tully and Quinn, 1985), suggesting that an adenyl cyclase-Rut-independent learning might exist. Moreover, ARM levels in *rut*, *amn* are close to normal (Folkers et al., 1993; Tamura et al., 2003; Isabel et al., 2004), while their labile memories are strongly affected. Thus ARM does not seem to depend on STM/MTM as

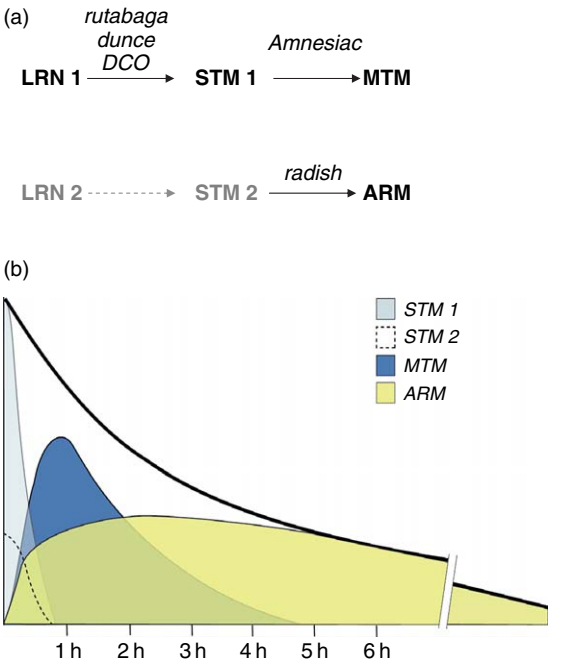


Figure 4 Model of associative memory phases (a) and temporal dynamics of memory phases (b) generated by a single cycle of conditioning (short protocol). LRN, learning; STM, short-term memory; MTM, middle-term memory; ARM, anesthesia-resistant memory; DCO, catalytic subunit gene.

previously suggested (Tully et al., 1994). Instead a second learning process could give rise to an STM 2 phase and later to ARM (**Figure 4**).

What are the relationships between ARM and LTM? To answer this question, the *ala* mutant was trained with the long protocol, and the memory of flies lacking vertical α/α' lobes was measured at 30 min and 5 h after the training. The 30-min memory was normal, but, surprisingly, the 5-h memory was close to zero. Memory performance was normal at 5 h when flies without vertical lobes were trained with the short protocol (Isabel et al., 2004) (**Figure 5**). Why does a longer training cycle give rise to weaker memory? The *ala* flies display no LTM because they lack the vertical lobes, the center for LTM. These flies show a normal ARM 5 h after the short protocol, but no ARM after the long protocol. This result suggests that ARM is erased after LTM conditioning. Thus the consolidated memory phases generated by olfactory conditioning are exclusive (**Figure 6**) (Isabel et al., 2004). Why is ARM erased after LTM conditioning? We propose that ARM could act as a gating mechanism for LTM formation, avoiding a heavy cascade of gene expression in absence of intensive spaced conditioning.

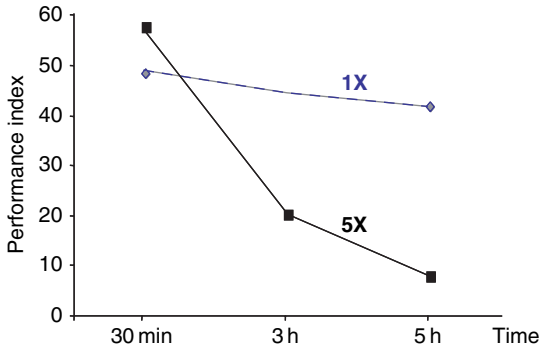


Figure 5 In flies without MB alpha lobes, which normally sustain long-term memory, the long protocol decreases memory performance at 5 h in comparison with the short protocol. Grey line, short protocol; black line, long protocol.

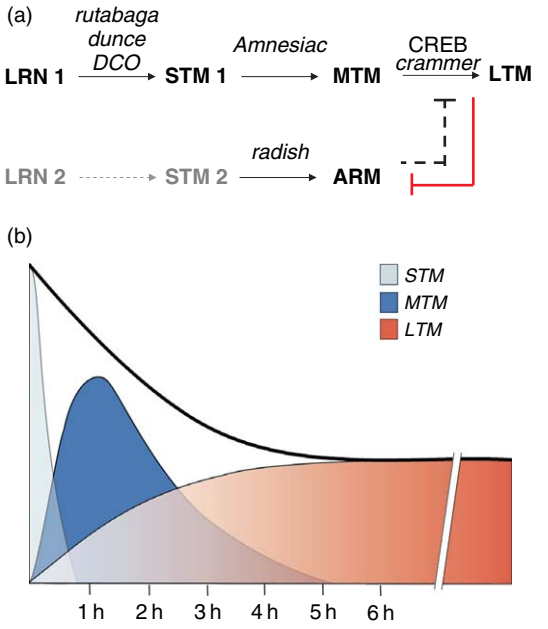


Figure 6 Model of associative memory phases (a) and temporal dynamics of memory phases (b) generated by five cycles of conditioning (long protocol). LRN, learning; STM, short-term memory; MTM, middle-term memory; ARM, anesthesia-resistant memory; LTM, long-term memory.

Despite the relative simplicity of the *Drosophila* brain, this model suggests a cognitive complexity more frequently associated with mammalian models. It supports the idea that *Drosophila* is a valid model to study some of the molecular and cellular mechanisms involved in normal or pathological human memory (Shulman et al., 2003).

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4.08 Molecular Mechanisms of Associative Learning in *Hermissenda*

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4.08.1 Introduction

A central focus in the history of studies of mentation is on how basic associations are formed and retained in memory. This characteristic of memory has been addressed from a descriptive perspective by the laws of association: similarity, contrast, and contiguity proposed by Aristotle, and further expanded by the philosophical school of British Associationism led by Locke, Berkeley, and Hartley (Watson, 1968). These early formulations led to a further elaboration of the role of contiguity in memory to include both simultaneous and successive associations. Within this historical context it is not surprising that the law of association by contiguity became the mainstay of several theories of learning proposed by behaviorists (Hilgard and Bower, 1966). Associations formed by contiguity are an essential feature of Pavlovian conditioning. Within the Pavlovian tradition, the response to the conditioned stimulus (CS) was conditional upon its pairing with the unconditioned stimulus (US), and the transfer of the response-evoking properties of the US to the CS as a result of pairing (contiguity) provided the critical evidence in support of the formation of an association in memory. In part, due to the detailed knowledge of the anatomy of sensory systems and the experimental control over the timing of stimulation of specific sensory pathways, Pavlovian conditioning paradigms have provided the opportunity to study the mechanisms underlying the formation of basic associations in memory at a cellular, synaptic, and molecular level. There is now a rich data base on Pavlovian conditioning generated from diverse species that represents sufficient complexity characteristic of different forms of memory.

The primary focus of cellular and molecular studies of Pavlovian conditioning has been on nondeclarative or procedural memory that is expressed by performance not dependent upon conscious recall. Recent studies of eyeblink conditioning employing trace procedures have explored the role of Pavlovian conditioning in declarative or conscious memory systems (Clark et al., 2002). The analysis of Pavlovian conditioning in the less complex nervous systems of higher invertebrates has been useful in elucidating the sites of memory storage, leading to an analysis of mechanisms of memory that underlie well-documented examples of associative learning.

The nudibranch mollusk *Hermissenda crassicornis* is one preparation that has contributed to an understanding of Pavlovian conditioning at the cellular, molecular, and systems levels. Pavlovian conditioning

in *Hermissenda* involves changes in intrinsic excitability and synaptic efficacy at multiple sites within the neural circuit supporting the generation of the conditioned response (CR). The modifications produced by Pavlovian conditioning involve the engagement of multiple cellular mechanisms within identified sensory neurons (photoreceptors) and interneurons that are expressed by alterations in the properties of channels in excitable membranes. Initial acquisition and long-term retention involve both presynaptic and postsynaptic mechanisms. The actions of several second messenger systems contribute to both acquisition and retention of associative memory. Short-term memory involves posttranslational modifications of proteins by several signaling pathways and is expressed by changes in both synaptic connections and intrinsic excitability. Intermediate-term memory requires translation and posttranslational modifications, but not transcription. Long-term memory requires posttranslational modifications, new mRNA and protein synthesis, and structural modifications and is expressed by long-term changes in intrinsic cellular excitability and synaptic efficacy.

4.08.2 Pavlovian Conditioning

Classical conditioning of *Hermissenda* follows the Pavlovian tradition where the CS and US elicit different responses prior to training. Stimulation of the CS and US pathways conveys different sensory information to the central nervous system. Before conditioning, light, the CS, does not elicit either of the unconditioned responses (UCRs) that have been studied in *Hermissenda:* foot-shortening and inhibition of forward ciliary locomotion. Stimulation of statocyst hair cells of the graviceptive system using rotation or orbital shaking, the US, elicits foot-shortening and a reduced rate of forward ciliary locomotion (Alkon, 1974; Crow and Alkon, 1978; Farley and Alkon, 1982; Lederhendler et al., 1986; Matzel et al., 1990b). Pavlovian conditioning produces both light-elicited inhibition of ciliary locomotion, which results in a suppression of *Hermissenda's* normal positive phototaxis (Crow and Alkon, 1978, 1980; Crow and Offenbach, 1983; Crow, 1985a), and CS-elicited foot-shortening (Lederhendler et al., 1986) (see **Figure 1**).

Both conditioned foot contraction and inhibition of ciliary locomotion involve the development or emergence of a new response to the CS, not the potentiation, through US presentations, of an already existing response to the CS referred to as alpha conditioning or reflex potentiation (e.g., Schreurs, 1989; Sahley and Crow, 1998). In both of the CRs there is a transfer of functional aspects of the response-evoking properties of the US to the CS (Crow and Alkon, 1978; Lederhendler et al., 1986;

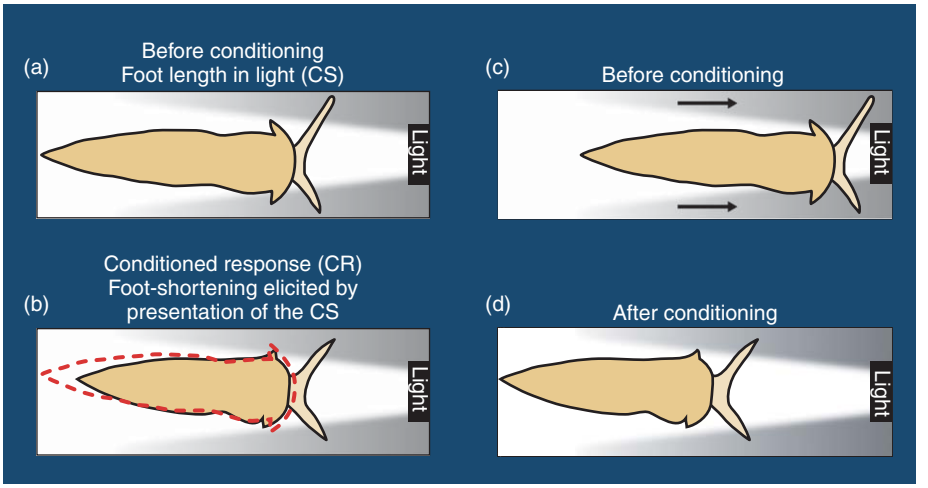


Figure 1 Pavlovian conditioning of foot-shortening and phototaxic inhibition in *Hermissenda.* (a) Foot length in light (CS) before conditioning. (b) CR foot-shortening elicited by the CS. Red outline indicates foot length in light before conditioning. (c) Light-elicited ciliary locomotion toward a light source (phototaxis) assessed before conditioning. (d) Inhibition of light-elicited ciliary locomotion detected after Pavlovian conditioning. Random or pseudorandom presentations of the CS and US do not produce either inhibition of ciliary locomotion or CS-elicited foot-shortening. Figure adapted from Crow T (2004) Pavlovian conditioning of *Hermissenda*: Current cellular, molecular, and circuit perspectives. *Learn. Mem.* 11: 229–238; used with permission from Cold Spring Harbor Laboratory Press.

Matzel et al., 1990b). The two CRs are proposed to develop independently (Matzel et al., 1990b), which is consistent with results showing that the neural circuitry supporting foot contraction and ciliary locomotion consists of different neuronal components (Crow and Tian, 2003a,b) (see the section titled 'Neural circuitry'). Retention of conditioned behavior persists for several days to weeks, depending upon the number of conditioning trials presented in initial acquisition (Crow and Alkon, 1978; Alkon, 1983; Harrigan and Alkon, 1985).

Pavlovian conditioning in *Hermissenda* has been shown to express many of the characteristics of Pavlovian conditioning in vertebrates, such as extinction (Richards et al., 1984), CS specificity (Crow and Offenbach, 1983), and conditioned inhibition (Britton and Farley, 1999). Conditioning is dependent upon the temporal association of the CS and US, involving both contiguity (Crow and Alkon, 1978) and contingency (Farley, 1987a,b). Extra CS and US presentations inserted into a sequence of CS-US pairings attenuate conditioning (Farley, 1987a). Conditioning in the two different behavioral response systems supporting the two CRs is sensitive to both CS-US contiguity and forward inter-stimulus-interval manipulations (Matzel et al., 1990c).

4.08.3 Neural Circuitry

The anatomy and synaptic organization of the two sensory structures (visual and graviceptive) mediating the CS and US have been described in detail (Alkon and Fuortes, 1972; Alkon, 1973a,b; Alkon and Bak, 1973; Detwiler and Alkon, 1973). In addition, many of the sites of convergence providing for synaptic interactions between the CS and US pathways have been identified (Alkon, 1973a,b; Alkon et al., 1978; Akaike and Alkon, 1980; Crow and Tian, 2000, 2002a,b, 2003a, 2004, 2006). Light produces a depolarizing generator potential and an increase in spike activity in the five photoreceptors in each eye (Dennis, 1967; Alkon and Fuortes, 1972). The primary sensory neurons of the pathway mediating the US consist of the 13 hair cells in each gravity-detecting statocyst. Rotation or gravity produces a depolarizing generator potential and an increase in the spike frequency of the stimulated hair cells (Alkon, 1975). There are multiple sites of convergence between the CS and US pathways.

1. The first site is between the primary sensory neurons, photoreceptors, and hair cells. Statocyst hair cells project monosynaptically to photoreceptors and receive monosynaptic input from photoreceptors (**Figure 2**). Stimulation of statocyst hair cells elicits a monosynaptic GABAergic (GABA: gamma-aminobutyric acid) inhibitory postsynaptic potential (IPSP) in type B photoreceptors (Alkon et al., 1993; Sakakibara et al., 1993; Blackwell, 2002a).
2. It has been proposed that hair cells also project polysynaptically to photoreceptors through a serotonergic modulatory pathway based upon behavioral, physiological, and immunohistochemical studies (Crow and Bridge, 1985; Land and Crow, 1985; Auerbach et al., 1989; Farley and Wu, 1989; Grover et al., 1989;

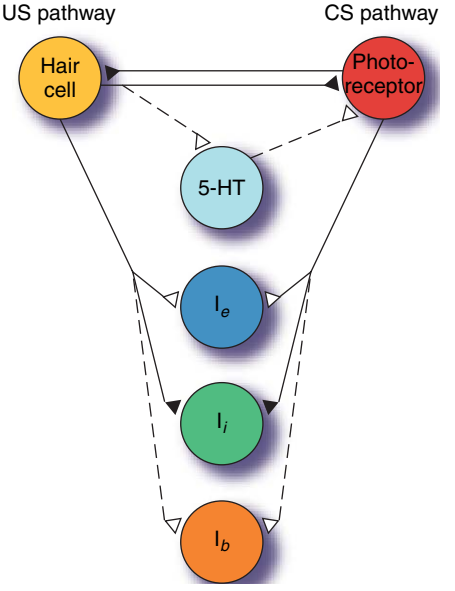


Figure 2 Sites of convergence between identified components of the CS and US pathways that result in intrinsic cellular plasticity in photoreceptors and type I_e interneurons, and proposed plasticity in type I_b interneurons. Statocyst hair cells project directly (monosynaptic) and indirectly (polysynaptically) through proposed serotonergic interneurons (5-HT) to identified photoreceptors. Caudal hair cells inhibit photoreceptors, and cephalic hair cells are inhibited by type B photoreceptors. Hair cells and photoreceptors form monosynaptic connections with type I_e and type I_i interneurons and form polysynaptic connections with type I_b interneurons. (▲) Inhibitory synaptic connections; (Δ) excitatory synaptic connections. Solid lines represent established monosynaptic connections, and dashed lines polysynaptic connections, with potential interneurons not yet identified. Figure adapted from Crow T (2004) Pavlovian conditioning of *Hermissenda*: Current cellular, molecular, and circuit perspectives. *Learn. Mem.* 11: 229–238; used with permission from Cold Spring Harbor Laboratory Press.

Crow and Forrester, 1991; Acosta-Urquidi and Crow, 1993; Rogers and Matzel, 1995; Yamoah and Crow, 1995, 1996; Tian et al., 2006). Serotonergic-immunoreactive varicosities encircle the optic nerve before entry into the cerebropleural ganglion (Land and Crow, 1985). However, the source of the serotonergic input to the photoreceptors has not yet been identified.

3. Statocyst hair cells and photoreceptors also form monosynaptic connections with type I_e and I_i interneurons (Akaike and Alkon, 1980; Crow and Tian, 2000, 2002a) and polysynaptic connections with type I_b interneurons (Crow and Tian, 2003a, 2004) (**Figure 2**).

The CS modulates ciliary locomotion through monosynaptic connections between photoreceptors and type I_e and I_i interneurons and polysynaptic connections between photoreceptors and type III_i inhibitory interneurons (see **Figure 3**). Type III_i inhibitory interneurons form monosynaptic connections with identified ciliary motor neurons located in the pedal ganglia. Activation of ciliary motor neurons is produced by a light-dependent reduction in the spike activity of type III_i inhibitory interneurons. Ciliary locomotion is reduced or inhibited by the US due to hair cell excitation of type I_e interneurons that in turn excite type III_i inhibitory interneurons, resulting in inhibition of ciliary motor neurons. An additional pathway that may modulate ciliary locomotion is the monosynaptic excitatory projections from type I_b interneurons to ciliary motor neurons (Crow and Tian, 2004). Interneurons projecting to motor neurons that innervate the foot have also been identified and have provided for the analysis of reflex movements of the foot modified by Pavlovian conditioning (Goh and Alkon, 1984, Goh et al., 1985; Crow and Tian, 2004).

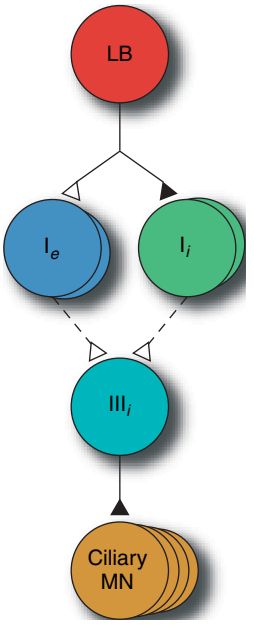


Figure 3 Components of the circuit involved in visually mediated ciliary locomotion in *Hermissenda.* Only synaptic connections with a single type B photoreceptor (LB) are shown. Monosynaptic connections are depicted by solid lines, and polysynaptic connections with dashed lines. Filled triangles denote inhibitory synapses, open triangles excitatory. As shown in the circuit diagram, identified photoreceptors project directly to aggregates of 'on' and 'off' neurons: type I_e and I_i interneurons. MN, motor neuron.

4.08.4 Cellular and Molecular Mechanisms Underlying Short-, Intermediate-, and Long-Term Memory Formation

Contemporary views of memory and its formation over time indicate that both declarative and nondeclarative forms of memory involve multiple stages with different underlying mechanistic requirements. A number of *in vivo* and *in vitro* procedures involving one or several training trials have been employed in Pavlovian conditioning studies of *Hermissenda* to examine the early events supporting the formation of short-, intermediate-, and long-term memory. The different protocols involving one or several conditioning trials produce behavioral changes and physiological modifications that can be detected within minutes following training (Crow, 1983; Farley and Alkon, 1987; Matzel et al., 1990a,b; Matzel and Rogers, 1993; Crow et al., 1998; Ramirez et al., 1998; Epstein et al., 2003; Kuzirian et al., 2006). Since the two sensory pathways mediating the CS and US are totally intact in the isolated nervous system, *in vitro* Pavlovian conditioning procedures can be applied to the isolated circumesophageal nervous system. Pairing the CS (light) with mechanical perturbations of the statocyst produced by piezoelectric stimulation (US) sufficient to depolarize hair cells, or rotation of the isolated nervous system (US), produces electrophysiological correlates in type B photoreceptors that are similar to correlates produced by multi-trial *in vivo* procedures (Matzel et al., 1990a; Matzel and Rogers, 1993; Gandhi and Matzel, 2000). A multi-trial *in vitro* procedure involving pairing the CS with extrinsic

current depolarization of identified statocyst hair cells (nominal US) also produces conditioning correlates in type B photoreceptors (Farley and Alkon, 1987). The results of these investigations depend upon the various conditioning protocols, the efficacy of the US, and the duration of CS-US stimulation.

4.08.4.1 One-Trial Conditioning

To more precisely control when the initial learning occurs and to not confound time after conditioning, when memory is tested, with varying numbers of conditioning trials, a one-trial *in vivo* conditioning procedure was developed that produces a pairing-specific long-term inhibition of normal light-elicited ciliary locomotion (Crow and Forrester, 1986). Pairing the CS (light) with the direct application of 5-hydroxytryptamine (5-HT, nominal US), one of the proposed transmitters of the US pathway to the exposed nervous system of otherwise intact *Hermissenda* produces inhibition of light-elicited ciliary locomotion when the animals are tested 24 h following the conditioning trial. An *in vitro* analog of the one-trial procedure involving pairing the CS with 5-HT application has been used with the isolated circumesophageal nervous system to examine mechanisms underlying the development of short-, intermediate-, and long-term memory. A one-trial *in vitro* procedure consisting of pairing the CS with mechanical perturbation of the statocyst produces a significant Ca^{2+}-dependent increase in input resistance of type B photoreceptors (a correlate of enhanced excitability) that is detected within minutes postconditioning (Matzel and Rogers, 1993). In addition, a one-trial *in vitro* procedure involving GABA application to the region of the photoreceptor terminal branches (nominal US) paired with a 10-s depolarization of the type B photoreceptors (nominal CS) produces an increase in the input resistance of the B photoreceptors that persists for at least 10 min (Matzel and Alkon, 1991). These studies indicate that the application of a neurotransmitter, when paired with depolarization resulting in a brief period of Ca^{2+} elevation, is sufficient to produce enhanced excitability, and the procedures may engage the essential components for the formation of associations underlying conditioning.

4.08.4.2 Long-Term Memory Following Multi-Trial Conditioning

The analysis of electrophysiological and biophysical modifications detected following multi-trial Pavlovian conditioning has focused upon two sites of convergence between the CS and US pathways. The first site is in the primary sensory neurons (photoreceptors) of the pathway mediating the CS (Crow and Alkon, 1980). Neural modifications in the primary sensory neurons of conditioned animals involve both enhanced excitability that is intrinsic to identified type A and type B photoreceptors (Crow and Alkon, 1980; Alkon et al., 1982, 1985; Farley and Alkon, 1982; West et al., 1982; Crow, 1985b; Frysztak and Crow, 1993, 1997) and facilitation of synaptic connections between identified photoreceptors (Frysztak and Crow, 1994, 1997; Gandhi and Matzel, 2000), and photoreceptors and interneurons (Crow and Tian, 2002b, 2003b). The intrinsic modifications in type B photoreceptors are expressed by enhancement of the amplitude of CS-elicited generator potentials and a concomitant increase in spike frequency, increased excitability to extrinsic current, decreased spike frequency accommodation, and a reduction in the peak amplitude of voltage-dependent (I_A, I_{Ca}) and Ca^{2+}-dependent ($I_{K(Ca)}$) currents (Crow and Alkon, 1980; Alkon et al., 1982, 1985, 1992, 1993; Farley and Alkon, 1982; Alkon, 1984; Crow, 1985b; Goh et al., 1985; Collin et al., 1988; Farley et al., 1990; Matzel et al., 1990a; Frysztak and Crow, 1993, 1994, 1997; Blackwell, 2000; 2002a,b; Muzzio et al., 2001).

The increase in the amplitude of CS-elicited generator potentials is in part the result of a reduction in I_A and $I_{K(Ca)}$. In type B photoreceptors of conditioned animals, the peak amplitude of I_A is significantly reduced and exhibits more rapid inactivation as compared to controls (Alkon et al., 1985). However, both the delayed rectifier (I_K) and inward rectifier (I_h) may play a role in conditioning-dependent enhanced excitability. The application of 5-HT to the isolated nervous system enhances the peak amplitude of I_h and decreases the peak amplitude of I_K and I_A in type B photoreceptors (Acosta-Urquidi and Crow, 1993). In addition, 5-HT reduces the amplitude of $I_{K(Ca)}$ and decreases I_{Ca} in type B photoreceptors (Yamoah and Crow, 1995). The reduction in $I_{K(Ca)}$ produced by 5-HT is a consequence of the decrease in I_{Ca} by 5-HT rather than a direct effect of 5-HT on $I_{K(Ca)}$. In conditioned animals, type A photoreceptors exhibit a decrease in the amplitude of light-elicited generator potentials, enhanced excitability to extrinsic current, increases in CS-elicited spike activity, and a significant increase in the magnitude of I_K (Farley et al., 1990; Frysztak and Crow, 1993, 1997; Farley and Han, 1997). Multi-trial conditioning does not result

in changes in either I_A or $I_{K(Ca)}$ in type A photoreceptors, in contrast to the modifications in type B photoreceptors (Farley and Han, 1997).

In addition to intrinsic enhanced excitability in sensory neurons produced by multi-trial conditioning, changes in synaptic strength between identified sensory neurons and interneurons occur following conditioning. The amplitude of the monosynaptic IPSP between the medial type B photoreceptor and medial type A photoreceptor is significantly enhanced in conditioned animals (Frysztak and Crow, 1994; Gandhi and Matzel, 2000). The second convergence site between the CS and US pathways is the monosynaptic connection between type B photoreceptors and type I interneurons. Multi-trial conditioning produces facilitation of monosynaptic and complex PSPs in identified type I_e and I_i interneurons (Crow and Tian, 2002b). In addition to conditioning-dependent synaptic facilitation, type I interneurons also express intrinsic enhanced excitability with conditioning. Extrinsic current pulses elicit significantly more spikes in type I_e interneurons of conditioned animals as compared to pseudorandom controls.

Therefore multi-trial conditioning in *Hermissenda* results in both presynaptic and postsynaptic modifications. The enhanced excitability of type B photoreceptors, expressed by an increase in both the amplitude of CS-elicited generator potentials and the number of action potentials elicited by the CS, may be a major contributor to changes in the duration and amplitude of CS-elicited complex PSPs and increased CS-elicited spike activity in type I interneurons of conditioned animals (Crow and Tian, 2002b). However, facilitation of the amplitude of the monosynaptic IPSP between type B photoreceptors and type I_i interneurons and the monosynaptic excitatory postsynaptic potential (EPSP) between type B photoreceptors and type I_e interneurons of conditioned animals may involve both pre- and postsynaptic mechanisms.

4.08.5 Second Messenger Systems

4.08.5.1 Protein Kinase C

Protein kinase C (PKC) activation contributes to enhanced excitability and synaptic facilitation underlying the formation of short- and long-term memory in *Hermissenda* (Farley and Auerbach, 1986; Neary et al., 1986; Matzel et al., 1990a; Crow et al., 1991; Farley and Schuman, 1991). PKC is translocated from the cytosol to membrane in the nervous system of *Hermissenda* by treatment with a phorbol ester (12-O-tetradecanoylphorbol-13-acetate (TPA)). The bath application of a phorbol ester (phorbol 12,13-dibutyrate (PDB)) and injection of PKC into type B photoreceptors results in a reduction in the peak amplitude of two K^+ currents, I_A and $I_{K,(Ca)}$, that resemble changes in conductances detected following multi-trial Pavlovian conditioning (Farley and Auerbach, 1986).

Nine conditioning trials produce a foot-shortening CR elicited by the CS that is detected within minutes after the last conditioning trial (Matzel et al., 1990a). An *in vitro* conditioning procedure consisting of nine training trials of the CS paired with rotation of the isolated circumesophageal nervous system (US) enhances type B photoreceptor excitability and increases the amplitude of the plateau phase of the CS-elicited generator potential. The conditioning-dependent change in excitability of type B photoreceptors is blocked by the broad-spectrum kinase inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperizine (H-7) applied in artificial seawater during *in vitro* conditioning. A second *in vitro* procedure involving pairing the CS with extrinsic current depolarization of the B photoreceptors (nominal US) produced enhanced excitability of the B photoreceptors which is also blocked by preconditioning application of H-7 or sphingosine (Matzel et al., 1990a).

One-trial *in vivo* conditioning consisting of pairing the CS with the application of 5-HT to the exposed but otherwise intact circumesophageal nervous system produces short-term enhanced excitability, intermediate-term enhanced excitability, and long-term enhanced excitability of type B photoreceptors (Crow and Forrester, 1991, 1993a; Crow et al., 1991). The induction of short-term enhanced excitability following one-trial conditioning is blocked by the protein kinase inhibitors H-7 and sphingosine and by downregulation of PKC produced by pretreatment with TPA (Crow et al., 1991; Crow and Forrester, 1993b). However, while H-7, sphingosine, or downregulation of PKC by TPA blocks short-term enhanced excitability, the same treatments do not block long-term enhanced excitability produced by one-trial conditioning (Crow and Forrester, 1993b). Therefore short- and long-term enhanced excitability produced by one-trial *in vivo* conditioning involve independent or parallel processes and differential contributions of second messengers. *Thus, the expression of long-term memory produced by one-trial conditioning does not depend upon the induction of short-term memory.*

Consistent with previous studies, the induction of enhanced excitability in type B photoreceptors produced by five *in vitro* conditioning trials involving the CS paired with depolarizing current stimulation of an identified statocyst hair cell is blocked by pretreatment with PKC inhibitors (Farley and Schuman, 1991). However, the contribution of PKC to the expression of long-term enhanced excitability depends upon the conditioning protocol and the number of conditioning trials. Previously established long-term enhancement produced by one-trial *in vivo* conditioning is not reversed by the broad-spectrum kinase inhibitor H-7 or the PKC inhibitors sphingosine or staurosporine (Crow and Forrester, 1993a). In contrast, long-term enhanced excitability in type B photoreceptors produced by multi-trial Pavlovian conditioning is attenuated by H-7 or sphingosine, suggesting that long-term enhanced excitability is dependent upon persistent kinase activity (Farley and Schuman, 1991).

Lateral type A photoreceptors exhibit an increase in the number of spikes elicited by the CS and extrinsic current following multi-trial Pavlovian conditioning (enhanced excitability) (Frysztak and Crow, 1993). Injection of the PKC inhibitor peptide PKC(19-36) into lateral type A photoreceptors 24–48 h following multi-trial conditioning reverses enhanced excitability within 16 min postinjection, suggesting that either a long-lived activator or a constitutively active kinase contributes to the expression of enhanced excitability in lateral A photoreceptors (Frysztak and Crow, 1997). Injection of the control noninhibitory peptide [glu^{27}] PKC(19-36) does not reverse enhanced excitability in lateral A photoreceptors of conditioned animals. PKC activation also contributes to the induction of 5-HT-dependent synaptic facilitation, but persistent PKC activity is not required for long-term synaptic facilitation. Short-term synaptic facilitation of the connection between type B and type A photoreceptors is produced by bath application of 5-HT (Schuman and Clark, 1994; Frysztak and Crow, 1997). Injection of the PKC inhibitor peptide PKC(19-36) into medial type B photoreceptors blocks 5-HT-induced synaptic facilitation of the IPSP recorded in the medial type A photoreceptor (Frysztak and Crow, 1997). However, injection of PKC(19-36) into medial type B photoreceptors following multi-trial Pavlovian conditioning does not reduce or reverse established synaptic facilitation of the IPSP recorded in medial type A photoreceptors. Thus PKC contributes to the induction of short-term synaptic facilitation of the monosynaptic connection between types B and A photoreceptors, but not to the expression of long-term synaptic facilitation of the same monosynaptic connection between type B and A photoreceptors.

4.08.5.2 Extracellular Signal-Regulated Protein Kinase

One-trial *in vitro* conditioning of the isolated nervous system involving the CS paired with 5-HT results in the increased $^{32}PO_4$ labeling of a protein with an apparent molecular weight consistent with extracellular signal-related kinase (ERK). The increased phosphorylation of the protein following one-trial conditioning is blocked by pretreatment with the MEK1 (MAPK/ERK kinase) inhibitor PD098059 (Crow et al., 1998). Assays of ERK activity with brain myelin basic protein as a substrate shows greater ERK activity for nervous systems from one-trial *in vitro* conditioned animals as compared to controls that received the CS and 5-HT unpaired. In addition, Western blot analysis of phosphorylated ERK with a phospho ERK antibody shows a significant increase in ERK phosphorylation after one-trial conditioning as compared with unpaired controls. The increased phosphorylation is blocked by pretreatment with a MEK1 inhibitor (PD098059). Following a multi-trial conditioning procedure consisting of 10–15 trials, circumesophageal nervous systems from conditioned animals exhibit significantly greater ERK phosphorylation as compared with pseudorandom controls (Crow et al., 1998).

PKC contributes to the 5-HT-dependent activation of the ERK pathway. The phorbol ester TPA increases ERK phosphorylation that is blocked by pretreatment with PKC inhibitors. TPA-dependent ERK phosphorylation is also blocked by the MEK1 inhibitors PD0988059 or U0126. The increased phosphorylation of ERK by 5-HT is attenuated, but not blocked, by pretreatment with the Ca^{2+} chelator BAPTA-AM or pretreatment with PKC inhibitors Gö6976 or GF109203X (Crow et al., 2001). This suggests that Ca^{2+}-dependent PKC activation contributes to ERK phosphorylation, although a PKC-independent pathway also contributes to 5-HT-dependent ERK phosphorylation and activation.

4.08.5.3 Memory Formation Is Ca^{2+}-Dependent

The photoreceptors in the eyes of *Hermissenda* exhibit a spatial segregation of function. Phototransduction takes place in the apical region where the rhabdomere

abuts the lens, and spike generation occurs near the distal end of the axon close to the location of synapses on the terminal processes. Therefore light and rotation have spatially separated physiological consequences in type B photoreceptors. Both light and depolarization increase cytosolic Ca^{2+} levels in photoreceptors (Connor and Alkon, 1989; Sakakibara et al., 1993; Blackwell, 2000, 2002a,b; Muzzio et al., 2001). Light activates phospholipase C (PLC) to produce an increase in inositol trisphosphate (IP_3) and diacylglycerol (DAG) (Sakakibara et al., 1986, 1994). IP_3 opens rhabdomeric Na^+ and Ca^{2+} channels, which result in a depolarizing generator potential and Ca^{2+} influx (Blackwell, 2000). IP_3 also binds to its receptor (IP_3R), which triggers Ca^{2+} release from the endoplasmic reticulum (Blackwell and Alkon, 1999). The Ca^{2+} influx from the rhabdomere and the IP_3R-gated storage compartment can cause Ca^{2+} release from the ryanodine receptor-gated (RyR) compartment (Blackwell and Alkon, 1999).

Rotation (US) produces a depolarizing generator potential in identified statocyst hair cells and elicits a monosynaptic GABAergic IPSP in the photoreceptors (Alkon et al., 1993; Sakakibara et al., 1993; Rogers et al., 1994; Blackwell, 2002a). The US is also proposed to activate a polysynaptic serotonergic pathway that projects to type B photoreceptors (Land and Crow, 1985; Crow and Forrester, 1986, 1991). Both 5-HT (Rogers and Matzel, 1995; Yamoah and Crow, 1996) and GABA (Yamoah and Crow, 1996) are linked to a pertussis toxin–sensitive G-protein. These proteins can activate multiple second messenger systems (see **Figure 4**), several of which have been implicated in one-trial and multi-trial classical conditioning. The primary focus of 5-HT effects has been on the modulation of membrane conductances in type B photoreceptors (e.g., Farley and Wu, 1989; Acosta-Urquidi and Crow, 1993; Yamoah and Crow, 1996). In addition, the induction of 5-HT-dependent enhanced excitability in type B photoreceptors is Ca^{2+} dependent, since BAPTA loading of photoreceptors before 5-HT application blocks the induction of enhanced excitability (Falk-Vairant and Crow, 1992). However, the precise role of 5-HT in the induction and expression of long-term intrinsic enhanced excitability and synaptic facilitation is poorly understood. In contrast, it is proposed that GABA binding to G-protein-coupled receptors on photoreceptors activates phospholipase A_2 (PLA_2) to liberate arachidonic acid (AA) that interacts with Ca^{2+} to synergistically stimulate PKC (Muzzio et al., 2001) and create a back-propagating wave of Ca^{2+}

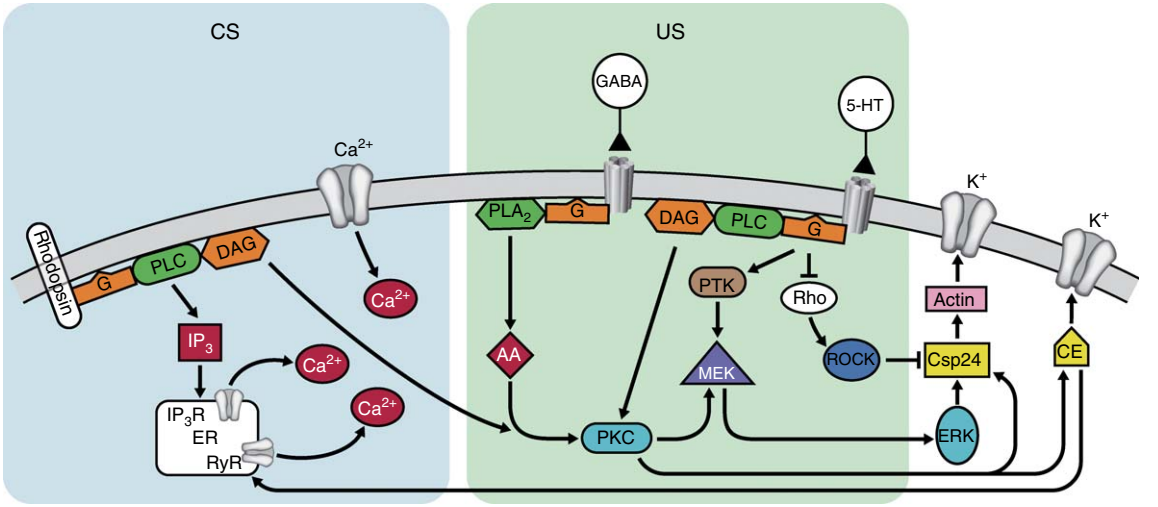


Figure 4 Mechanisms of memory formation produced by Pavlovian conditioning in *Hermissenda.* Acquisition involves the interaction of Ca^{2+} with the second messenger pathways regulated by neurotransmitter release in the unconditioned stimulus (US) pathway. Light (CS) activates phospholipase C (PLC) to produce an increase in inositol trisphosphate (IP_3) and diacylglycerol (DAG). The depolarizing generator potential and IP_3 effects on endoplasmic reticulum (ER) result in an increase in intracellular Ca^{2+}. Transmitters in the US pathway bind to G-protein-coupled receptors (G) to activate phospholipase A_2 (PLA_2), increase arachidonic acid (AA), and activate protein kinase C (PKC), nonreceptor protein tyrosine kinase (PTK), extracellular signal-regulated kinase (ERK), and the Rho GTPase/Rho-associated protein kinase (Rho/ROCK) pathway. Enhanced excitability is a consequence of short-term and long-term modification of K^+ channels by calexcitin (CE) and conditioned stimulus pathway protein 24 (Csp24). MEK, MAPK/ERK kinase; 5-HT, serotonin; GABA, gamma-aminobutyric acid.

released from intracellular stores (Ito et al., 1994; Blackwell, 2002a). When the CS and US are repeatedly paired, the Ca^{2+} influx due to light, IP_3R stores, RyR stores, and voltage-gated Ca^{2+} channels sums together (Blackwell and Alkon, 1999). The large increase in cytosolic Ca^{2+} combined with DAG and AA acts to synergistically activate PKC by translocation of PKC to the membrane (Lester et al., 1991). Each pairing of the CS and US has been proposed to incrementally increase the proportion of PKC translocated to the membrane that would contribute to the phosphorylation of K^+ channels (Muzzio et al., 1997, 2001; Alkon et al., 1998).

4.08.5.4 Long-Term Memory Depends Upon Translation and Transcription

The existence of mechanistic differences between short- and long-term enhanced excitability are illustrated by studies showing that inhibition of protein synthesis during one-trial *in vivo* conditioning blocks long-term enhanced excitability without affecting the induction or expression of short-term enhanced excitability (Crow and Forrester, 1990). Moreover, long-term enhanced excitability produced by one-trial conditioning is blocked by inhibition of mRNA synthesis, which does not affect the induction of short-term enhanced excitability (Crow et al., 1997). This result indicates that long-term memory following one-trial *in vivo* conditioning is dependent upon both translation and transcription.

The time-dependent development of enhanced excitability following one-trial *in vivo* conditioning is biphasic; enhancement reaches a peak at 3 h, decreases toward baseline control levels at 5–6 h, and increases to a plateau at 16 to 24 h postconditioning (Crow and Siddiqi, 1997). Enhanced excitability following one-trial conditioning involves an intermediate phase of memory consolidation that requires protein synthesis but not mRNA synthesis (Crow et al., 1999). The phosphorylation of a cytoskeletal-related protein, Csp24 (see the section titled 'Proteins regulated by Pavlovian conditioning') is associated with the intermediate phase, but not the short-term phase. Reducing the concentrations of 5-HT used in one-trial conditioning produces a short-term (< 1 h) associative enhancement of excitability that does not involve the posttranslational modification of Csp24 (Crow and Xue-Bian, 2000).

The conditioned foot contraction CR is expressed at a retention interval of 5 min following two or nine conditioning trials (Ramirez et al., 1998). However, nine conditioning trials are required for 90-min retention. *In vivo* incubation of animals with the protein synthesis inhibitor anisomycin during conditioning does not affect the expression of the CR at the 5-min retention interval, but attenuates conditioning at the 90-min interval for the group that received nine conditioning trials. A protocol involving *in vitro* conditioning of the isolated nervous system produces similar results to the effects of anisomycin on conditioned behavior. Two conditioning trials produce a short-term protein synthesis–independent increase in excitability that decreases within 45 min, and nine conditioning trials produce a persistent protein synthesis–dependent increase in type B photoreceptor excitability detected at 90 min (Ramirez et al., 1998). Applying anisomycin 5 min after the ninth conditioning trial does not affect the retention of enhanced excitability.

However, a recent study has challenged the view that protein synthesis occurring after the learning event is necessary and sufficient for the formation of long-term memory. PKC activation produced by bryostatin application on days before conditioning leads to the expression of proteins that can support long-term memory produced by later Pavlovian conditioning. Two conditioning trials typically result in a short-term (~7 min) foot-shortening CR. A 4-h exposure to bryostatin on two days preceding conditioning results in a long-term (>1 week) CR produced by two conditioning trials that is not blocked by anisomycin (Alkon et al., 2005).

4.08.6 Morphological Modifications in the Sensory Neurons of Conditioned Stimulus Pathway

Ultrastructural and electrophysiological analyses indicate that synaptic interactions between photoreceptors, other sensory neurons, and interneurons is in the neuropil of the cerebropleural ganglion (Crow et al., 1979). Changes in the morphology of secondary and terminal photoreceptor processes within the neuropil are produced by conditioning. Structural changes characterized by a contraction or reduction of dendritic boundary volumes enclosing labeled medial-type-B photoreceptor arborizations occur in conditioned animals as compared to unpaired controls (Alkon et al., 1990). This suggests that selective synaptic pruning may be a correlate of Pavlovian conditioning. Three-dimensional reconstructions of the volume of the terminal arborizations at the

synaptic connections between all pairs of identified photoreceptors double-labeled with different fluorescent dyes revealed that most project to the intermediate and lateral segments in the ventral region of the lateral B photoreceptor, a region of the lateral type B photoreceptor that is not contracted by conditioning (Kawai and Crow, 2004). The structural changes in type B photoreceptor dendritic volume observed with multi-trial conditioning have also been observed following an *in vitro* conditioning procedure. As compared to unpaired controls, five *in vitro* conditioning trials produce a contraction of the terminal branches along a central lateral axis of fluorescently labeled type B photoreceptors imaged with confocal microscopy (Kawai et al., 2002). The change in terminal branch morphology occurs within an hour after *in vitro* conditioning and is not observed at the synaptic connections between hair cells and photoreceptors (Kawai et al., 2002). The structural remodeling of the type B photoreceptor terminal branches following *in vitro* conditioning is blocked with anisomycin pretreatment (Kawai et al., 2003). In addition to changes in dendritic volume, changes in the volume of photoreceptor somas occur following activation of PKC with a phorbol ester (Lederhendler et al., 1990). Phorbol-induced changes involve outgrowths from the cell surface, similar to blebs, that alter the soma volume.

4.08.7 Proteins Regulated by Pavlovian Conditioning

Different *in vivo* and *in vitro* conditioning protocols have been used to study proteins regulated by conditioning. Multi-trial conditioning produces posttranslational modifications in a number of proteins; however, only a few of the full-length cDNAs have been cloned and the phosphoproteins fully characterized. Calexcitin (CE) is a guanosine triphosphate (GTP) and Ca^{2+}-binding protein found in *Hermissenda* photoreceptors (Neary et al., 1981; Alkon et al., 1998; Kuzirian et al., 2001). CE is activated by elevated Ca^{2+} and binds to the RyR to increase cytosolic Ca^{2+} concentrations (Ascoli et al., 1997; Nelson et al., 1996, 1999). CE is phosphorylated by PKC, which results in translocation of CE to membrane compartments where it decreases K^+ currents. Phosphorylation of CE also results in binding to the Ca^{2+}-ATPase transporter to increase the rate of Ca^{2+} removal from the cytosol (Alkon et al., 1998). Multi-trial conditioning increases the phosphorylation of CE (Neary et al., 1981) and increases CE in B photoreceptors, specifically in Ca^{2+} sequestering organelles such as endoplasmic reticulum (ER) and within mitochondria and photopigments (Kuzirian et al., 2001). The increased CE levels in B photoreceptors of conditioned animals results in increased excitability via K^+-channel inactivation and internal Ca^{2+} release from ER due to increased CE binding to ryanodine receptors.

In addition to CE, one-trial and multi-trial conditioning regulates other proteins found in the CS pathway and circumesophageal nervous system (Crow et al., 1996, 1997, 1999; Crow and Siddiqi, 1997; Crow, 2004). The phosphorylation of conditioned stimulus pathway protein 24 (Csp24) is regulated by Pavlovian conditioning and is involved in both intermediate-term and long-term memory. Csp24 is a cytoskeleton-related protein that is homologous to members of the family of multi-domain β-thymosin repeat proteins (Crow and Xue-Bian, 2000, 2002; Crow et al., 2003). Actin co-precipitates with Csp24 and is colocalized with Csp24 in the cytosol of B photoreceptor cell bodies (Crow and Xue-Bian, 2002). In addition, recombinant Csp24 binds to and sequesters G-actin *in vitro,* and phosphorylation of Csp24 by one-trial *in vitro* conditioning increases the co-precipitation of actin with anti-Csp24 (Redell et al., 2007).

Csp24 is phosphorylated by procedures that produce intermediate-term and long-term enhanced excitability, but not after *in vitro* procedures that result in only short-term enhanced excitability of photoreceptors (Crow and Xue-Bian, 2000). Several signaling pathways regulate Csp24 phosphorylation; thus it can integrate a number of signals that result in cytoskeletal remodeling. Inhibitors of PKC and MEK1 reduce Csp24 phosphorylation produced by *in vitro* conditioning. In addition to PKC and ERK regulation of Csp24, Rho GTPase activity and its downstream target Rho-associated protein kinase (ROCK) contribute to the posttranslational regulation of Csp24 through an inhibitory pathway (Crow et al., 2004). The ROCK inhibitor Y-27632 significantly increases Csp24 phosphorylation, and the Rho activator lysophosphatidic acid decreases Csp24 phosphorylation (Crow et al., 2004). In addition, the application of 5-HT to the isolated nervous system decreases Rho activity and increases the phosphorylation of Csp24. Inhibition of cyclin-dependent kinase 5 by butyrolactone also reduces Csp24 phosphorylation. Incubation of isolated *Hermissenda* nervous systems with *Csp* antisense oligonucleotides decreases Csp24 expression, and treatment with antisense oligonucleotides before

one-trial *in vitro* conditioning blocks intermediate-term enhanced excitability without affecting the induction of short-term immediate enhanced excitability (Crow et al., 2003).

Since Csp24 is associated with the actin cytoskeleton, its regulation by conditioning may influence K^+ channel activity by the spatial and temporal control of actin dynamics. One-trial *in vitro* conditioning of isolated type B photoreceptors produces a significant reduction in the amplitude of I_A and a depolarized shift in the steady-state activation curve of I_A without altering the inactivation curve (Yamoah et al., 2005). The conditioning-dependent changes in I_A are blocked by incubation of the isolated photoreceptors with *Csp* antisense oligonucleotide. Therefore Csp24 contributes to the regulation of voltage-gated channels associated with intrinsic enhanced excitability underlying Pavlovian conditioning.

Interestingly, the distribution of Csp24-like immunoreactivity in lateral type B photoreceptors is changed by one-trial *in vitro* conditioning. Conditioning results in a significant decrease in immunoreactivity in the soma and a significant increase in immunoreactivity in the terminal arborizations of identified lateral type B photoreceptors (Kawai and Crow, 2005).

4.08.8 Overview

Pavlovian conditioning in *Hermissenda* results in both intrinsic enhanced cellular excitability and modifications in synaptic efficacy at multiple loci within the neural circuit responsible for the generation of the CR. The first site of storage for the memory of the associated experience is in the primary sensory neurons of the CS pathway. The modifications in the sensory neurons are spatially segregated. There are alterations in the properties of K^+ channels in the soma that result in an enhancement of the amplitude of the CS-elicited generator potential and a concomitant change in channels in the spike-generating zone that results in a decrease in spike frequency accommodation. In addition, changes in synaptic efficacy result in facilitation of the monosynaptic connections between identified type B and type A photoreceptors and between identified photoreceptors and interneurons. Since the second site of memory storage is in the type I interneurons, the memory for Pavlovian conditioning involves both presynaptic and postsynaptic mechanisms.

Acquisition of short-term, intermediate-term, and long-term memory for Pavlovian conditioning involves the activation of several second messenger cascades, posttranslational modification of proteins, and the synthesis of mRNA and proteins. Acquisition engages the interaction of elevated intracellular Ca^{2+} and arachidonic acid to activate PKC and ERK that is dependent upon CS-US pairings. The mechanism for intrinsic enhanced excitability is different from the mechanisms supporting modifications in synaptic efficacy since long-term synaptic facilitation detected following multi-trial conditioning is not PKC-dependent.

Two proteins that have been fully characterized and are regulated by Pavlovian conditioning are CE and Csp24. The binding of CE to the plasma membrane decreases K^+ conductances and releases Ca^{2+} from internal stores. Csp24 phosphorylation is regulated by one-trial and multi-trial conditioning, is associated with actin, and contributes to long-term intrinsic enhanced excitability produced by the depolarized shift in the steady-state activation of I_A and the concomitant reduction in peak I_A. Therefore, the expression of Csp24 is important in both intermediate-term and long-term memory involving intrinsic enhanced excitability.

The analysis of mechanisms of memory in *Hermissenda* raises a number of questions that are important to an understanding of memory produced by Pavlovian conditioning. How are posttranslational modifications in proteins supporting short-term memory transformed into long-term memory involving both intrinsic enhanced excitability and changes in synaptic efficacy? What are the contributions of presynaptic and postsynaptic modifications to short-term, intermediate-term, and long-term memory? How does the regulation of CE and Csp24 by conditioning result in an alteration in the properties of K^+ channels in excitable membranes? Finally, how are modifications in intrinsic excitability and synaptic strength at several loci integrated within a neural circuit to reconfigure the circuit to support the generation of the conditioned response?

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4.09 Molecular Mechanism of Associative Learning in *Lymnaea*

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4.09.1 Introduction

4.09.1.1 Reductionist and Top-Down Approaches to Studying Molecular Mechanisms of Learning and Memory in Mollusks

Historically, there have been two different approaches to the analysis of the molecular mechanisms of learning and memory (*See* Chapters 4.06, 4.07, 4.08, 4.10, 4.38). One, often referred to as a reductionist or simple systems approach, is based on the investigation of molecular pathways involved in long-term neuronal plasticity (heterosynaptic facilitation, long-term potentiation, long-term depression) in neuronal cell cultures, invertebrate ganglia, or mammalian brain slices (Kandel, 2001). This approach rests on the assumption that these forms of synaptic plasticity underlie some forms of learning, such as sensitization or classical conditioning. The other type of approach, commonly known as a top-down approach, starts from the investigation of behavioral aspects of learning and memory and aims to establish causal links between learning-induced molecular changes and the learned behavior itself (Benjamin et al., 2000; Sadile, 1993; McGuire et al., 2005).

Gastropod mollusks (slugs and snails), such as *Aplysia californica, Hermissenda crassicornis, Helix pomatia, Lymnaea stagnalis, Pleurobranchaea californica,* and *Tritonia diomedia,* have been providing extremely useful experimental models for both types of approach since the 1970s (early reviews: Abraham et al., 1972; Willows, 1973; Kandel, 1979; Kandel and Schwartz, 1982; Carew and Sahley, 1986; Byrne, 1987). The main reason for the continuing success of gastropod preparations in learning and memory research is that snails and slugs have easily accessible central nervous systems with unusually large (up to 500 μm) neurons and a variety of well-defined reflex behaviors that can undergo plastic changes during both nonassociative (habituation, sensitization) and associative learning (classical and operant conditioning). These are known as implicit or nondeclarative forms of learning (Milner et al., 1998), during which the memory trace is formed and stored in the same network of neurons, making cellular-level analyses of both memory acquisition and storage feasible in the same system. There is

also an increasing amount of genomic and proteomic information available on mollusks, which has facilitated more in-depth molecular analyses of learning and memory in molluscan models, including the pond snail *Lymnaea stagnalis*.

4.09.1.2 The Roots of Top-Down Analyses of Associative Memory in *Lymnaea*

The predominantly top-down approach to investigations into the cellular and molecular mechanisms of associative learning in *Lymnaea* is rooted in two seemingly disparate fields of research, one almost exclusively physiological, the other purely behavioral (**Figure 1**). In the 1970s *Lymnaea* became a major experimental model system used for the analysis of the organization of central pattern generator (CPG) networks underlying the generation of rhythmic behaviors, such as feeding and respiration (**Figure 2**). Pioneering work in laboratories at Sussex University, Leeds University, and the University of Calgary led to a detailed understanding of how the feeding and respiratory CPGs worked in *Lymnaea* (Benjamin and Rose, 1979; Rose and Benjamin, 1979; McCrohan and Benjamin, 1980a,b; Elliott and Benjamin, 1985a,b; Kyriakides and McCrohan, 1989; Syed et al., 1990, 1992; Syed and Winlow, 1991; Elliott, 1992; Elliott and Kemenes, 1992; Elliott et al., 1992; Kemenes and Elliott, 1994). These studies, together with later work identifying serotonergic (mediated by the transmitter serotonin) and nitrergic (mediated by the gaseous transmitter nitric oxide, NO) modulatory mechanisms in the feeding system (Yeoman et al., 1994a,b, 1996; Elphick et al., 1995; Park et al., 1998; Kobayashi, 2000; Straub and Benjamin, 2001; Korneev et al., 2002), provided a firm physiological foundation for subsequent work aimed at understanding how learning can affect these CPG-driven behaviors (**Figure 1**).

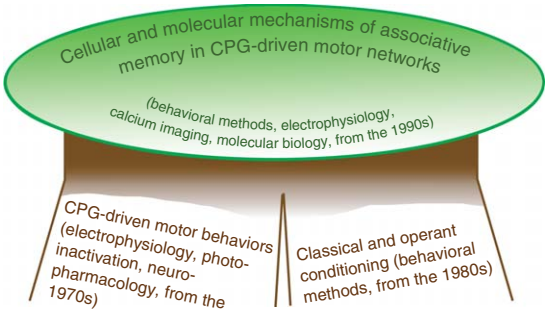


Figure 1 The two roots of current cellular and molecular studies of associative memory in *Lymnaea*.

Shortly after the publication of the first papers describing CPG interneurons, motor neurons, and modulatory neurons of the feeding network (Benjamin and Rose, 1979; Rose and Benjamin, 1979; McCrohan and Benjamin, 1980a,b), seminal behavioral work established the ability of *Lymnaea* to form long-term associative memory after a few pairings or even just a single pairing of a nonfood chemical stimulus and a food stimulus (Alexander et al., 1982, 1984; Audesirk et al., 1982). It was subsequently shown that *Lymnaea* is also capable of forming a positive association between tactile stimuli and food, but only after multiple trials (Kemenes and Benjamin, 1989a). Both the chemical and tactile conditioning paradigms revealed a number of characteristics of associative learning in *Lymnaea* that were shared by learning in vertebrates, such as dependence on age and motivational states, stimulus generalization, discriminative learning, and classical-operant interactions (Audesirk et al., 1982; Kemenes and Benjamin, 1989a,b, 1994). Later on it was also demonstrated that *Lymnaea* can form negative associations between a food conditioned stimulus (CS) and an aversive chemical unconditioned stimulus (US) (classical conditioning) (Kojima et al., 1996) or between a behavior and an aversive tactile stimulus (operant conditioning) (Lukowiak et al., 1996). The wealth of knowledge about the behavioral features of both operant and classical, aversive and reward conditioning, together with a detailed understanding of the neuronal networks underlying the unconditioned behaviors used in these paradigms (feeding and respiration, **Figure 2**), has made *Lymnaea* a very attractive experimental model for top-down analyses of learning and memory.

The two fields of research, physiological analysis of CPGs and behavioral analysis of associative conditioning, started to converge in the mid- to late 1990s when neuronal correlates of both classical and operant conditioning were first described (Whelan and McCrohan, 1996; Kemenes et al., 1997; Kojima et al., 1997; Staras et al., 1998, 1999; Spencer et al., 1999). The most important neuronal aspects of associative learning in *Lymnaea* are discussed in another chapter (*See* Chapter 1.30) as well as several review articles (Kemenes, 1999; Lukowiak and Syed, 1999; Benjamin et al., 2000; Lukowiak et al., 2003b). Detailed analyses of molecular mechanisms of associative memory started in the late 1990s after sufficient information had been obtained on both the behavioral and neuronal aspects of associative learning (**Figures 1** and **2**).

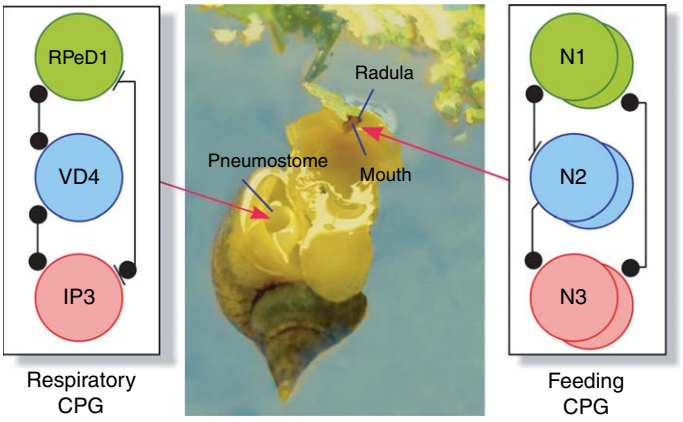


Figure 2 The respiratory and feeding behavior and the underlying CPG networks in *Lymnaea.* The photograph (courtesy of Dr. I. Kemenes) shows a snail hanging upside down on the water surface in an aquarium, simultaneously performing aerial respiration through the open pneumostome and feeding on a piece of lettuce. The cartoon to the left of the photograph shows the three-neuron respiratory CPG responsible for movements of the pneumostome. The cartoon on the right shows the feeding CPG responsible for the synchronized rhythmic movements of the snail's mouth parts, including the radula, which performs rasping movements during feeding. The feeding CPG consists of three main types of CPG neurons, each type having two different subtypes. The respiratory CPG produces a two-phase motor pattern (closure and opening of the pneumostome), whereas the feeding CPG produces a three-phase pattern (radula protraction, rasping, and swallowing). The arrows pointing from the CPG diagrams to the pneumostome and mouth parts, respectively, indicate causal relationships between CPG activity and motor functions performed by these organs. For more details on the two *Lymnaea* CPG networks see the references quoted in the text. Data sources: Benjamin PR and Elliott CJ (1989) Snail feeding oscillator: The central pattern generator and its control by modulatory interneurons. In: Jacklet J (ed.) *Neuronal and Cellular Oscillators*, p. 173. New York: Dekker; Lukowiak K (1991) Central pattern generators: Some principles learned from invertebrate model systems. *J. Physiol. (Paris)* 85: 63–70; Lukowiak K (2001) The *Lymnaea* respiratory system: Where are we going with learning? *Adv. Esp. Med. Biol.* 499: 321–326; Lukowiak K and Syed N (1999) Learning, memory and a respiratory central pattern generator. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 124: 265–274; Benjamin PR, Staras K, and Kemenes G (2000) A systems approach to the cellular analysis of associative learning in the pond snail, *Lymnaea. Learn. Mem.* 7: 124–131; Benjamin PR, Kemenes G, and Staras K (2005) Molluscan nervous systems. In: *Encyclopedia of Life Sciences*. London: John Wiley; Elliott CJ and Susswein AJ (2002) Comparative neuroethology of feeding control in molluscs. *J. Exp. Biol.* 205: 877–896; Lukowiak K, Sangha S, Scheibenstock A, et al. (2003b) A molluscan model system in the search for the engram. *J. Physiol. Paris* 97: 69–76; Lukowiak K, Martens K, Orr M, Parvez K, Rosenegger D, and Sangha S (2006b) Modulation of aerial respiratory behaviour in a pond snail. *Respir. Physiol. Neurobiol.* 154: 61–72.

4.09.2 Molecular Analyses of Associative Memory in *Lymnaea*

4.09.2.1 Reward Classical Conditioning of Feeding

Classical (or Pavlovian) reward conditioning in *Lymnaea* is based on forming an association between a nonfood tactile or chemical CS and an unconditioned food stimulus. Associations are formed in the feeding network and lead to a learned change in the behavior, with trained animals producing stronger feeding responses to the CS compared to control animals. **Figure 2** shows a *Lymnaea* feeding on a piece of lettuce (an unconditioned food stimulus) and the CPG network underlying the generation of the three-phase rhythmic snail feeding behavior called rasping. A more detailed description of the *Lymnaea* feeding behavior and underlying neuronal circuitry is presented in another chapter (*See* Chapter 1.30) and previous review articles (Benjamin and Elliott, 1989; Elliott and Susswein, 2002; Benjamin et al., 2005).

4.09.2.1.1 Single-trial reward conditioning

To date, the most detailed information on the molecular mechanisms of associative long-term memory (LTM) in *Lymnaea* has been gained from experiments using single-trial food-reward (also known as appetitive) classical conditioning. In this paradigm, snails are subjected to a conditioning protocol using a single pairing of amyl acetate (pear drops) as a neutral CS with sucrose as a rewarding or appetitive US. In subsequent memory tests with the CS alone, the explicitly paired (CS/US) experimental group shows significantly greater feeding responses to amyl acetate over their own naive responses and all the standard control groups (random, explicitly unpaired, CS alone, US alone) (Alexander et al., 1984; Kemenes et al., 2002; also see **Figure 4**).

The most important finding from the original behavioral studies was that a single pairing of the amyl acetate CS and sucrose US resulted in LTM, which lasted for several weeks (Alexander et al., 1984). This is a remarkable example of robust single-trial learning, which is now very effectively used for analyses of the time course of the molecular mechanisms underlying memory processes. The use of this single-trial paradigm for the analysis of the molecular mechanisms of memory consolidation and reconsolidation has two main advantages. First, after single-trial conditioning, translation- and transcription-dependent memory emerges in a matter of hours (Fulton et al., 2005), allowing this type of memory to be studied on a timescale of a few hours to several weeks. This early emergence of LTM was utilized in recent *in vitro* conditioning experiments investigating the cellular and molecular mechanisms of memory formation in semi-intact preparations, which are only viable for up to 6 h (Ireland, 2006). Second, unlike multi-trial paradigms, single-trial conditioning allows the analyses of the amnestic effects of sharply timed manipulations of key molecular pathways during both memory consolidation (Kemenes et al., 2002; Fulton et al., 2005; Ribeiro et al., 2005) and reconsolidation after the retrieval of memory (Kemenes G et al., 2006).

In semi-intact preparations made from conditioned animals, electrophysiological correlates of the rhythmic conditioned feeding response to amyl acetate were recorded in nerves, motor neurons, and command-like neurons of the feeding system (Kemenes et al., 2002; Straub et al., 2004, 2006; Kemenes I et al., 2006). Moreover, in attempts to localize sites of plasticity, electrical changes, such as maintained depolarization of the modulatory neuron type cerebral giant cells (CGCs) after conditioning, have also been recorded (Kemenes I et al., 2006).

At the molecular level, the most important question to be addressed was whether or not LTM formation after single-trial conditioning (similar to 'flash-bulb' memory known in other systems, including humans, cf. Carew, 1996) is based on the same conserved pathways that were originally described using multi-trial paradigms in other systems. An important clue to suggest that this might be the case is that the broad-spectrum protein synthesis inhibitor anisomycin (ANI), injected in an early time window (10 min to 1 h) after conditioning, blocks the 24-h memory trace (conventionally regarded as LTM) (Fulton et al., 2005). An earlier (5 h) memory trace is blocked by both ANI and actinomycin-D (Act-D), an RNA synthesis blocker, confirming that LTM, defined as memory dependent on early post-training translation and transcription, is present as early as 5 h after single-trial conditioning (Fulton et al., 2005). This observation indicates that single-trial reward conditioning triggers molecular cascades that are involved in the rapid consolidation of long-lasting memory traces. Interestingly, there is only a single early time window of sensitivity to transcription and translation blockers (Fulton et al., 2005) (**Figure 3**), unlike in a number of other studies, which described a temporally distinct second window of protein synthesis-dependent LTM in both vertebrates and invertebrates (Grecksch and Matthies, 1980; Freeman et al., 1995;

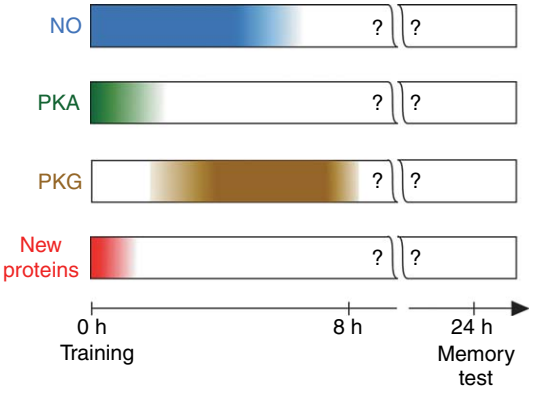


Figure 3 Time windows of requirement for NO, protein kinase A (PKA), protein kinase G (PKG), and new proteins for the consolidation of LTM after single-trial reward classical conditioning. Time windows were only investigated for up to 8 h post-training, so at present it cannot be ruled out (question marks) that there are further time windows between 8 h and 24 h. Data sources: Kemenes I, Kemenes G, Andrew RJ, Benjamin PR, and O'Shea M (2002) Critical time-window for NO-cGMP-dependent long-term memory formation after one-trial appetitive conditioning. *J. Neurosci.* 22: 1414–1425; Kemenes G, Kemenes I, Michel M, Papp A, and Müller U (2005) Early memory retrieval interferes with late memory expression in a PKA-dependent manner. In: *Abstracts of the 18th National Meeting of the British Neuroscience Association:* 39:10. Brighton, UK: British Neuroscience Association; Kemenes G, Kemenes I, Michel M, Papp A, and Müller U (2006) Phase-dependent molecular requirement for memory reconsolidation: Differential roles for protein synthesis and protein kinase A activity. *J. Neurosci.* 26:6298–6302; Fulton D, Kemenes I, Andrew RJ, and Benjamin PR (2005) A single time-window for protein synthesis-dependent long-term memory formation after one-trial appetitive conditioning. *Eur. J. Neurosci.* 21: 1347–1358; Korneev SA, Straub V, Kemenes I, et al. (2005) Timed and targeted differential regulation of nitric oxide synthase (NOS) and anti-NOS genes by reward conditioning leading to long-term memory formation. *J. Neurosci.* 25: 1188–1192; Ribeiro MJ, Schofield MG, Kemenes I, O'Shea M, Kemenes G, and Benjamin PR (2005) Activation of MAPK is necessary for long-term memory consolidation following food-reward conditioning. *Learn. Mem.* 12: 538–545.

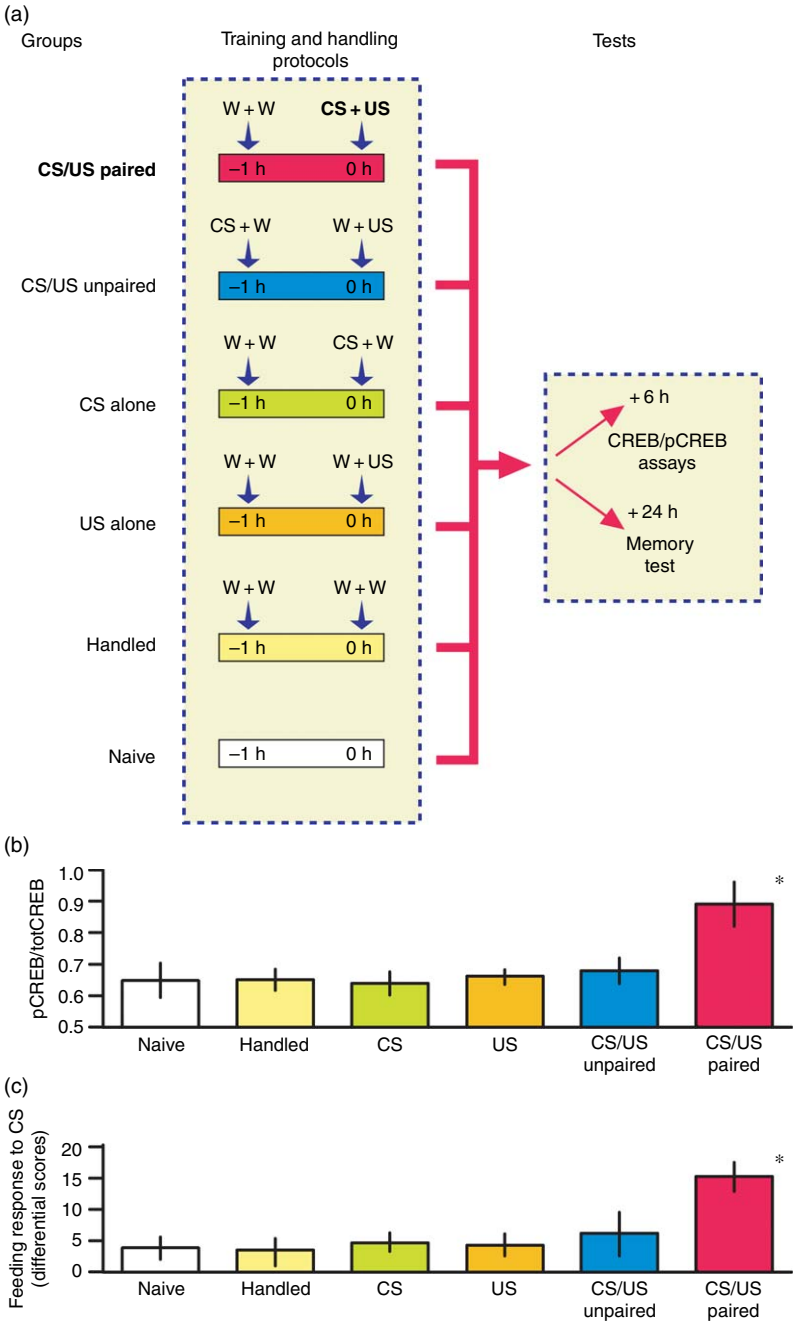


Figure 4 CREB is only phosphorylated after single-trial conditioning with contingent CS (amyl acetate) and US (sucrose) application. (a) The experimental design. An experimental (CS/US paired) and five different control groups were used in the same experiment. Water (W) stimuli were given to balance for disturbance in the experimental dish caused by applications of the CS and/or US solutions. After training or control treatment, each group was randomly divided into two subgroups. One of these groups was dissected for CREB/pCREB assays at 6 h posttraining; the other group was tested with the CS at 24 h posttraining to confirm that LTM had formed. (b) Significant (*, at least $p < .05$, analysis of variance (ANOVA) and post hoc tests) upregulation of pCREB levels in the experimental vs. all the control groups, indicating CS/US pairing-specific activation of CREB. (c) Significant (*, at least $p < .05$, ANOVA and post hoc tests) increase in the feeding response to the CS in the experimental vs. the control groups, indicating associative memory. The feeding response is shown as a difference score obtained by subtracting background feeding responses to water from the CS-induced feeding responses, both measured in 2-min observation periods. Data source: Michel M, Daniels M, Mueller U, Kemenes G (2007) Involvement of PKA and CREB in single-trial reward learning in *Lymnaea. Br. Neurosci. Assoc. Abstr.* 19: 57.

Bourtchouladze et al., 1998; Epstein et al., 2003). It has been suggested that 'strong' (i.e., effective) training protocols lead to rapid memory consolidation involving only a single wave of protein synthesis-dependent events, whereas 'weak' (i.e., less effective) training protocols are followed by a more prolonged consolidation phase, containing two or more windows of requirement for new protein synthesis (Bourtchouladze et al., 1998). According to this categorization, single-trial reward conditioning in *Lymnaea* certainly qualifies as a 'strong' training protocol, explaining both the rapid emergence and persistence of the associative memory trace, characteristic of 'flash-bulb' memory.

4.09.2.1.1.(i) CREB and LTM after single-trial reward conditioning Detailed molecular analyses identified highly conserved cyclic adenosine monophosphate (cAMP)-responsive element binding protein (CREB) genes (*Lym-CREB1* and *Lym-CREB2*) and CREB-like proteins in *Lymnaea* (Ribeiro et al., 2003; Sadamoto et al., 2004). A highly conserved CREB-binding protein (CBP) gene (*Lym-CBP*) also has been cloned in *Lymnaea* (Hatakeyama and Kemenes, 2005), further indicating the existence of functional CREB-dependent transcriptional mechanisms. These were important findings because regulation of gene expression during memory consolidation is known to involve a variety of transcription factors, with CREB playing a particularly important role in the switch between short-term and long-term memory storage in a variety of different species and paradigms (Frank and Greenberg, 1994; Stevens, 1994; Goda, 1995; Carew, 1996; Yin and Tully, 1996; Fletcher, 1997; Pittenger and Kandel, 1998; Silva et al., 1998; Alberini, 1999; Lamprecht, 1999; Scott et al., 2002; Tully et al., 2003; Josselyn and Nguyen, 2005). Consistent with a role for CREB in *Lymnaea* LTM is the observation that levels of phosphorylated CREB1 are increased in neurons of the feeding network following reward conditioning (Ribeiro et al., 2003). More recent experiments (Michel et al., 2007) showed that pCREB1 levels only increased in the CS/US paired group, and they remained unaffected in all the standard control groups (**Figure 4**).

4.09.2.1.1.(ii) NO and LTM after single-trial reward conditioning Historically, the first important discovery of the detailed molecular studies of learning and memory in *Lymnaea* was that consolidation of LTM after single-trial reward conditioning is dependent on the NO-cyclic guanosine monophosphate signaling pathway. There is a critical period of sensitivity up to 5 h after conditioning (**Figure 3**) when blocking this pathway by drug injection prevents LTM formation, which was established at both the level of whole animals and the neuronal network responsible for generating the feeding behavior (Kemenes et al., 2002).

Further evidence for a role of NO in LTM came from experiments on single isolated CGCs (Korneev et al., 2005). These giant modulatory neurons express mRNA transcripts from two related nNO synthase (nNOS) genes (*Lym-nNOS1* and *Lym-nNOS2*). Six hours after single-trial conditioning, *Lym-nNOS1* is upregulated compared with controls. This upregulation of the NOS-encoding transcript may be due to an earlier downregulation of the *Lymnaea anti-nNOS* transcript at 4 h that is known to be inhibitory on NOS transcript production. This is a rare example of analysis where the role of a specific signaling molecule in memory formation could be traced from the behavioral to the network and single neuronal level.

4.09.2.1.1.(iii) PKA, MAPK, and LTM after single-trial reward conditioning Other highly conserved molecular pathways that have been implicated in LTM after single-trial reward conditioning in *Lymnaea* are the protein kinase A (PKA) and mitogen-activated protein kinase (MAPK)-dependent signaling cascades. Inhibition of PKA catalytic subunit activity or MAPK phosphorylation blocked 24-h LTM without blocking sensory or motor pathways (Kemenes et al., 2005; Ribeiro et al., 2005). When measured 30 min after conditioning, increased levels of both PKA activity and MAPK phosphorylation were found (Ribeiro et al., 2005), with increased PKA activation also detected when measured in an earlier (5 min) and a later (1 h) time window (Kemenes et al., 2005), indicating a more prolonged dependence of LTM on PKA compared to protein synthesis (**Figure 3**).

A summary of the observations concerning the role of PKA, MAPK, CREB, and NOS/NO in 24-h LTM forming after reward conditioning is presented in **Table 1**. This direct comparison shows an important difference between MAPK and the other signaling molecules investigated in reward conditioning. Unlike the other factors, MAPK is activated not only in response to contingent CS/US application, but also when the CS or US were applied alone or in an unpaired manner (Ribeiro et al., 2005). This observation, together with the fact that preventing MAPK phosphorylation after training blocks LTM, shows that MAPK is necessary but definitely not sufficient for the consolidation of associative LTM

Table 1 A summary of the role of various molecular pathways in the consolidation of associative memory after single-trial reward classical conditioning in *Lymnaea*[a]

Molecule/pathway	*CS alone*	*US alone*	*CS/US unpaired*	*CS/US paired*	*Inhibition blocks associative LTM*
PKA	–	–	–	↑	Yes
MAPK	↑	↑	↑	↑	Yes
CREB	–	–	–	↑	?
NOS/NO	–	–	–	↑	Yes

[a]Abbreviations: – , no change after training; ↑, activation/upregulation after training; ?, no data.
Data source: Kemenes I, Kemenes G, Andrew RJ, Benjamin PR, and O'Shea M (2002) Critical time-window for NO-cGMP-dependent long-term memory formation after one-trial appetitive conditioning. *J. Neurosci.* 22: 1414–1425; Ribeiro MJ, Serfozo Z, Papp A, et al. (2003) Cyclic AMP response element-binding (CREB)-like proteins in a molluscan brain: Cellular localization and learning-induced phosphorylation. *Eur. J. Neurosci.* 18: 1223–1234; Ribeiro MJ, Schofield MG, Kemenes I, O'Shea M, Kemenes G, and Benjamin PR (2005) Activation of MAPK is necessary for long-term memory consolidation following food-reward conditioning. *Learn. Mem.* 12: 538–545; Kemenes G, Kemenes I, Michel M, Papp A, and Müller U (2005) Early memory retrieval interferes with late memory expression in a PKA-dependent manner. In: *Abstracts of the 18th National Meeting of the British Neuroscience Association:* 39:10. Brighton, UK: British Neuroscience Association; Kemenes G, Kemenes I, Michel M, Papp A, and Müller U (2006) Phase-dependent molecular requirement for memory reconsolidation: Differential roles for protein synthesis and protein kinase A activity. *J. Neurosci.* 26:6298–6302; Korneev SA, Straub V, Kemenes I, et al. (2005) Timed and targeted differential regulation of nitric oxide synthase (NOS) and anti-NOS genes by reward conditioning leading to long-term memory formation. *J. Neurosci.* 25: 1188–1192.

after single-trial reward conditioning. PKA, CREB, and NOS/NO are selectively activated/upregulated by the associative training protocol, so potentially each of these factors could be sufficient for memory consolidation. However, it is more likely to be the case that these and other signaling molecules make a synergistic contribution to the memory consolidation process, with each molecule and pathway playing an important role in LTM but none of them alone being sufficient for its consolidation.

Interestingly, unlike early inhibition of protein synthesis, inhibition of PKA catalytic subunit activity did not block the expression of memory at 6 h post-training (Kemenes et al., 2005), indicating the involvement of a different signaling molecule (possibly Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), Wan et al., 2007) in the protein synthesis-dependent consolidation of this earlier memory trace (**Figure 5** and **Table 2**).

Treatment of isolated cerebral ganglia with the adenylate cyclase activator forskolin resulted in massively increased CREB phosphorylation in neuronal nuclei (Ribeiro et al., 2003), indicating a potential link between training-induced PKA activation in the cerebral ganglia (Kemenes et al., 2005) and CREB phosphorylation in conditioned animals. Whether there is any linkage between the NO, PKA, and MAPK signaling pathways has yet to be determined.

4.09.2.1.1.(iv) Time windows of sensitivity of LTM to amnestic treatments after single-trial reward conditioning A summary of the known time windows of sensitivity of the 24-h LTM to inhibition of NO, PKA, protein kinase G (PKG), and protein synthesis is shown in **Figure 3**. Time windows of sensitivity were only investigated in the first 8 h after training, so we cannot rule out that there are also later time windows when these molecular processes are required for the consolidation of the 24-h memory trace. The observation that *Lym-nNOS1* mRNA levels increase at 6 h posttraining (Korneev et al., 2005) strongly suggests an additional later role for NO, perhaps in memory maintenance. The level of phosphorylated CREB1 was also found to be high at 6 h posttraining (Ribeiro et al., 2003), indicating that new transcription and translation still take place after this period. The time windows of requirement for NO and PKA are both wider (Kemenes et al., 2002, 2005) than the time window for requirement for early protein synthesis (Fulton et al., 2005), indicating that the functions of NO and PKA go beyond being upstream components of molecular pathways leading to new protein synthesis shortly after training.

Recently, we have found that, unlike PKA, NO, and new proteins, PKG is not involved in the early processes of memory consolidation (**Figure 3**). This kinase enzyme is, however, required for later memory consolidation processes (between 2 and 8 h posttraining), hardly overlapping with the temporal requirement for PKA (up to 2 h posttraining, Kemenes et al., 2005) but linked to the requirement for NO/soluble guanylyl cyclase (10 min to 6 h posttraining (Kemenes et al., 2002) in a delayed manner.

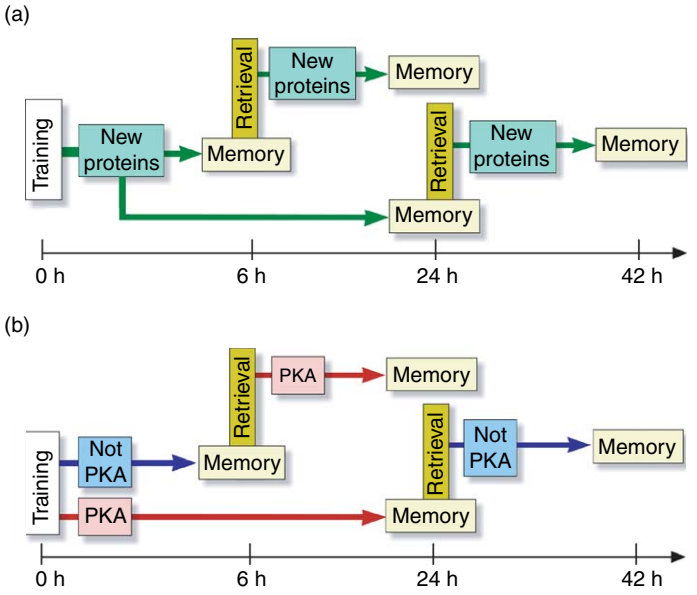


Figure 5 Time-dependent differential requirements for protein synthesis and PKA after training and memory retrieval. (a) New proteins are required for both 6-h and 24-h memory as well as memory reconsolidation after memory retrieval at both time points. (b) By contrast, PKA is only required for 24-h but not 6-h memory, whereas reconsolidation of the retrieved memory is PKA-dependent at 6 h but not at 24 h posttraining. These observations indicate that not PKA, but other signaling molecules are important for memory consolidation at 6 h and for memory reconsolidation at 24 h posttraining. Data sources: Kemenes G, Kemenes I, Michel M, Papp A, and Müller U (2005) Early memory retrieval interferes with late memory expression in a PKA-dependent manner. In: *Abstracts of the 18th National Meeting of the British Neuroscience Association:* 39:10. Brighton, UK: British Neuroscience Association; Kemenes G, Kemenes I, Michel M, Papp A, and Müller U (2006) Phase-dependent molecular requirement for memory reconsolidation: Differential roles for protein synthesis and protein kinase A activity. *J. Neurosci.* 26: 6298–6302.

Table 2 Differential time-dependent requirements for PKA activity and translation for memory consolidation after single-trial classical reward conditioning and reconsolidation after the retrieval of associative memory in *Lymnaea*

		Time of memory test or retrieval	
Requirement for	***Process required for***	***6 h***	***24 h***
Translation	Memory consolidation after training	Yes	Yes
	Memory reconsolidation after retrieval	Yes	Yes
PKA	Memory consolidation after training	No	Yes
	Memory reconsolidation after retrieval	Yes	No

Data sources: Kemenes G, Kemenes I, Michel M, Papp A, and Müller U (2005) Early memory retrieval interferes with late memory expression in a PKA-dependent manner. In: *Abstracts of the 18th National Meeting of the British Neuroscience Association:* 39:10. Brighton, UK: British Neuroscience Association; Kemenes G, Kemenes I, Michel M, Papp A, and Müller U (2006) Phase-dependent molecular requirement for memory reconsolidation: Differential roles for protein synthesis and protein kinase A activity. *J. Neurosci.* 26: 6298–6302.

4.09.2.1.1.(v) cAMP-dependent molecular cascades and neuronal plasticity contributing to LTM after single-trial reward conditioning

An important link between cAMP-dependent molecular cascades and neuronal plasticity contributing to LTM was found in voltage-clamp experiments, which demonstrated a long-lasting cAMP-induced increase in a low-threshold tetrodotoxin-resistant persistent sodium current of the CGCs (Nikitin et al., 2006). This current makes an important contribution to the CGC somal membrane potential (Staras et al., 2002; Nikitin et al., 2006), which in turn becomes

persistently depolarized after single-trial reward conditioning (Kemenes I et al., 2006). The CGC's synaptic output also became similarly enhanced by somal injection of cAMP (Nikitin et al., 2006) and artificial depolarization of the soma membrane (Kemenes I et al., 2006), lending further support to the notion that cAMP-dependent cascades might support long-lasting plastic changes in ion channel number and/or function, contributing to learning-induced maintained depolarization. The mechanism by which persistent somal depolarization affects synaptic output is likely to be based on a maintained increase in background calcium levels in axon terminals of the CGCs presynaptic to their target neurons (Kemenes I et al., 2006), similar to what was demonstrated previously in mammalian neurons (Awatramani et al., 2005).

4.09.2.1.1.(vi) PKA, protein synthesis, and memory reconsolidation after single-trial reward conditioning An interesting observation is that memory reconsolidation after retrieval at 6 h posttraining is both PKA- and protein synthesis-dependent, whereas reconsolidation after retrieval at 24 h depends on protein synthesis but not on PKA activity (Kemenes G et al., 2006) (**Figure 5** and **Table 2**). This finding indicates that, depending on how recent or remote consolidated memory is relative to the time of training, different molecular pathways are activated by memory retrieval and contribute differentially to memory reconsolidation (*See* Chapters 1.24, 4.14). At a more general level, this phase-dependent differential molecular requirement for reconsolidation supports the notion that even seemingly fully consolidated memories undergo further selective molecular maturation processes (the 'lingering consolidation' hypothesis, Dudai and Eisenberg, 2004), which however may only be revealed by analyzing the role of a variety of different specific pathways in memory reconsolidation after retrieval.

A key question concerning the molecular mechanisms of memory reconsolidation is whether or not they are a recapitulation of the processes active during memory consolidation (Nader et al., 2000; Sara, 2000; Dudai, 2002; Nader, 2003; Dudai and Eisenberg, 2004; Morris et al., 2006). If they are, memory retrieval should reactivate the same molecular cascades that were activated by training, and therefore the same amnestic treatments should impede both processes. Direct comparisons of the PKA and protein synthesis dependence of the consolidation of the 6-h and 24-h memory trace and the reconsolidation of memory after retrieval at these two time points (**Figure 5** and **Table 2**) support the notion that reconsolidation is a recapitulation of consolidation, only at a more general molecular level (protein synthesis), but not at the level of a specific signaling molecule (PKA). In fact, it seems that with regard to PKA, the molecular requirements for consolidation and reconsolidation are completely different: consolidation of the 6-h memory trace does not require PKA, but reconsolidation after memory retrieval at 6 h does, whereas reconsolidation after retrieval at 24 h takes place even when PKA activity is inhibited, despite the dependence of the consolidation of the 24-h memory on early posttraining PKA activity (Kemenes G et al., 2005, 2006). It remains to be elucidated if other specific signaling molecules already known to contribute to the consolidation of the 24-h memory trace (MAPK, NO) are also important for the 6-h memory trace and whether or not they are activated by retrieval at these two different time points.

4.09.2.2 Aversive Classical Conditioning of Feeding

Aversive classical conditioning of feeding in *Lymnaea* is based on pairing sucrose as a CS with an aversive chemical US such as KCl, which inhibits feeding and evokes a withdrawal response (conditioned taste aversion, CTA). After eight or more trials, trained animals showed a significantly weaker feeding response to sucrose than did controls, and this associative memory lasted for over 1 month (Kojima et al., 1996; Ito et al., 1999).

A neural analysis of CTA was carried out on isolated brains dissected from conditioned and control animals (Kojima et al., 1997, 2001). Like reward chemical conditioning, aversive chemical conditioning leads to specific changes in the feeding network, described in another chapter (*See* Chapter 1.30) and previous reviews (Ito et al., 1999; Benjamin et al., 2000; Lukowiak et al., 2003a).

4.09.2.2.1 PKA, CREB, and LTM after aversive conditioning

The transcription and translation dependence of aversive behavioral LTM or the necessity of the activation of specific molecular cascades for its consolidation have not been tested in intact *Lymnaea,* but *in vitro* experiments were performed to

investigate the role of the transcription factor CREB and its upstream activators in synaptic plasticity that may be linked to CTA. The injection of cAMP or PKA into the soma of the CGCs in isolated nervous system preparations led to a long-term enhancement of the synapse between the CGC and a follower motor neuron, B1 (Nakamura et al., 1999). The injection of a CRE oligonucleotide into the CGCs prevented this cAMP-induced long-lasting synaptic plasticity (Sadamoto et al., 2004). These *in vitro* experiments showed that one of the mechanisms of the previously described enhancement in the CGC's synaptic output induced by CTA learning could be the activation of the cAMP/PKA/CREB cascade. However, synaptic plasticity induced by CTA learning in the CGCs could depend on molecular mechanisms other than cAMP-dependent cascades (e.g., CaMKII), and cAMP/PKA and CREB-mediated synaptic plasticity in the CGCs could be involved in the formation of a variety of different memory traces arising from different training paradigms using food stimuli (e.g., visual conditioning (Andrew and Savage, 2000)). Thus, unlike LTM forming after reward conditioning, CTA has not yet been conclusively linked to PKA or the transcription factor CREB in *Lymnaea*.

4.09.2.2.2 C/EBP and LTM after aversive conditioning

Unlike in the case of PKA and CREB, a link has been found between CTA in intact *Lymnaea* and another conserved transcription factor, CCAAT/enhancer binding protein (C/EBP), and this was shown in the buccal ganglia and, specifically, the B2 gut motor neuron (Hatakeyama et al., 2006). Both the phosphorylated and total levels of the *Lym-C/EBP* protein increased in the buccal ganglia and neuron B2 during CTA consolidation, although *Lym-C/EBP* mRNA levels were reduced in the same time window (1 h posttraining) when the increases in protein levels were measured. One explanation for this paradoxical observation could be that the existing pool of *Lym-C/EBP* mRNA is rapidly translated and degraded early after CTA learning, resulting in fast turnover of newly transcribed mRNA, which may be required for a prolonged *de novo* synthesis of large amounts of *Lym-C/EBP* necessary for long-term memory after CTA learning. A more detailed analysis of the time course of changes in *Lym-C/EBP* mRNA and protein levels will be necessary to elucidate the relationship between *Lym-C/EBP* gene transcription, mRNA turnover, and protein synthesis in this system.

Although the CRE element upstream of *Lym-C/EBP* has not been investigated, based on data from *Aplysia*, *Helix*, and mammals (Niehof et al., 1997; Alberini, 1999; Grinkevich, 2002), the expression of *Lym-C/EBP* is likely to be regulated by *Lymnaea* CREB. It is not known which genes are targeted by C/EBP in *Lymnaea*. However, likely candidate downstream targets of C/EBP are the *Lym-nNOS* genes (Korneev et al., 2005). *Lym-C/EBP* and *Lym-nNOS* are colocalized in the B2 motor neurons (Hatakeyama et al., 2006), and in addition, *Lym-nNOS* genes have three putative *Lym-C/EBP* binding sites (Korneev et al., 2005), providing the necessary structural conditions for the interaction of C/EBP with NOS genes in the *Lymnaea* feeding network.

An interesting but so far unanswered question concerns the differences in the molecular mechanisms underlying reward and aversive conditioning of feeding. Plastic changes after both types of conditioning were found in the CGCs (Kojima et al., 1997; Kemenes I et al., 2006), which are therefore regarded as key neurons for both reward and aversive conditioning. In the case of reward conditioning, the plastic change (a persistent somal depolarization) is nonsynaptic in nature and increases the probability of a feeding response to the CS by recruiting feeding command-like neurons into the network of cells activated by the CS (Kemenes I et al., 2006). In the case of aversive conditioning, the plastic change increases the amplitude of a CGC-driven inhibitory synaptic input to the N1M-type CPG neurons (Kojima et al., 1997), whose inhibition suppresses feeding (Kemenes et al., 2001). It will be important to determine what molecular differences underlie the different cellular changes induced in the same neuron by reward versus appetitive conditioning and contributing either to the activation or suppression of the same behavior after classical conditioning.

4.09.2.3 Operant Conditioning of the Suppression of Aerial Respiration

The aerial respiratory behavior of *Lymnaea stagnalis* has been used in a series of experiments investigating the behavioral, neuronal, and molecular mechanisms of operant conditioning. The respiratory behavior and underlying neuronal circuitry are extremely well characterized and are reviewed in another chapter (*See* Chapter 1.30) as well as several previous reviews (Lukowiak, 1991, 2001; Lukowiak and Syed, 1999; Benjamin et al., 2000; Lukowiak et al., 2003b, 2006b). While single-trial classical conditioning has

provided detailed information on specific molecular mechanisms of LTM, work based on the operant conditioning of the suppression of aerial respiration has provided comprehensive information on the general molecular requirements for different memory phases (intermediate-term memory (ITM) and LTM) and active processes related to associative memory, such as forgetting, extinction, and reconsolidation.

The biological basis for operant conditioning in *Lymnaea* lies in the fact that these pond snails are bimodal breathers; they can breathe both through the skin (cutaneous respiration) and via a simple lung (aerial respiration), which is supplied with air through a respiratory orifice called the pneumostome (**Figure 2**). Hypoxia triggers pneumostome opening, the CPG-driven motor mechanism of aerial respiration (**Figure 2**). Tactile stimulation of the pneumostome area evokes reflexive pneumostome closure and thus suppresses aerial respiratory behavior. In an operantly trained group, a tactile stimulus was applied to the pneumostome area each time aerial respiration was attempted by the animal or semi-intact preparation in an artificially created hypoxic environment (Lukowiak et al., 1996). Suitable yoked and hypoxic control groups were also used. The number of openings, latency to first opening, and total breath durations were recorded in pre- and posttraining periods. Only the operantly conditioned group showed significant changes between the pre- and posttraining behaviors, with significant reductions in openings and total breathing time and significant increases in the latency to first breath. A memory for the operantly conditioned suppression of aerial respiration could persist for at least 4 weeks (Lukowiak et al., 1998), indicating the formation of LTM.

4.09.2.4 Intermediate and Long-Term Memory, Reconsolidation, and Extinction after Operant Conditioning

At the behavioral level, both ITM and LTM have been described based partly on the length of time the memory persists (Lukowiak et al., 2000) but also on the sensitivity to the protein and mRNA synthesis blockers ANI and Act-D, respectively (Sangha et al., 2003c). ANI prevents the formation of both ITM and LTM, whereas Act-D only prevents LTM. Both reconsolidation and extinction have been studied following operant conditioning, and both have been shown to be dependent on new RNA and protein synthesis (Sangha et al., 2003b,d). Extinction is viewed as a new type of associative memory that temporarily masks but does not replace the original memory. Thus following extinction trials, the loss of memory at 2 h is followed by full spontaneous recovery at 24 h.

4.09.2.4.1 Inhibition of macromolecular synthesis and dynamics of memory consolidation and forgetting after operant conditioning

In a series of intriguing experiments, Ken Lukowiak and colleagues showed that cooling, which inhibits macromolecular synthesis, can be used as a tool to prevent not only memory consolidation, but also forgetting, which is thought to be an active process itself, requiring both new learning and memory. When snails were kept at room temperature after operant conditioning, LTM was absent 7 days after training (i.e., forgetting occurred) (Sangha et al., 2005). However, when snails were placed into 4 °C water 1 h after the end of training and kept there for 7 days, LTM was preserved. Thus, unlike cooling for 1 h immediately after training, which impedes LTM (Sangha et al., 2003a), delayed and prolonged post-training cooling impeded forgetting instead (Sangha et al., 2005). Another interesting observation was that ITM can be boosted into LTM by cooling snails immediately after the first bout of a training protocol (two 30-min operant conditioning training sessions in hypoxic water separated by a 30-min rest interval in water with normal oxygen levels) that normally only results in ITM, but not LTM (Parvez et al., 2005).

4.09.2.4.2 Single-neuronal contribution to LTM, forgetting, extinction, and reconsolidation after operant conditioning

Neural changes associated with associative memory have been identified in the isolated central nervous system derived from operantly conditioned animals (Spencer et al., 1999; Spencer et al., 2002). For example, a higher percentage of right pedal dorsal 1 (RPeD1) CPG interneurons, which are important in the onset of the respiratory cycle (**Figure 2**), were silent in conditioned versus control preparations. This neuron became the main focus of work aimed at understanding the molecular processes involved in LTM, forgetting, extinction, and reconsolidation at the level of single identified neurons.

Evidence for the role of molecular processes active in the nucleus and somal cytoplasm of RPeD1 during memory formation came from both somal ablation experiments in intact animals

(Scheibenstock et al., 2002) and spike activity perturbation experiments in semi-intact preparations, where LTM formation was shown *in vitro* (Lowe and Spencer, 2006). Removal of the soma 2 h prior to conditioning of intact snails prevented LTM but had no effect on ITM, suggesting that the RPeD1 soma was necessary for LTM formation (Scheibenstock et al., 2002). Removal of the RPeD1 soma 1 h after conditioning had no effect on LTM, indicating that the effects of soma ablation were not related to memory access or retrieval. In semi-intact preparations, preventing RPeD1 spike activity between training sessions can directly augment memory formation (Lowe and Spencer, 2006), again emphasizing the important role this respiratory CPG neuron plays in memory consolidation.

Interestingly, forgetting, extinction, and reconsolidation also require the presence of the soma of RPeD1 (Sangha et al., 2003b,d, 2005), indicating both that these are all active processes and that RPeD1 is involved in all of them. The observations regarding the role of macromolecular synthesis and RPeD1 are summarized in **Table 3**.

Recent work has shown that snails with axotomized and subsequently regenerated RPeD1 axons can both form new LTM and retain already existing LTM (Lukowiak et al., 2006a). An important implication of this observation is that, despite the fact that similar second messenger cascades are utilized to elicit gene activity in both memory consolidation and regeneration (Feng et al., 1997; Zhang and Ambron, 2000; Carew and Sutton, 2001; cited in Lukowiak et al., 2006a), the effect of the complex molecular cascades activated during regeneration does not abolish the molecular processes necessary for memory consolidation or maintenance.

Table 3 Both macromolecular synthesis and the presence of the soma of RPeD1 (a CPG neuron of the respiratory network) are required for a variety of LTM-related processes after operant conditioning in *Lymnaea*

Memory-related process	*Protein/RNA synthesis required*	*RPeD1 soma required*
Consolidation of LTM	Yes	Yes
Reconsolidation of LTM	Yes	Yes
Forgetting of LTM	Yes	Yes
Extinction of LTM	Yes	Yes
Boosting ITM into LTM	Yes	Yes

Data sources: Scheibenstock A, Krygier D, Haque Z, Syed N, and Lukowiak K (2002) The soma of RPeD1 must be present for long-term memory formation of associative learning in *Lymnaea*. *J. Neurophysiol.* 88: 1584–1591; Sangha S, Morrow R, Smyth K, Cooke R, and Lukowiak K (2003a) Cooling blocks ITM and LTM formation and preserves memory. *Neurobiol. Learn. Mem.* 80: 130–139; Sangha S, Scheibenstock A, and Lukowiak K (2003b) Reconsolidation of a long-term memory in *Lymnaea* requires new protein and RNA synthesis and the soma of Right Pedal Dorsal 1. *J. Neurosci.* 23:8034–8040; Sangha S, Schiebenstock A, Mccomb C, and Lukowiak K (2003c) Intermediate and long-term memories of associative learning are differentially affected by transcription versus translation blockers in *Lymnnaea*. *J. Exp. Biol.* 206: 1605–1613; Sangha S, Schiebenstock A, Morrow R, and Lukowiak K (2003d) Extinction requires new RNA and protein synthesis and the soma of the cell right pedal dorsal 1 in *Lymnaea stagnalis*. *J. Neurosci.* 23: 9842–9851; Sangha S, Schiebenstock A, Martens K, Varshney N, Cooke R, and Lukowiak K (2005) Impairing forgetting by preventing new learning and memory. *Behav. Neurosci.* 119: 787–796; Parvez K, Stewart O, Sangha S, and Lukowiak K (2005) Boosting intermediate-term into long-term memory. *J. Exp. Biol.* 208: 1525–1536; Lowe MR and Spencer GE (2006) Perturbation of the activity of a single identified neuron affects long-term memory formation in a molluscan semi-intact preparation. *J. Exp. Biol* 209: 711–721.

4.09.3 Conclusions

Studies based on the associative conditioning paradigms described in this article have yielded valuable information on a variety of general and specific molecular mechanisms contributing to memory consolidation, reconsolidation, forgetting, and extinction. Reconsolidation and extinction in operant conditioning have been shown to have similar general molecular requirements to memory consolidation. Memory consolidation after classical conditioning and reconsolidation after retrieval also share the same general molecular requirement (synthesis of new proteins), but the requirement for specific signaling molecules (such as PKA) for reconsolidation depends on how recent or remote the consolidated memory is relative to the time of training.

Studies of LTM in classical conditioning have emphasized the importance of regulation of gene expression by transcription factors such as CREB and C/EBP and the role of the PKA and MAPK signaling pathways, with the most detailed information gained from experiments using the single-trial classical conditioning paradigm. The importance of these molecular pathways in *Lymnaea* provides further evidence for the generality of these highly conserved mechanisms in learning, both across phylogenetic groups and across learning paradigms (nonassociative or associative, single- or multi-trial,

aversive or reward, operant or classical). NO has been shown to be important for memory consolidation in classical reward conditioning, and this appears to involve regulation of nNOS gene expression in a specific modulatory cell type, the CGCs. Work using *Lymnaea* as an experimental model has thus both provided important insights into the molecular mechanisms of associative learning and memory at the behavioral level and linked these mechanisms to learning-induced cellular and molecular changes in single identified neurons, hallmarks of a successful top-down approach to the study of the molecular mechanisms of associative memory.

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Dedicated to the memory of my father.

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4.10 Cellular Mechanisms of Associative Learning in *Aplysia*

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4.10.1 *Aplysia* Classical Conditioning and Operant Conditioning

The simple nervous system and the relatively large identifiable neurons of the marine mollusk *Aplysia* provide a useful model system to examine the cellular and molecular mechanisms of the two major forms of associative learning, classical conditioning and operant (instrumental) conditioning. The ability to associate a predictive stimulus with a subsequent salient event (i.e., classical conditioning) and the ability to associate an expressed behavior with the consequences (i.e., operant conditioning) allow for a predictive understanding of a changing environment. Although operationally distinct, there has been considerable debate whether at some fundamental level classical and operant conditioning are mechanistically distinct or similar (e.g., Rescorla and Solomon, 1967; Gormezano and Tait, 1976; Dayan and Balleine, 2002). Studies utilizing the defensive withdrawal reflexes of *Aplysia* have provided much information on the mechanisms underlying classical conditioning. Recent studies utilizing the feeding behavior of *Aplysia* are providing for a comparative analysis of the mechanisms underlying classical and operant conditioning, using the same behavior and studying the same neuron. This comparative analysis can help to resolve the issue of whether similar or different mechanisms underlie these two forms of associative learning.

4.10.2 Classical Conditioning

4.10.2.1 Behavioral Studies

The initial studies of classical conditioning in *Aplysia* focused on defensive reflex behaviors and used aversive conditioning procedures. A tactile or electrical stimulus delivered to the siphon of the animal resulted in a reflex withdrawal of the gill and siphon, a reaction which presumably protects sensitive structures from harmful stimuli. Aversive classical conditioning in this species can be demonstrated by presenting a conditioned stimulus (CS), a brief, weak tactile stimulus to the siphon which produced a small siphon withdrawal, and an unconditioned stimulus (US), a short-duration noxious electric shock to the tail which produced a large withdrawal of the siphon (the unconditioned response, UR). After repeated pairings of the CS and US, the CS alone produced a large siphon withdrawal (the conditioned response, CR). This withdrawal was enhanced beyond that produced by the US alone (sensitization control) or unpaired or random presentations of the CS or the US (Carew et al., 1981), and this conditioning persisted for as long as 4 days. Carew et al. (1983) also found that this reflex exhibited differential classical conditioning. CS (tactile stimulation) were delivered to either the siphon or to the mantle region. One CS (the CS^+) is paired with the US (electric shock to the tail) and the other is explicitly unpaired (the CS^-). After conditioning, the CS^+ produced a greater withdrawal than the CS^-.

In addition to the classical conditioning of defensive reflexes, classical conditioning can also be applied to feeding behavior. *Aplysia* feed by protracting a toothed structure called the radula into contact with seaweed. The radula grasps seaweed by closing and retracting, which results in the ingestion of the seaweed. Inedible objects can be rejected if the radula protracts while closed (grasping the object) and then opens as it retracts to release the object. Thus, the timing of radula closure determines which behavior will occur. Feeding behavior can be classically conditioned with an appetitive protocol (Colwill et al., 1997; Lechner et al., 2000a). This appetitive protocol (Lechner et al., 2000a) consisted of tactile stimulation of the lips with a fine-tipped paint brush (CS), and the US was a small piece of seaweed, which the animals were allowed to eat. The animals were trained by repeatedly pairing the CS and the US. After training, presentation of the CS elicited an increase in ingestive behavior (CR).

4.10.2.2 Neural Mechanisms of Aversive Classical Conditioning in *Aplysia*

A cellular mechanism called activity-dependent neuromodulation contributes to associative learning in *Aplysia* (Hawkins et al., 1983; Walters and Byrne, 1983; Antonov et al., 2001). A general cellular scheme of activity-dependent neuromodulation is illustrated in **Figure 1**. Two sensory neurons (SN1 and SN2) constitute the pathways for the conditioned stimuli (CS^+ and CS^-) and make weak subthreshold connections to a motor neuron. Delivering a reinforcing stimulus or US alone has two effects. First, the US activates the motor neuron and produces the UR. Second, the US activates a diffuse modulatory system that nonspecifically enhances transmitter release from all the sensory neurons. This nonspecific enhancement contributes to sensitization. Temporal specificity, which is characteristic of associative learning, occurs when there is pairing of the CS (spike activity in SN1) with the US, which causes a selective amplification of the modulatory effects in SN1. Unpaired activity does not amplify the effects of the US in SN2. The amplification of the modulatory effects in SN1 leads to an enhancement of the ability of SN1 to activate the motor neuron and produce the CR.

A reduced preparation for the siphon-withdrawal reflex was developed that consists of the isolated tail, siphon, and central nervous system (CNS) of the animal (Antonov et al., 2001). A classical conditioning

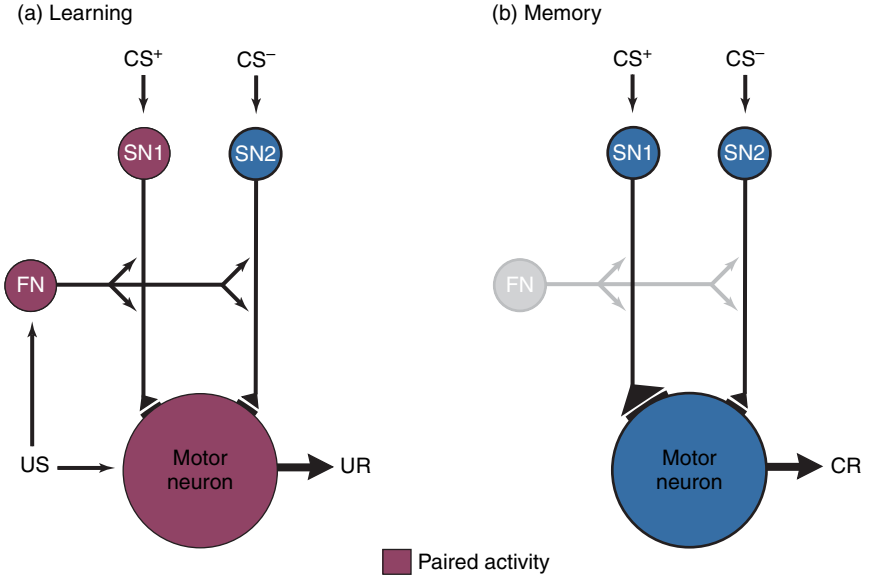


Figure 1 General model of activity-dependent neuromodulation. (a) Learning. A motivationally potent reinforcing stimulus (US) activates a motor neuron to produce the unconditioned response (UR) and a facilitatory neuron (FN) or modulatory system that regulates the strength of the connection between sensory neurons (SN1 and SN2) and the motor neuron. Increased spike activity in one sensory neuron (SN1) immediately before the modulatory signal amplifies the degree and duration of the modulatory effects, perhaps through the Ca^{+2} sensitivity of the modulatory evoked second messenger, with contributions from the postsynaptic neuron. The unpaired sensory neuron (SN2) does not show an amplification of the modulatory effects. (b) Memory: The amplified modulatory effects cause increases in transmitter release and/or excitability of the paired neuron, which in turn strengthens the functional connection between the paired sensory neuron (SN1) and the motor neuron. The associative enhancement of synaptic strength represents the conditioned response (CR).

training protocol was performed with tactile stimulation of the siphon as the CS and an electric shock to the tail as the US. Paired training significantly increased the amplitude of the siphon withdrawal, indicating successful conditioning. The classical conditioning protocol also produced a pairing-specific increase in the strength of the sensorimotor neuron synapse.

Experimental analyses of sensitization of defensive reflexes in *Aplysia* have shown that the neuromodulator released by the reinforcing stimulus, which is believed to be serotonin, activates the enzyme adenylyl cyclase in the sensory neuron. The activation of adenylyl cyclase increases the synthesis of the second messenger cyclic adenosine monophosphate (cAMP), which activates the cAMP-dependent protein kinase, and the subsequent protein phosphorylation leads to a modulation in several properties of the sensory neurons. These changes include modulation of membrane conductances and other processes which facilitate synaptic transmission. This facilitation results in the increased activation of the motor neuron and the sensitization of the reflex. The pairing specificity of the associative conditioning is at least partly due to an increase in the level of cAMP beyond that produced by serotonin alone (Ocorr et al., 1985; Abrams and Kandel, 1988). The influx of Ca^{+2} associated with the CS (spike activity) amplifies the US-mediated modulatory effect by interacting with a Ca^{+2}-sensitive component of the adenylyl cyclase (Abrams and Kandel, 1988). A critical role for Ca^{+2}-stimulated cyclase is also suggested by studies of *Drosophila* showing that the adenylyl cyclase of a mutant deficient in associative learning exhibits a loss of Ca^{+2}/calmodulin sensitivity.

The postsynaptic cell (i.e., motor neuron) also contributes to the plasticity of the synapse (Murphy and Glanzman, 1997; Bao et al., 1998). The postsynaptic membrane of the motor neuron contains *N*-methyl-D-aspartate (NMDA)-like receptors. If these receptors are blocked, then the associative modification of the synapse is disrupted. NMDA receptors require concurrent delivery of glutamate and depolarization in order to allow the entry of calcium. Activity in the sensory neuron (CS) provides the glutamate, and the US depolarizes the cell. The subsequent increase in intracellular Ca^{+2} may release a retrograde signal from the postsynaptic cell to the presynaptic terminal. This retrograde signal would then act to further enhance the cAMP cascade in the sensory neuron. Presynaptically blocking protein kinase A (PKA) by injecting a peptide inhibitor into the sensory neuron, or postsynaptically blocking Ca^{+2} by injecting BAPTA (1,2-bis-(o-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid) into the motor neuron, also blocked the pairing-specific strengthening of the sensorimotor neuron synapse (Antonov et al., 2003) using the simplified preparation for classical conditioning mentioned earlier. The plasticity in the sensorimotor neuron synapse can be blocked by an injection to the postsynaptic motor neuron alone, suggesting that a retrograde signal is an integral part of the process.

This mechanism for associative learning appears to be an elaboration of a process already in place that mediates sensitization, which is a simpler form of learning (*See* Chapters 4.02, 4.03 for a review of the mechanisms of sensitization). This finding raises the interesting possibility that even more complex forms of learning may use simpler forms as building blocks, an idea that has been suggested by psychologists for many years but has only recently become testable at the cellular level.

4.10.2.3 Neural Mechanisms of Appetitive Classical Conditioning in *Aplysia*

The feeding system of *Aplysia* has many advantages. For example, much of the cellular circuitry controlling feeding behavior has been identified, so it is possible to study neurons with known behavioral significance. The *in vivo* training protocol for classical conditioning (Lechner et al., 2000a) has been used to examine correlates of classical conditioning in several neurons that are important for the expression of feeding behavior. Classical conditioning led to the pairing-specific strengthening of the CS-evoked excitatory synaptic input to pattern-initiating neuron B31/32, although no changes were observed in the membrane properties, such as input resistance or the threshold for bursting (Lechner et al., 2000b). If B31/32 receives a greater excitatory input from the CS, then this neuron would be more likely to initiate a feeding motor pattern, and the feeding motor patterns evoked by the CS would be mostly ingestive (Lechner et al., 2000b). However, B31/32 promotes the initiation of feeding motor patterns without defining which type of pattern (i.e., ingestive or egestive) is expressed (Hurwitz et al., 1996). Thus, the pairing-specific increase in excitatory input to B31/32 alone cannot account for the specific increase in the ingestive behavior that is observed following *in vivo* classical conditioning (Lechner et al., 2000a).

Correlates of classical conditioning were measured in neuron B51 following the *in vivo* classical

conditioning protocol (Lorenzetti et al., 2006). Neuron B51 is pivotal for the expression of ingestive motor patterns, and B51 exhibits a characteristic all-or-nothing sustained level of activity (i.e., plateau potential; Plummer and Kirk, 1990) during ingestive motor patterns (Nargeot et al., 1999a). Classical conditioning induced a significant pairing-specific increase in the CS-evoked excitatory synaptic input to B51, as well as an increase in the number of CS-elicited plateau potentials. The pairing-specific strengthening of the excitatory synaptic input can increase the likelihood that B51 is recruited into a motor pattern, which can contribute to the pairing-specific increase in the number of CS-evoked plateau potentials. An increase in the recruitment of B51 should bias the feeding central pattern generator (CPG) toward the expression of ingestive motor patterns (Nargeot et al., 1999b). Thus, the effects produced by classical conditioning appear to be distributed among elements of the feeding CPG such as B31/32 and B51, with the pairing-specific plasticity in B31/32 contributing to the increased number of motor patterns, while the pairing-specific plasticity in B51 is biasing the nature of the motor patterns toward ingestion.

In addition to the pairing-specific enhancement of the CS-evoked excitatory synaptic input to B51, classical conditioning can alter the intrinsic biophysical properties of B51. Classical conditioning raised the threshold for eliciting a burst (i.e., plateau potential) in B51 without affecting either the resting membrane potential or input resistance. This result adds to an increasing body of evidence that, in addition to changes in synaptic efficacy, changes in the intrinsic neuronal excitability also contribute to the storage of memory (for reviews see Daoudal and Debanne, 2003; Zhang and Linden, 2003; *See also* Chapter 4.40). The pairing-specific decrease in the excitability would make B51 less likely to be active, whereas the increase in the excitatory synaptic input would facilitate the recruitment of the neuron. However, the training produced more CS-evoked plateau potentials in B51 in the paired group as compared to the unpaired group. Thus, the factors that enhance the recruitment of B51 overpower the diminished excitability and bias B51 toward producing more plateau potentials, resulting in a greater number of ingestive motor patterns. This pairing-specific decrease in the excitability of B51 could be an adaptive mechanism to help shape the CS specificity produced by classical conditioning.

An *in vitro* analog of classical conditioning has been developed for the feeding system of *Aplysia* (Mozzachiodi et al., 2003). This preparation used isolated ganglia from naive animals. Stimulation of the anterior tentacular nerve was chosen as the analog of the CS. Specifically, the fourth branch of the anterior tentacular nerve was used because this branch innervates the lip region, which is the site of stimulation for the *in vivo* CS. Stimulation of the esophageal nerve was used as the analog of the US. The ganglia were trained by repeatedly pairing the CS with the US. After training, the ganglia produced more motor patterns (analogs of the CR) after CS delivery, indicating that conditioning was successful in this reduced analog. The *in vitro* analog of classical conditioning was then performed while monitoring the membrane properties and CS-elicited synaptic input to B31/32, CBI-2 (Mozzachiodi et al., 2003), and B51 (Lorenzetti et al, 2006). The *in vitro* training protocol produced an increase in the CS-elicited synaptic input to both B31/32 and to B51, an increase in the burst threshold of B51, and an increased number of CS-elicited plateau potentials in B51, similar to what was observed following *in vivo* training. In addition, *in vitro* classical conditioning led to a pairing-specific enhancement of the CS-elicited synaptic input to CBI-2 (Mozzachiodi et al., 2003), which is one of the command-like interneurons controlling the activity of the feeding CPG (Rosen et al., 1991). Synergism among these effects can help produce the pairing-specific increase in the number of CS-evoked bites observed following *in vivo* classical conditioning.

These results provide further support that memory can be distributed among multiple sites of plasticity, similar to what has been observed with other animal model systems. In *Lymnaea*, appetitive classical conditioning strengthened the CS-evoked excitatory synaptic drive to feeding motor neurons (Staras et al., 1999) and induced a persistent depolarization in the modulatory neuron CV1a (Jones et al., 2003). In *Aplysia*, empirical studies (e.g., Trudeau and Castellucci, 1993; for review see also Cleary et al., 1995) and theoretical work (White et al., 1993; Lieb and Frost, 1997) on the neural circuits controlling defensive withdrawal reflexes emphasize the role of both sensory neurons and interneurons as sites of learning-related plasticity underlying behavioral sensitization. In vertebrates, the plasticity produced by delay classical conditioning of the eyelid response is distributed between the cerebellar cortex and the deep cerebellar nuclei (for reviews see Raymond et al., 1996; Kim and Thompson, 1997). Trace

classical conditioning of the same reflex can also involve the hippocampus (for review see Christian and Thompson, 2003). Therefore, studies in both invertebrate and vertebrate neural circuits support the concept that multiple sites of plasticity contribute to the storage of information for associative and nonassociative forms of memory.

4.10.3 Operant Conditioning

4.10.3.1 Behavioral Studies

Feeding behavior in *Aplysia* can be modified by pairing feeding with an aversive stimulus. If food is wrapped in a tough plastic net, *Aplysia* bite and attempt to swallow the food. However, netted food cannot be swallowed, and so it is rejected. The inability to consume the food appeared to be an aversive stimulus that modified the feeding behavior, because the trained animals no longer attempted to bite the netted food (Susswein et al., 1986).

Feeding behavior can also be operantly conditioned with an appetitive stimulus (Brembs et al., 2002). The reinforcement signal for the *in vivo* training protocol was a brief shock to the esophageal nerve. The esophageal nerve is believed to be part of the pathway mediating food reward because bursts of activity in this nerve occur when the animal successfully ingests food (Brembs et al., 2002). In addition, lesions to this nerve blocked *in vivo* appetitive classical conditioning (Lechner et al., 2000a). Also, the *in vitro* analog of classical conditioning discussed earlier successfully increased the number of CS-elicited motor patterns when esophageal nerve shock was used as the US (Mozzachiodi et al., 2003). In the operant conditioning paradigm, the contingent reinforcement of biting behavior by a shock to the esophageal nerve produced an increase in the frequency of biting, when measured both immediately after training and 24 h after training, as compared to animals trained with a yoke-control procedure (Brembs et al., 2002).

4.10.3.2 Neural Mechanisms of Appetitive Operant Conditioning in *Aplysia*

We have previously discussed B51 (Plummer and Kirk, 1990) as being implicated in the expression of ingestive behavior and as a correlate of classical conditioning. B51 is active predominantly during the retraction phase (Nargeot et al., 1997), and when B51 is recruited into a pattern, it recruits radula closure motor neurons (see **Figure 2**).

The *in vivo* training protocol for operant conditioning was used to examine correlates in neuron B51 (Brembs et al., 2002). Operant conditioning led to changes in the membrane properties of B51. The input resistance was increased and the threshold for bursting was decreased. These changes increase the likelihood of B51 activation and thereby contribute to the conditioned increase in the ingestive response.

An *in vitro* analog of operant conditioning was developed using only the isolated buccal ganglia, which is responsible for generating the motor patterns involved in feeding (Nargeot et al., 1999a). These motor patterns can either be ingestive or egestive. In this analog of operant conditioning, motor patterns corresponding to ingestion were used as the analog of the behavior. The ingestive motor pattern was selectively reinforced by contingently shocking the esophageal nerve, which was the analog of the reinforcement. The conditioning procedure resulted in an increase in the likelihood of ingestive patterns being produced (Nargeot et al., 1999a). The contingent reinforcement also resulted in the modulation of the membrane properties of neuron B51 (Nargeot et al., 1999a). The input resistance increased and the threshold for eliciting a burst decreased in a manner similar to the *in vivo* operant conditioning protocol. These changes in the membrane properties of B51 make the cell more excitable and more likely to be recruited into a motor pattern, thus helping to explain the increase in the frequency of expression of the ingestive motor patterns following the *in vitro* analog of operant conditioning. Furthermore, these results for the membrane properties of B51 can be replicated when induced electrical activity in B51 was substituted for the analog of the behavior, instead of an ingestive motor pattern, which was then contingently reinforced with a shock to the esophageal nerve (Nargeot et al., 1999b).

The esophageal nerve, which is used to send both a reinforcement signal with operant conditioning and a US signal with classical conditioning, contains dopaminergic processes (Kabotyanski et al., 1998). Esophageal nerve stimulation produced a postsynaptic potential (PSP) in B51 and this PSP was blocked by the dopamine antagonist ergonovine (Nargeot et al., 1999c). This dopamine antagonist also blocked the acquisition of the associative changes induced by *in vitro* analogs of both operant conditioning (Nargeot et al., 1999c) and classical conditioning (Reyes et al., 2005).

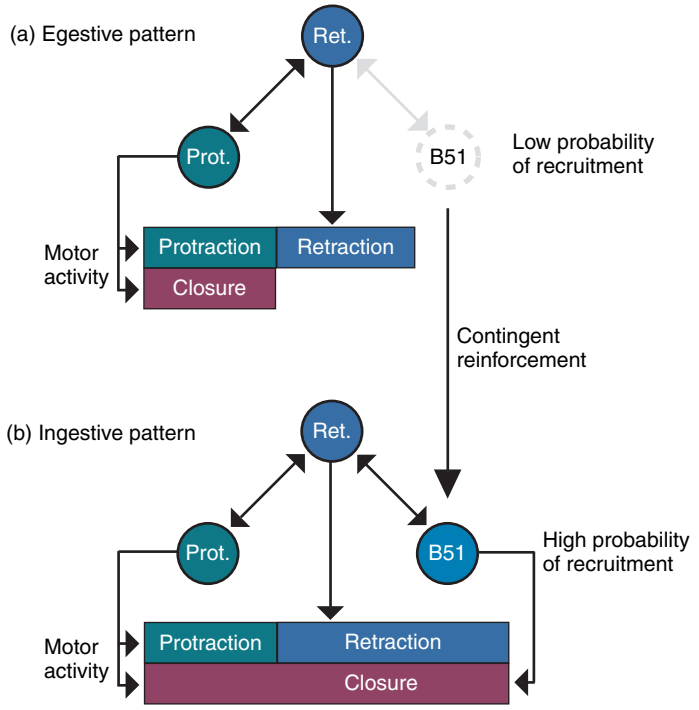


Figure 2 Model of operant conditioning of feeding in *Aplysia.* The cellular network that mediates feeding behavior is represented by the elements in circles. Motor activity comprising two basic feeding patterns is depicted below. (a) At first, the radula protraction-generating element (Prot.) is active, followed by the radula retraction element (Ret.). In the naive state, neuron B51 has a low probability for recruitment and thus does not take part in the feeding motor program. Radula closure occurs during the protraction phase. Consequently, the pattern elicited is egestive. (b) Neuron B51 now has a higher probability for recruitment following contingent reinforcement. B51 is now active during the motor program, leading to radula closure occurring primarily during the retraction phase. Thus, the pattern elicited will now be ingestive.

The analog was further reduced by removing neuron B51 from the ganglia and placing it in culture (Lorenzetti et al., 2000; Brembs et al., 2002). This single, isolated neuron was conditioned by contingently reinforcing induced electrical activity (the analog of behavior) with a direct and temporally discrete application of dopamine (the analog of reinforcement). After conditioning, the input resistance of B51 increased and the threshold for bursting decreased, similar to the *in vivo* and *in vitro* analogs of operant conditioning described above. The membrane properties of B51 were modulated such that the cell was more likely to be active in the future. Such a highly reduced preparation is a promising candidate to study the mechanisms of dopamine-mediated reward and the conditioned expression of behavior at the level of the intracellular signaling cascades.

The operant conditioning of the feeding behavior of *Aplysia* increased the expression of the ingestive responses. The excitability of B51 was also increased by the operant protocol, accounting for the bias in the output of the CPG toward ingestion. However, increasing the excitability of B51 is not likely to increase the total number of patterns expressed. Thus, another site of plasticity is probably induced by the operant protocol. A likely candidate for this additional site of plasticity is in a cell or synapse that is responsible for pattern initiation (e.g., B31). Though these possible sites of plasticity have not yet been explored, it seems likely that both operant and classical conditioning lead to the distribution of memory at multiple sites within the neural circuit.

4.10.4 Conclusions

The feeding system of *Aplysia,* with its relatively simple circuitry, provides a model system for a systematic comparison of the mechanisms underlying classical and operant conditioning. Some interesting similarities as well as differences are beginning to emerge (**Table 1**). One similarity is the nature of the reinforcement pathway and its neurotransmitter. The esophageal nerve mediates the reinforcement signal

Table 1 Comparative analysis between appetitive classical and operant conditioning of feeding in *Aplysia*

	Classical conditioning	*Operant conditioning*
Change in the number of bites	Increase	Increase
Pathway mediating US/reinforcement	Esophageal nerve	Esophageal nerve
Transmitter mediating US/reinforcement	Dopamine	Dopamine
B51 plateau potentials	Increase	Increase
B51 resting membrane potential	No change	No change
B51 input resistance	No change	Increase
B51 burst threshold	Increase	Decrease

for appetitive operant conditioning (Nargeot et al., 1997) and the US pathway for appetitive classical conditioning (Lechner et al., 2000a; Mozzachiodi et al., 2003). Also, this pathway appears to use dopamine as a transmitter, which is consistent with the long-held view that dopamine can mediate the US/reinforcement for appetitive forms of both classical and operant conditioning in both vertebrates and invertebrates (for review see Schultz, 2002).

Appetitive classical conditioning of feeding behavior in *Aplysia* produced two major changes in neuron B51. The synaptic input along the CS pathway into B51 was increased. This increase in the CS pathway suggests that the conditioned sensory pathway receives a preferential boost, while the other sensory pathways could remain unchanged. The second change seen with B51 was an increase in the burst threshold. This change acts on the level of the pattern generation machinery and makes the expression of ingestive feeding responses less likely. Thus, the animal would be less likely to feed unless the CS was present. Identical changes in the properties of B51 were expressed in intact animals trained with a classical conditioning protocol and in an *in vitro* analog of classical conditioning using isolated ganglia (Lorenzetti et al., 2006).

Appetitive operant conditioning of feeding behavior in *Aplysia* also produced two major changes in neuron B51. Both changes were made to the intrinsic membrane properties of the cell. First, the input resistance was increased. Second, the burst threshold was decreased. Both of these changes act in the same direction and would make B51 more likely to be active, thus accounting for the increased expression of the behavior following reinforcement. Identical changes in the membrane properties of B51 were expressed in intact animals trained with an operant conditioning protocol (Brembs et al., 2002), in an *in vitro* analog of operant conditioning using isolated ganglia (Nargeot et al., 1999a,b), and in a single-cell analog consisting of neuron B51 in culture (Brembs et al., 2002).

B51 is a cellular locus for the changes induced by both operant and classical conditioning. No pairing-specific changes in the input resistance were observed following classical conditioning, which was in contrast to the contingent-dependent increase in this parameter measured in B51 following both *in vivo* and *in vitro* operant conditioning. Both operant and classical conditioning modified the threshold level for activation of neuron B51, but in opposite directions, revealing key differences in the cellular mechanisms underlying these two forms of associative learning and suggesting a difference at the molecular level. B51 appears to be a coincidence detector for both the CS–US association (classical conditioning) and the contingency between ingestive behavior and reinforcement (operant conditioning). Because dopamine likely mediates both the US and the reinforcement, a key problem is the elucidation of the mechanisms that lead to the induction of the opposite effects on the burst threshold. One possibility is that the coincidence detector for classical conditioning involves an association between a transmitter released by the CS and dopamine, whereas for operant conditioning it involves an association between the cellular effects of B51 burst activity and dopamine.

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4.11 Neural and Molecular Mechanisms of Fear Memory

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4.11.1 An Overview of Pavlovian Fear Conditioning

Classical or Pavlovian fear conditioning has long been a tool of behavioral psychology to study simple forms of associative learning in the mammal. In this paradigm, an animal (or human) learns to fear an initially emotionally neutral stimulus (the *conditioned stimulus,* CS) that acquires aversive properties after being paired with a noxious stimulus (the *unconditioned stimulus,* US). First used by J. B. Watson and his colleague Rosalie Rayner in the now infamous studies of "Little Albert" (Watson and Rayner, 2000), fear conditioning is now most widely studied in rodents, where a discrete cue (such as a tone, light, or odor; CS) is paired with a brief electric shock to the feet (US). Before conditioning, the CS does not elicit fearful behavior. After as little as one CS-US pairing, however, the animal begins to exhibit a range of *conditioned responses* (CRs), both to the tone CS and to the context in which conditioning occurs (e.g., the conditioning chamber). In rats, these CRs include 'freezing' or immobility (the rat's species-typical behavioral response to a threatening stimulus), autonomic and endocrine alterations (such as changes in heart rate and blood pressure, defecation, and increased levels of circulating stress hormones), and potentiation of reflexes like the acoustic startle response (Blanchard and Blanchard, 1969; Kapp et al., 1979; LeDoux et al., 1988; Roozendaal et al., 1991; Davis, 1997).

4.11.2 The Amygdala and Fear Conditioning

4.11.2.1 The Neuroanatomy of Fear

There are few associative learning paradigms that have been better characterized at the neuroanatomical level than Pavlovian fear conditioning (see **Figure 1**). This is particularly true for the 'auditory fear conditioning' paradigm, where an animal learns to fear a tone (CS) that is paired with foot shock (US). In this review, we will therefore emphasize the findings from the auditory fear conditioning literature, although similar mechanisms have also been proposed for conditioning to visual stimuli (Davis, 1992, 1997).

Auditory fear conditioning involves transmission of auditory CS and somatosensory US information to the lateral nucleus of the amygdala (LA), an area that lesion and functional inactivation studies have shown to be critical for learning (LeDoux et al., 1990; Helmstetter and Bellgowan, 1994; Campeau and Davis, 1995; Muller et al., 1997; Wilensky et al., 2000). Anatomical tract tracing studies have shown that cells in the LA receive direct glutamatergic projections from areas of the auditory thalamus and cortex, specifically from the medial division of the medial geniculate body and the posterior intralaminar nucleus (MGm/PIN) and cortical area TE3, respectively (LeDoux et al., 1985; LeDoux and

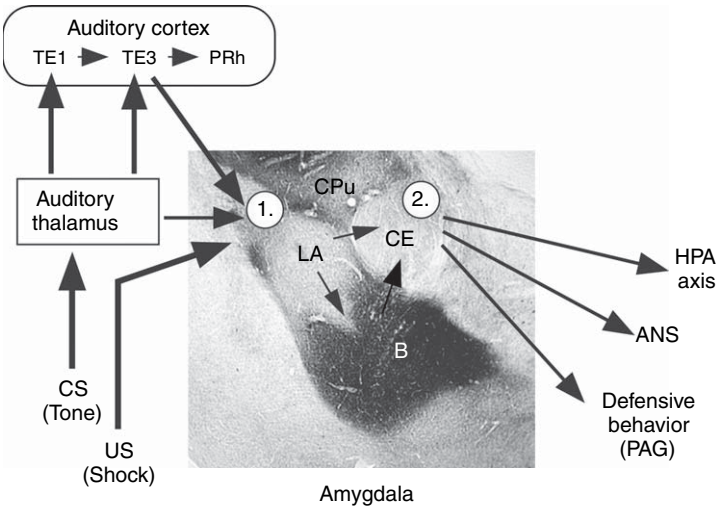


Figure 1 Anatomy of the fear system. (1) Auditory fear conditioning involves the transmission of CS sensory information from areas of the auditory thalamus and cortex to the lateral amygdala (LA), where it can converge with incoming somatosensory information from the foot shock US. It is in the LA that alterations in synaptic transmission are thought to encode key aspects of the learning. (2) During fear expression, the LA engages the central nucleus of the amygdala (CE), which projects widely to many areas of the forebrain and brainstem that control the expression of fear CRs, including freezing, hypothalamic-pituitary-adrenal (HPA) axis activation, and alterations in cardiovascular activity. CPu, caudate/putamen; B, basal nucleus of amygdala; ANS, autonomic nervous system; PRh, perirhinal cortex; PAG, periaqueductal gray.

Farb, 1991; Bordi and LeDoux, 1992; Romanski and LeDoux, 1993; McDonald, 1998; Doron and LeDoux, 1999). Neurophysiological evidence has indicated that inputs from each of these auditory areas synapse onto single neurons in the LA (Li et al., 1996), where they converge with inputs from the somatosensory US (Romanski et al., 1993). Individual cells in the LA are thus well suited to integrate CS and US information during fear conditioning, and it is here, as we will see, that alterations in synaptic transmission are thought to encode key aspects of the memory.

Thalamic and cortical inputs to the LA, while both capable of mediating fear learning (Romanski and LeDoux, 1992a), are believed to carry different types of information to the LA. The thalamic route (often called the 'low road') is believed to be critical for rapidly transmitting crude aspects of the CS to the LA, while the cortical route (known as the 'high road') is believed to carry highly refined information to the amygdala (LeDoux, 2000). Interestingly, while lesions of the MGm/PIN impair auditory fear conditioning (LeDoux et al., 1984, 1986), lesions of the auditory cortex do not (LeDoux et al., 1984; Romanski and LeDoux, 1992b). Thus, the thalamic pathway between the MGm/PIN and the LA appears to be particularly important for auditory fear conditioning. This is not to say, however, that the cortical input to the LA is not involved. Indeed, when conditioning depends on the ability of the animal to make fine discriminations between different auditory CSs, or when the CS is a complex auditory cue such as an ultrasonic vocalization, then cortical regions appear to be required (Jarrell et al., 1987; Lindquist et al., 2004).

During retrieval or expression of a fear memory, the LA, both directly and by way of the adjacent basal nucleus of the amygdala, engages the central nucleus of the amygdala (CE). The CE has traditionally been thought of as the principal output nucleus of the fear learning system, projecting to areas of the forebrain, hypothalamus, and brainstem that control behavioral, endocrine, and autonomic CRs associated with fear learning (Blanchard and Blanchard, 1969; Kapp et al., 1979; LeDoux et al., 1988; Roozendaal et al., 1991; Davis, 1997). Projections from the CE to the midbrain periaqueductal gray, for example, have been shown to be particularly important for mediating behavioral and endocrine responses such as freezing and hypoalgesia (LeDoux et al., 1988; Helmstetter and Landeira-Fernandez, 1990; Helmstetter and Tershner, 1994; De Oca et al., 1998), while projections to the lateral hypothalamus have been implicated in the control of conditioned cardiovascular responses (Iwata et al., 1986; LeDoux et al., 1988). Importantly, while lesions of these individual areas can selectively impair expression of individual CRs, damage to the CE interferes with the expression of all fear CRs (LeDoux, 2000). Thus, the CE acts to coordinate the collection of hard-wired, and typically species-specific, responses that underlie defensive behavior.

4.11.2.2 Synaptic Plasticity in the Amygdala and Fear Conditioning

In addition to being the recipient of CS and US information, the LA is also thought to be a critical site of synaptic plasticity underlying fear learning (LeDoux, 2000; Blair et al., 2001; Maren, 2001). In support of this view, numerous studies have shown that individual cells in the dorsal regions of the LA (LAd) alter their neurophysiological response properties when CS and US are paired during fear conditioning (**Figures 2(a)** and **2(b)**). For example, LAd neurons that are initially weakly responsive to auditory input respond vigorously to the same input after fear conditioning (Quirk et al., 1995, 1997; Rogan et al., 1997; Maren, 2000; Repa et al., 2001; Blair et al., 2003). This change in the responsiveness of LAd cells that occurs as the result of training has contributed to the view that neural plasticity in the LA encodes key aspects of fear learning and memory storage (Fanselow and LeDoux, 1999; Blair et al., 2001; Maren, 2001; Schafe et al., 2001), a topic to which we will return in a later section.

In the next several sections, we will discuss the biochemical and molecular mechanisms that likely underlie plasticity and memory formation at LA synapses. We begin with a discussion of long-term potentiation (LTP), as it has been proposed that this type of synaptic plasticity is the most likely type of mechanism that underlies memory formation in the mammalian brain (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999), including in the LA (Maren, 1999; Blair et al., 2001; Schafe et al., 2001).

4.11.3 LTP as a Mechanism of Fear Learning

4.11.3.1 Why is LTP Important?

The change in the responsiveness of LA cells during fear conditioning suggests that alterations in excitatory synaptic transmission in the LA might be critical for fear conditioning. Accordingly, many of the recent studies that have examined the biochemical

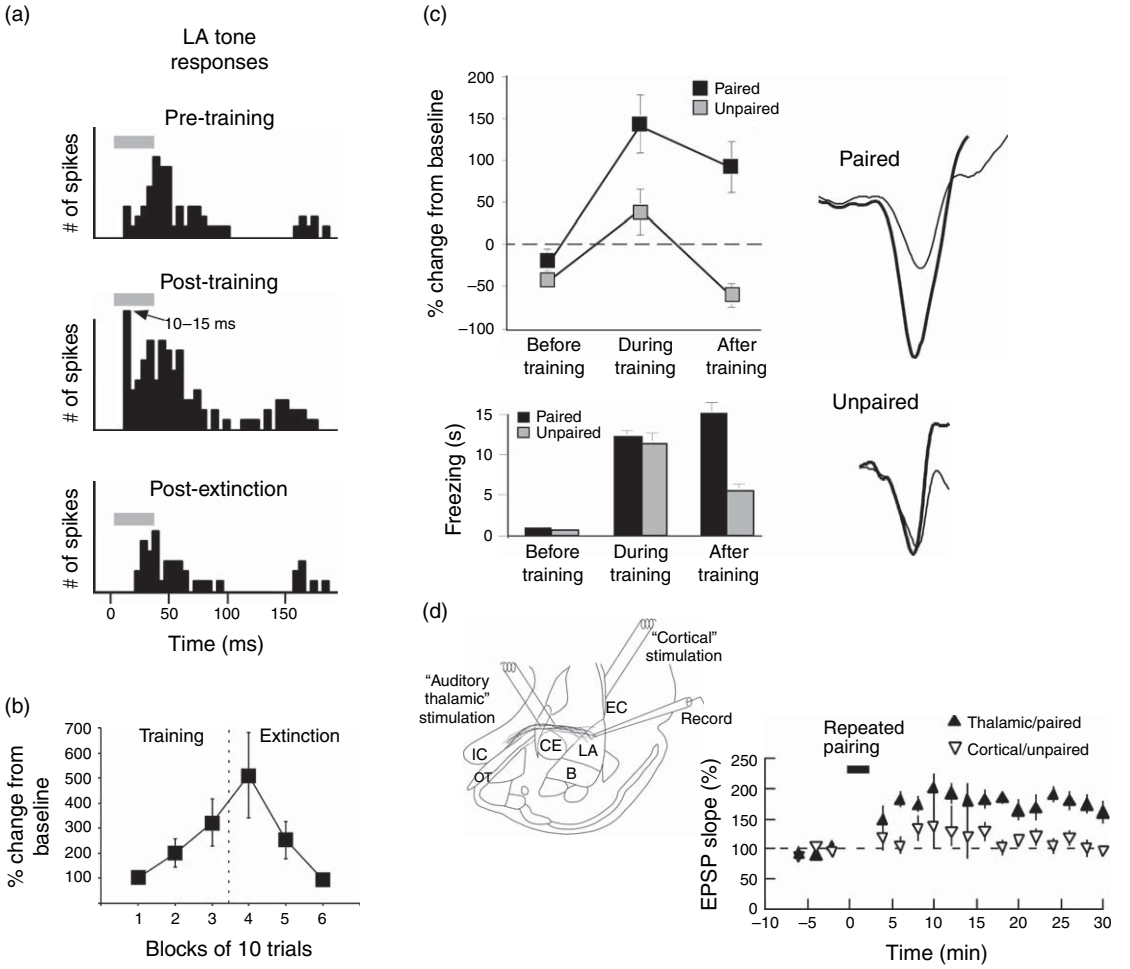


Figure 2 Synaptic plasticity in the lateral amygdala (LA) and fear conditioning. Pairing of CS and US during fear conditioning leads to changes in the responsiveness of LA cells to auditory stimuli, as measured by electrophysiological recording of single cells in the LA. (a) TOP: Prior to auditory fear conditioning, LA cells are weakly responsive to auditory stimuli. MIDDLE: Immediately after conditioning, the same cells respond vigorously, especially in the first few milliseconds of the tone (arrow). BOTTOM: Tone conditioning of LA cells decreases with extinction. In each figure, the onset of the tone stimulus is depicted by a gray bar. (b) Change in firing rate of LA cells over the course of training and extinction. The values represent averaged responses of 16 cells within the first few milliseconds of the tone stimulus and are expressed as percent change from preconditioning firing rates. (c) TOP: Fear conditioning leads to electrophysiological changes in the LA in a manner similar to long-term potentiation (LTP). The figure represents percent change in the slope of the auditory-evoked field potential in the LA before, during, and after conditioning in both paired and unpaired rats. BOTTOM: Freezing behavior across training and testing periods. Note that both paired and unpaired groups show equivalent freezing behavior during training, but only the paired group shows an enhanced neural response. Representative traces can be seen in the inset. (d) LEFT: Associative LTP is induced in the amygdala slice by pairing trains of presynaptic stimulation of fibers coming from the auditory thalamus with depolarization of LA cells. Stimulation of fibers coming from cortical areas serves as a control for input specificity. RIGHT: LTP induced by pairing as measured by the change in the slope of the excitatory postsynaptic potential (EPSP) over time. In this case, the thalamic pathway received paired stimulation, whereas the cortical pathway received unpaired stimulation (i.e., trains and depolarizations, but in a noncontingent manner). The black bar represents the duration of the pairing. IC, internal capsule; OT, optic tract; CE, central nucleus of amygdala; B, basal nucleus of amygdala; EC, external capsule.

basis of fear conditioning have drawn upon a larger literature that has focused on the biochemical events that underlie LTP, an activity-dependent form of synaptic plasticity that was initially discovered in the hippocampus (Bliss and Lømo, 1973). There are several good reasons behind this strategy, including the fact that LTP has been demonstrated in thalamic and cortical auditory input pathways to the

LA (Chapman et al., 1990; Clugnet and LeDoux, 1990; Rogan and LeDoux, 1995; Huang and Kandel, 1998; Weisskopf et al., 1999; Weisskopf and LeDoux, 1999), and that auditory fear conditioning itself has been shown to lead to neurophysiological changes in the LA that resemble artificial LTP induction (McKernan and Shinnick-Gallagher, 1997a; Rogan et al., 1997). Collectively, these findings provide strong support for the hypothesis that an LTP-like process in the LA may underlie fear conditioning (**Figures 2(c)** and **2(d)**). This, in turn, suggests that fear memory acquisition and consolidation may share a common biochemical and molecular substrate with LTP.

4.11.3.2 The 'Consolidation' of LTP – E-LTP Versus L-LTP

There are several pharmacologically distinct forms of LTP, most of which have been identified in the hippocampus. One form critically involves the *N*-methyl-D-aspartate receptor (NMDAR), which is normally blocked by Mg^{2+}, but which can be opened following sufficient depolarization of the postsynaptic cell during LTP induction (Malenka and Nicoll, 1993). The other, less widely studied form involves the L-type voltage-gated calcium channel (L-VGCC). Other forms require a combination of both NMDARs and L-VGCCs (Grover and Teyler, 1990; Cavus and Teyler, 1996). Importantly, both NMDAR and L-VGCC-mediated forms of LTP have been discovered in the LA (Weisskopf et al., 1999; Bauer et al., 2002).

Regardless of how it is induced, the hallmark of each form of LTP is the entry of Ca^{2+} into the postsynaptic spine, which initiates a biochemical cascade of events that leads to strengthening of the synapse. Some of these biochemical cascades lead to a transient change in synaptic strength known as early LTP (E-LTP) that is independent of *de novo* RNA and protein synthesis. This type of LTP is thought to involve the activation of protein kinase signaling pathways near the postsynaptic density and the alteration in the conductance of number of a number of key synaptic proteins involved in glutamatergic signaling, including the NMDAR and the closely related alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor (Soderling and Derkach, 2000). Other intracellular cascades lead to a more permanent alteration in cell excitability known as late LTP (L-LTP). Unlike E-LTP, L-LTP requires *de novo* RNA and protein synthesis and different classes of protein kinase signaling cascades that are thought to promote long-term synaptic plasticity by engaging activators of transcription in the nucleus (Nguyen et al., 1994; Nguyen and Kandel, 1996). Thus, the two forms of LTP have features in common with the traditional phases of memory consolidation. L-LTP is conceptually similar to long-term memory (LTM) formation, which is known to be dependent on *de novo* mRNA transcription and protein synthesis, while E-LTP is conceptually similar to short-term memory (STM), which is known to be short-lasting and independent of transcription and translation (Davis and Squire, 1984; Milner et al., 1998; Schafe et al., 2000). This pattern of findings suggests, in turn, that the consolidation process can be represented at the cellular level and understood through studies of LTP (Milner et al., 1998).

An exhaustive review of the biochemical mechanisms underlying LTP is beyond the scope of this chapter (for a more exhaustive review of this topic, see Milner et al., 1998). In the next several sections, however, we will review some of the key membrane-bound receptors and intracellular signaling pathways that have been most widely implicated in LTP and in memory formation of fear conditioning.

4.11.4 Biochemical Mechanisms of Fear Memory Formation and Consolidation

The fact that LTP is characterized by phases that differ as a function of the requirement for transcription and translation suggests that it is an excellent cellular model by which to study the biochemical mechanisms of memory consolidation. Accordingly, inspired by the success of studies that have defined the contribution of different cellular and molecular signaling cascades underlying both E-LTP and L-LTP, a number of recent studies have asked whether these same mechanisms might underlie short- and long-term formation of fear memories in the amygdala. In this section, we will summarize these findings, beginning with recent studies that have focused on glutamatergic signaling pathways and their contribution to fear acquisition and STM formation.

4.11.4.1 Short-Term Fear Memory Formation – Glutamatergic Signaling, CaMKII Activation, and AMPAR Trafficking in the Amygdala

Like E-LTP, STM is a short-lasting form of memory that does not require new protein or RNA synthesis (Milner et al., 1998). While no consistent time frame of STM has been defined in the literature, it is generally tested shortly after training, usually within 1 h. Further, deficits in STM formation are typically assumed to reflect deficits in memory acquisition, although it should be emphasized that acquisition and STM formation are likely subserved by distinct molecular mechanisms (Rodrigues et al., 2004b). In this section, we will examine how glutamatergic transmission, αCaMKII, and AMPA receptor (AMPAR) regulation and trafficking might contribute to fear memory acquisition and STM formation in the LA.

4.11.4.1.1 NMDA receptors

The NMDAR has a long history in the fear conditioning literature. Early pharmacological studies showed that blockade of NMDARs in the LA using the NMDAR antagonist D-2-amino-5-phosphonovaleric acid (APV) reliably impaired fear conditioning (Miserendino et al., 1990; Kim et al., 1991; Campeau et al., 1992), suggesting that an NMDAR-dependent form of synaptic plasticity was critical for fear learning. Later reports, however, indicated that infusion of APV into the LA also impaired the expression of previously acquired fear responses (Maren et al., 1996). These findings are consistent with neurophysiological evidence showing that NMDARs are involved, at least in part, in routine synaptic transmission in the LA (Weisskopf and LeDoux, 1999; Bauer et al., 2002). As such, it has been difficult to conclude unambiguously that NMDARs are required for fear acquisition independently of a role in routine synaptic transmission.

Several years ago, the role of NMDARs in fear conditioning was revisited by examining the effects of selective blockade of the NR2B subunit of the NMDA receptor in the LA. NMDARs are heteromeric complexes composed of several subunits, including the NR1 subunit, which is essential for channel function, as well as a range of NR2 subunits which regulate channel function (Monyer et al., 1992; Nakanishi, 1992). *In vitro* studies have shown that the NR1-NR2B complex exhibits longer excitatory postsynaptic potentials (EPSPs) than the NR1-NR2A complex (Monyer et al., 1992). This characteristic of NR2B-containing NMDARs is thought to provide a longer time window for coincidence detection, which is thought to be especially important during synaptic plasticity (Tsien, 2000). Indeed, recent molecular genetic studies have implicated the NR2B subunit in both synaptic plasticity and memory formation; overexpression of NR2B in the forebrain of mice results in enhanced LTP and memory formation for a variety of tasks, including fear conditioning (Tang et al., 1999).

In the amygdala, blockade of the NR2B by ifenprodil, a selective antagonist of the NR2B subunit of the NMDAR, dose-dependently impairs formation of both STM and LTM of fear conditioning (Rodrigues et al., 2001); that is, memory is impaired both at 1 h and 24 h after infusion and training (**Figures 3(a) and 3(b)**). In contrast, infusions of ifenprodil prior to testing at either time point have no effect on fear expression. These results suggest that ifenprodil lacks the nonspecific effects on routine transmission that are characteristic of the more global NMDAR antagonist APV. In support of this hypothesis, bath application of ifenprodil to amygdala slices also impairs LTP at thalamic inputs to LA neurons but has no effect on routine synaptic transmission (Bauer et al., 2002; **Figure 3(c)**). These results are also consistent with those of a recent study that examined the effects of APV on acquisition of fear-potentiated startle (Walker and Davis, 2000), showing that APV can, under certain circumstances, have selective effects on plasticity. Collectively, findings suggest that the NMDA receptor in the amygdala plays an essential role in both the acquisition and STM of conditioned fear.

4.11.4.1.2 Ca^{2+}/calmodulin-dependent protein kinase

One of the immediate downstream consequences of NMDAR-mediated activity-dependent increases in Ca^{2+} at the time of LTP induction is the activation of Ca^{2+}/calmodulin (CaM)-dependent protein kinase II (CaMKII). The alpha isoform of CaMKII has been widely implicated in synaptic plasticity and memory formation (Fukunaga and Miyamoto, 1999; Soderling and Derkach, 2000; Fink and Meyer, 2002; Lisman et al., 2002), in part for its ability to undergo a rapid 'autophosphorylation,' a state in which this enzyme can remain active in the absence of further Ca^{2+} entry (Soderling and Derkach, 2000). In this state, αCaMKII can phosphorylate and transiently enhance the conductance of a variety of membrane proteins, including AMPARs (Barria et al., 1997;

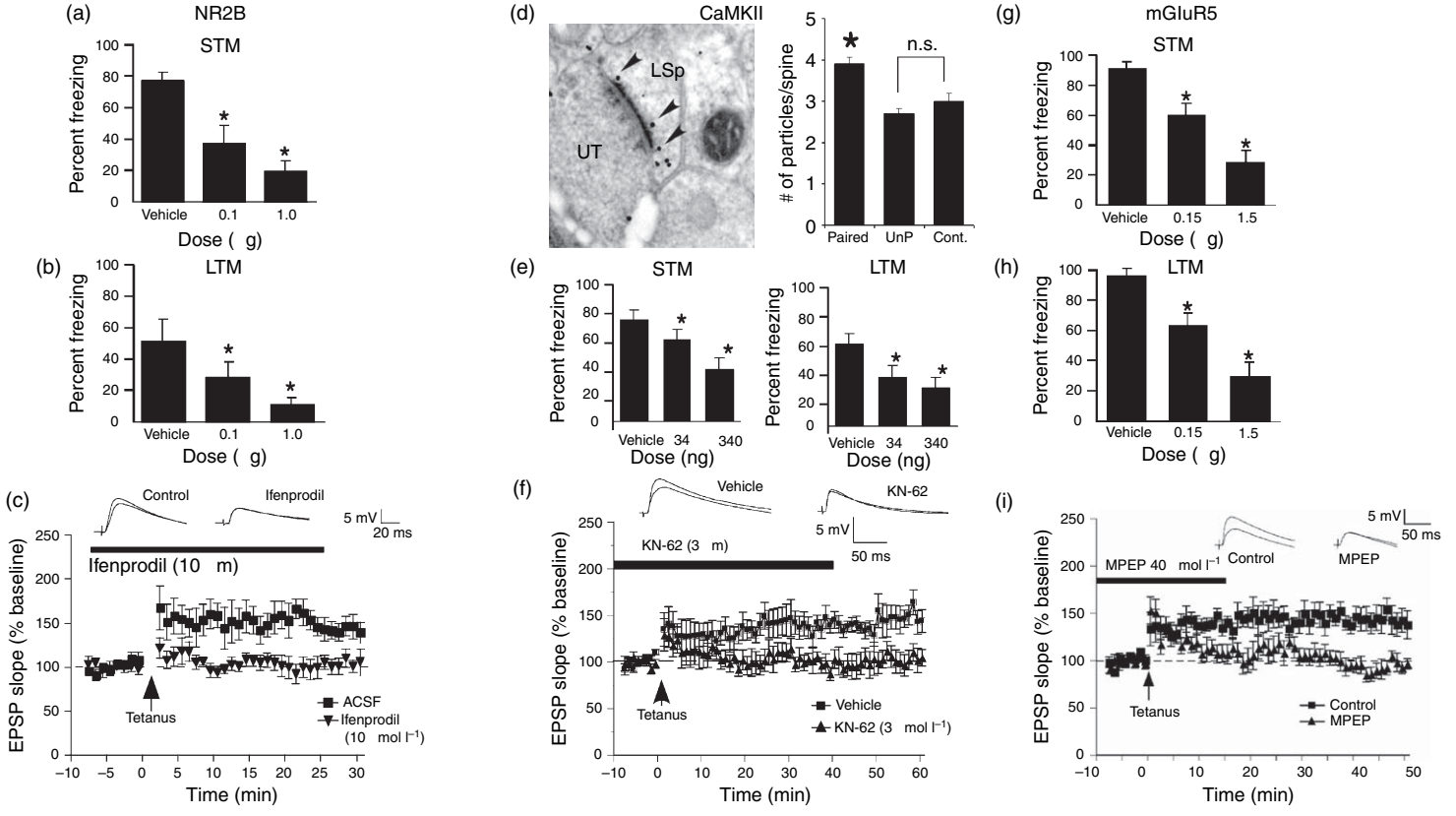


Figure 3 Glutamatergic mechanisms of fear acquisition and STM formation in the LA. (a–b) Both STM and LTM of auditory fear conditioning are dose-dependently impaired by intra-LA infusions of ifenprodil, a selective NR2B antagonist. Adapted from Rodrigues SM, Schafe GE, and LeDoux JE (2001) Intraamygdala blockade of the NR2B subunit of the NMDA receptor disrupts the acquisition but not the expression of fear conditioning. *J. Neurosci.* 21(17): 6889–6896, with permission from the Society for Neuroscience. (c) LTP at thalamic inputs to the LA is also impaired by ifenprodil. Adapted from Bauer EP, Schafe GE, and LeDoux JE (2002) NMDA receptors and L-type voltage-gated calcium channels contribute to long-term potentiation and different components of fear memory formation in the lateral amygdala. *J. Neurosci.* 22: 5239–5249, with permission from the Society for Neuroscience. (d) Fear conditioning results in an increase in autophosphorylated alpha Ca^{2+}/calmodulin-dependent protein kinase II (αCaMKII) in LA spines. Here, rats were conditioned, and activated αCaMKII was detected in LA spines using electron microscopy and an antibody against autophosphorylated αCaMKII at Thr^{286}. The image on the left shows a labeled spine (LSp) in the LA that contains numerous αCaMKII-immunogold labeled particles (arrowheads). The graph on the right shows that paired, but not unpaired (UnP), training leads to significant elevations in αCaMKII-labeled particles in LA spines. UT, unlabeled terminal. $p < .05$. (e) Both STM and LTM of auditory fear conditioning are impaired after intra-LA infusion of KN-62, a CaMKII antagonist. (f) LTP at thalamic inputs to the LA is also impaired by KN-62. (d–f) Adapted from Rodrigues SM, Farb CR, Bauer EP, LeDoux JE, and Schafe GE (2004a) Pavlovian fear conditioning regulates Thr286 autophosphorylation of Ca^{2+}/calmodulin-dependent protein kinase II at lateral amygdala synapses. *J. Neurosci.* 24: 3281–3288, with permission from the Society for Neuroscience. (g–h) Both STM and LTM of auditory fear conditioning are dose-dependently impaired by intra-LA infusions of 2-methyl-6-(phenylethynyl)-pyridine (MPEP), a selective mGluR5 antagonist. (i) LTP at thalamic inputs to the LA is also impaired by MPEP. (g–i) Adapted from Rodrigues SM, Bauer EP, Farb CR, Schafe GE, and LeDoux JE (2002) The group I metabotropic glutamate receptor mGluR5 is required for fear memory formation and long-term potentiation in the lateral amygdala. *J. Neurosci.* 22: 5219–5229, with permission from the Society for Neuroscience.

Mammen et al., 1997; Soderling and Derkach, 2000). Autophosphorylation of αCaMKII on Thr^{286}, for example, promotes the translocation of the kinase to synaptic sites (Shen and Meyer, 1999) and results in phosphorylation of the AMPAR subunit GluR1 on Ser^{831} (Barria et al., 1997; Mammen et al., 1997), an event which increases excitatory current influx into the postsynaptic cell (Derkach et al., 1999) and which is critical for LTP induction (Lee et al., 2000, 2003). Transgenic mice with a deletion of the αCaMKII gene display deficits in hippocampal LTP and hippocampal-dependent spatial memory (Silva et al., 1992a,b). Similarly, pharmacological inhibition of CaMKII blocks the induction of LTP in hippocampal area CA1 (Ito et al., 1991; Stanton and Gage, 1996) and impairs hippocampal dependent learning and memory (Tan and Liang, 1996).

Recent studies have also implicated αCaMKII in fear conditioning. Anatomical studies have shown that αCaMKII is robustly expressed in LA pyramidal neurons (McDonald et al., 2002), where it coexists with NR2B in LA spines postsynaptic to terminals that originate in the auditory thalamus (Rodrigues et al., 2004a). Fear conditioning leads to increases in the autophosphorylated form of αCaMKII at Thr^{286} in spines of LA neurons (**Figure 3(d)**). Further, intra-amygdala infusion or bath application of an inhibitor of CaMKII (KN-62) impairs acquisition and STM formation of fear conditioning and LTP at thalamic inputs to LA neurons, respectively (**Figures 3(e)** and **3(f)**). This latter finding is consistent with molecular genetic experiments indicating that induced overexpression of active αCaMKII by a transgene that replaces Thr^{286} with an aspartate residue in the amygdala and striatum results in a reversible deficit in fear conditioning (Mayford et al., 1996).

4.11.4.1.3 Metabotropic glutamate receptors and protein kinase C

While activation of αCaMKII and resultant GluR1 phosphorylation and receptor trafficking appears to be NMDAR dependent (Hayashi et al., 2000; Fu et al., 2004), it appears that Group I metabotropic glutamate receptors (mGluRs), including mGluR1 and mGluR5, are critical for the potentiation of NMDAR function via the Ca^{2+}/phospholipid-dependent protein kinase (PKC) (Ben-Ari et al., 1992; Kelso et al., 1992). Both mGluR1 and mGluR5, for example, are positively coupled to phospholipase C, activation of which leads to the hydrolysis of phosphatidylinositol 4,5-biphosphate into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), two substances that are directly upstream of PKC. In mGluR5 knockout mice, LTP of NMDAR currents in CA1 is absent, but can be rescued by activators of PKC (Jia et al., 1998). Further, an mGluR5 antagonist (CHPG) has been reported to induce a slowly developing, long-lasting potentiation of NMDAR currents via PKC (Doherty et al., 1997). Studies have suggested that two serine residues on the C-terminal domain of the NR2B subunit of the NMDAR, Ser^{1303} and Ser^{1323}, are the critical structural domain for PKC-mediated current potentiation (Liao et al., 2001). However, removal of all the PKC phosphorylation sites on NR1 and NR2 does not alter the PKC-induced potentiation of NMDAR currents (Zheng et al., 1999). Thus, it has been hypothesized that there is an intermediate step between PKC activation and NR2 subunit activation. One hypothesis is that this involves Src kinases (Ali and Salter, 2001; MacDonald et al., 2001). Src is the lead member of a family of protein tyrosine kinases, which also includes Fyn, Lyn, Lck, and Yes. It is thought that these kinases regulate the activity of NMDARs during LTP induction by phosphorylating tyrosine residues that, in turn, are responsible for increased channel conductance (Ali and Salter, 2001; MacDonald et al., 2001). The phosphorylation of NR2B on Tyr^{1472} is increased after tetanic stimulation in area CA1 (Nakazawa et al., 2001), and this appears to be Fyn mediated (Nakazawa et al., 2001, 2002). Further, mice lacking Fyn have impaired LTP in hippocampal area CA1 (Grant et al., 1992).

Several recent studies have examined the role of mGluRs in fear conditioning. Transgenic mice lacking mGluR5 are impaired in fear conditioning tasks (Lu et al., 1997), as are rats injected systemically with the selective mGluR5 antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) prior to fear conditioning (Fendt and Schmid, 2002). In a recent study, Rodrigues et al. showed that mGluR5 was localized in LA spines postsynaptic to auditory thalamic inputs and required for synaptic plasticity at thalamic inputs to LA neurons (Rodrigues et al., 2002). Further, in behavioral experiments, intra-amygdala infusion of MPEP prior to fear conditioning impaired formation of both STM and LTM of fear conditioning (Rodrigues et al., 2002; **Figures 3(g)** amd **3(h)**), and also impaired LTP at thalamic inputs to the LA (**Figure 3(i)**). Similar to the results with the NR2B antagonist ifenprodil, infusion of MPEP prior to training blocked both STM and

LTM, while infusion immediately prior to testing at either time point had no effect.

These findings suggest that mGluRs, and in particular mGluR5, are required for fear conditioning and STM formation in the amygdala. Future experiments, however, will be required to understand the exact mechanisms by which mGluRs contribute to fear conditioning. One attractive hypothesis, suggested by the LTP literature, is that activation of mGluR5 in the amygdala recruits the PKC signaling pathway and leads to modulation and trafficking of NMDARs via tyrosine phosphorylation of NR2B (Doherty et al., 1997; Anwyl, 1999; Liao et al., 2001). The role of PKC or tyrosine kinases in fear acquisition and STM formation has not been explicitly tested, although mice with a specific deletion of the β isoform of PKC have impaired fear conditioning when tested 24 h after training (Weeber et al., 2000). Additional experiments will be necessary to examine the role of mGluR5-mediated signaling in the LA in fear conditioning.

4.11.4.1.4 AMPA receptor regulation and trafficking

Alterations in the conductance properties of glutamatergic receptors are thought to be only one mechanism underlying LTP induction and E-LTP. Ample evidence, for example, has accumulated indicating that new AMPARs and NMDARs are trafficked and inserted into synapses during and after LTP (Grosshans et al., 2002; Malenka, 2003; Malinow, 2003). The insertion of GluR1 into synapses, for example, appears to be αCaMKII dependent, and blockade of αCaMKII-mediated synaptic delivery of GluR1 prevents LTP (Hayashi et al., 2000). Further, activation of PKC has been shown to drive NMDAR subunits into synapses, an effect which is blocked by tyrosine kinase inhibitors (Grosshans et al., 2002). Together with alterations in receptor conductance, these rapid physical alterations in the distribution of AMPARs and NMDARs represent one mechanism by which LTP might persist in the short term.

A recent study by Malinow and colleagues elegantly showed that intra-amygdala expression of a viral vector that prevents GluR1 from being inserted into synaptic sites impairs fear acquisition and synaptic plasticity in the LA (Rumpel et al., 2005). Thus, while additional studies are needed, these findings collectively suggest that activation of αCaMKII during fear acquisition may regulate the insertion of AMPARs at LA synapses and thereby contribute to the formation and maintenance of STM. It is currently unknown how long-lasting this effect is; e.g., whether it persists only over the course of hours or is also evident days after fear learning. Further, no studies have to date examined how fear conditioning might similarly regulate the trafficking of NMDARs. Additional experiments will be critical to examine each of these questions.

4.11.4.2 Long-Term Fear Memory Formation – Protein Kinase Signaling and Transcriptional Regulation in the Amygdala

As its name implies, LTM is a long-lasting phenomenon that can last many hours, days, weeks, or even years (Milner et al., 1998). Accordingly, LTM is typically tested at longer intervals after training, usually starting at 24 h. In this section, we discuss what is known about the mechanisms of LTM formation of fear conditioning in the amygdala. We begin with a discussion of L-VGCCs, as recent work has suggested that these channels play an essential role in promoting LTM formation in the LA.

4.11.4.2.1 L-VGCCs

Recent experiments have shown that LTP at thalamic input synapses to the LA is, under certain conditions, L-VGCC dependent and NMDAR independent (Weisskopf et al., 1999). These experiments used a pairing protocol in which subthreshold presynaptic stimulation of auditory afferents was paired with brief postsynaptic depolarizations (Magee and Johnston, 1997; Markram et al., 1997; Johnston et al., 1999). In this protocol, back-propagating action potentials (BPAPs) originating in the soma are thought to invade the dendrites and interact with EPSPs leading to Ca^{2+} influx through VGCCs (Magee and Johnston, 1997; Johnston et al., 1999; Stuart and Hausser, 2001). Accordingly, LTP induced by pairing in the thalamic pathway is blocked by application of the L-VGCC blockers nifedipine or verapamil (Weisskopf et al., 1999; Bauer et al., 2002).

Until recently, the contribution of L-VGCCs to fear conditioning had not been established. Bauer et al., however, examined the effect of intra-amygdala infusion of the L-VGCC blocker verapamil on the acquisition and consolidation of auditory fear conditioning (Bauer et al., 2002). The findings revealed that blockade of L-VGCCs prior to conditioning selectively impaired LTM formation of fear conditioning at 24 h after training; acquisition and STM, assessed at 1 h, were left intact. These findings,

together with those of studies that examined the role of NMDAR function in fear conditioning discussed earlier, suggest that there are two sources of Ca^{2+} in the LA that are critical for fear memory formation. One, mediated by NMDARs, appears to be selectively involved in fear acquisition and STM formation of fear conditioning (Walker and Davis, 2000; Rodrigues et al., 2001). The second, mediated by L-VGCCs, is selectively involved in LTM formation. While the effects of L-VGCC blockade are not apparent in fear conditioning for many hours after training, it is important to note that this is likely due to interference with a process that is set in motion at the time of CS-US pairing and fear acquisition. Consistent with that notion, recent reports have demonstrated that L-VGCCs play a selective role in signaling to the nucleus and initiating cyclic adenosine monophosphate (cAMP) response element (CRE)-mediated transcription, which is known to be required for long-term synaptic plasticity and memory formation (Dolmetsch et al., 2001). Additional experiments will be necessary to determine the contribution of L-VGCCs to activation of protein kinases and CRE-driven gene expression in the LA following fear conditioning.

4.11.4.2.2 Protein kinase A and mitogen-activated protein kinase

Activity-dependent increases in intracellular Ca^{2+} in LA neurons during fear acquisition is thought to lead, either directly or indirectly, to the activation of both protein kinase A (PKA) and the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK). There has been a great deal of recent interest in each of these kinases, in part because they have been shown to be essential for the late phase of multiple forms of synaptic plasticity and memory (Milner et al., 1998; Sweatt, 2004). Once activated by stimulation that promotes L-LTP, each of these kinases is thought to engage activators of transcription. While PKA is directly capable of regulating transcription, recent evidence suggests that PKA may play a permissive role in transcriptional regulation by promoting the activation and nuclear translocation of ERK/MAPK (Roberson et al., 1999). As a result, it has been suggested that ERK/MAPK may represent a final common pathway through which different upstream kinases regulate transcription, long-term plasticity, and memory formation (Adams and Sweatt, 2002).

Both PKA and ERK/MAPK have also been implicated in fear conditioning. Mice that overexpress an inhibitory form of PKA, R(AB), exhibit impaired L-LTP in hippocampal area CA1 and selective deficits in LTM, but not STM, of contextual fear conditioning (Abel et al., 1997). Similarly, mice that lack *Ras-GRF,* an upstream regulator of ERK/MAPK, have impaired memory consolidation of auditory and contextual fear conditioning, as well as impaired amygdala LTP (Brambilla et al., 1997).

Recent pharmacological experiments have examined the role of PKA and ERK/MAPK in amygdala LTP and in fear conditioning. Huang et al. showed that bath application of inhibitors of PKA or ERK/MAPK to amygdala slices impairs LTP at thalamic and cortical inputs to the LA but has no effect on E-LTP (Huang et al., 2000). Consistent with those findings, infusion of a PKA inhibitor or of a peptide that blocks the association of PKA with the A-kinase anchoring protein (AKAP) in the LA impairs LTM, but not STM of fear conditioning (Schafe and LeDoux, 2000; Moita et al., 2002; **Figure 4(e)**). Further, fear conditioning results in a transient activation of ERK/MAPK in the LA (**Figures 4(a)–4(c)**), and infusion of an inhibitor of MEK, an upstream regulator of ERK/MAPK, into the LA prior to fear conditioning impairs memory consolidation; that is, rats have intact STM and impaired LTM (Schafe et al., 2000; **Figure 4(d)**). Collectively, these findings support the hypothesis that both PKA and ERK/MAPK contribute to fear memory formation by engaging cellular processes, possibly those in the nucleus, that are necessary for long-term synaptic plasticity and memory formation.

4.11.4.2.3 Neurotrophin signaling

In addition to Ca^{2+}-mediated signaling, neurotrophins have been widely implicated in driving protein kinase signaling pathways necessary for long-term synaptic plasticity and memory formation, including fear conditioning. In hippocampal neurons, direct application of brain-derived neurotrophic factor (BDNF) produces a long-lasting, transcription-dependent form of LTP (Kang and Schuman, 1995; Figurov et al., 1996). Further, blockade or genetic deletion of BDNF or its membrane-bound receptor tyrosine kinase, TrkB, impairs L-LTP in the hippocampus (Figurov et al., 1996; Patterson et al., 1996; Korte et al., 1998; Fanselow and LeDoux, 1999), and L-LTP is impaired in hippocampal slices in mice that lack BDNF (Patterson et al., 1996; Korte et al., 1998). Consistent with the importance of ERK/MAPK signaling in long-term synaptic plasticity and memory formation, recent studies have suggested that

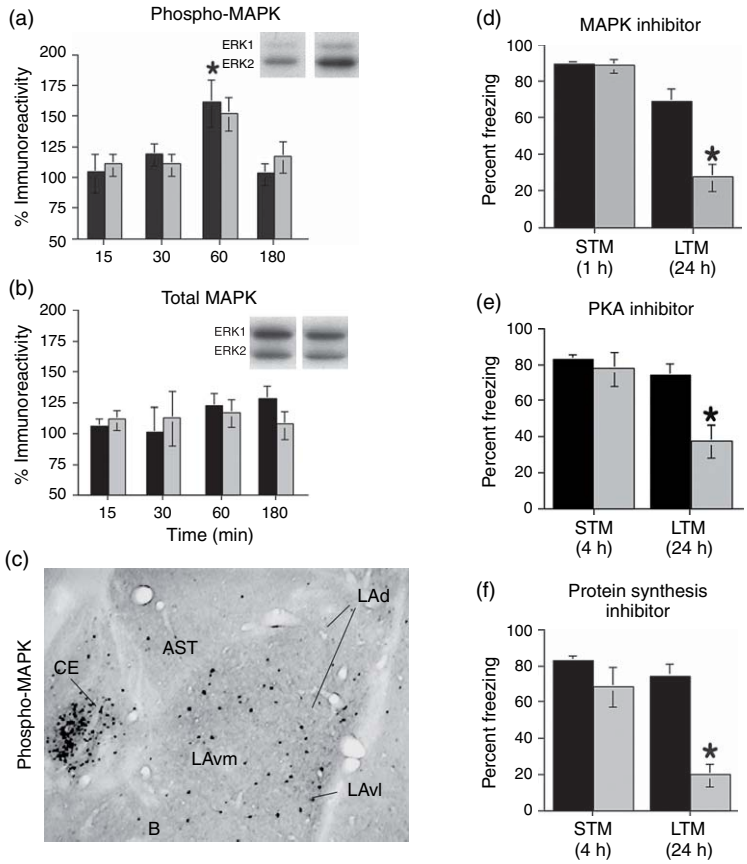


Figure 4 Protein kinase signaling pathways involved in long-term memory (LTM) formation in the lateral amygdala (LA). (a) Fear conditioning leads to an increase in phosphorylated extracellular signal-related kinase 1 (ERK1) and ERK2 at t = 60 min after training. In these experiments, rats were trained and sacrificed at different time points after conditioning, and LA homogenates were probed with antibodies that recognize phosphorylated ERK/mitogen-activated protein kinase (MAPK). ERK1 (black bars) and ERK2 (gray bars) are the two isoforms of ERK/MAPK recognized by the anti-phospho-ERK antibody. $p < .05$. (b) The increase in activated ERK/MAPK is not accounted for by a change in the amount of total (unphosphorylated) ERK/MAPK. (c) Immunocytochemical localization of phosphorylated ERK/MAPK in the LA after fear conditioning. The image shows ERK-labeled cells in three different regions of the LA (dorsal, LAd; ventromedial, LAvm; and ventrolateral, LAvl), with most of the label concentrated in the ventral portions of the LAd and throughout the LAvm and LAvl. Activated ERK/MAPK is also highly expressed in the nearby central nucleus (CE) and the amygdala-striatal transition zone (AST). B, basal nucleus of the amygdala. (a–c) Adapted from Schafe GE, Atkins CM, Swank MW, Bauer EP, Sweatt JD, and LeDoux JE (2000) Activation of ERK/MAP kinase in the amygdala is required for memory consolidation of Pavlovian fear conditioning. *J. Neurosci.* 20: 8177–8187, with permission from the Society for Neuroscience. (d–f) LTM, but not short-term memory (STM), in the LA requires MAPK, protein kinase A (PKA), and protein synthesis. In these studies, rats received intra-amygdala infusions of (d) U0126 (a MEK inhibitor, which is an upstream regulator of ERK/MAPK activation), (e) Rp-cAMPS (a PKA inhibitor), or (f) anisomycin (a protein synthesis inhibitor) at or around the time of training and were assayed for both STM (1–4 h later) and LTM (24 h later) of auditory fear conditioning. In each figure, vehicle-treated rats are represented by the gray bars, while drug-treated animals are represented by the black bars. $^{*}p < .05$ relative to vehicle controls. (d–f) Adapted from Schafe GE and LeDoux JE (2000) Memory consolidation of auditory Pavlovian fear conditioning requires protein synthesis and protein kinase A in the amygdala. *J. Neurosci.* 20: RC96, with permission from the Society for Neuroscience.

BDNF-TrkB-mediated signaling promotes long-term synaptic plasticity by engaging the ERK/MAPK signaling pathway (Patterson et al., 2001). Application of BDNF potently activates ERK/MAPK in hippocampal neurons (Ying et al., 2002), and treatment with an inhibitor of ERK/MAPK activation impairs BDNF-induced LTP (Ying et al., 2002). Collectively, these findings suggest that BDNF-induced ERK signaling plays an essential role in long-term synaptic plasticity.

A recent study has shown that BDNF-mediated signaling in the amygdala is critical to fear learning

(Rattiner et al., 2004, 2005). In that study, fear conditioning led to increases in both TrkB receptor phosphorylation and decreases in TrkB receptor immunoreactivity in the LA during the consolidation period, which is typically indicative of bound BDNF. Further, disruption of TrkB receptor signaling in the amygdala using either a Trk receptor antagonist or lentiviral overexpression of a dominant negative TrkB isoform impaired fear memory formation (Rattiner et al., 2004). While this study did not distinguish between acquisition and consolidation phases of fear learning, the assumption is that BDNF signaling in the LA plays a critical role in the establishment of long-term fear memories, possibly by promoting the activation and nuclear translocation of protein kinases such as ERK (Patterson et al., 2001; Ying et al., 2002). Additional experiments will be necessary to define the signaling pathways through which BDNF acts during fear learning.

4.11.4.2.4 Transcriptional regulation and macromolecular synthesis

Both L-LTP in the LA (Huang and Kandel, 1998; Huang et al., 2000) and LTM of fear conditioning (Bailey et al., 1999; Schafe and LeDoux, 2000) are known to require new RNA and protein synthesis in the LA (**Figure 4(f)**). The requirement for *de novo* RNA synthesis is particularly important, because it suggests that a nuclear event is required for the transition between short- and long-term memory formation.

As previously discussed, signaling via ERK/MAPK plays a critical role in memory formation by engaging activators of transcription in the nucleus. ERK/MAPK is thought to promote transcription by binding to and activating transcription factors, including, by way of the Rsk and MSK1 signaling pathways (Xing et al., 1996; Adams and Sweatt, 2002), the cAMP-response-element binding protein (CREB) (Impey et al., 1996, 1998a). It is the activation of CREB and CRE-mediated genes that ultimately leads to the protein and RNA synthesis-dependent functional and/or structural changes that are thought to underlie L-LTP (Frank and Greenberg, 1994; Yin and Tully, 1996; Silva et al., 1998; Stevens, 1998; Holt and Maren, 1999). While many of these genes and their functional roles remain to be elucidated, it has been suggested that the regulation of a number of CRE- and serum response element (SRE)-mediated immediate early genes (IEGs) plays a critical intermediate role in regulating the expression of late-response genes. These have included *Zif-268,* and its protein product EGR-1, and the activity-regulated cytoskeletal-associated protein (Arc). Importantly, each of these IEGs is known to be regulated by hippocampal LTP (Richardson et al., 1992; Abraham et al., 1993; Worley et al., 1993; Link et al., 1995) and required for hippocampal-dependent LTM formation (Guzowski et al., 2000; Jones et al., 2001).

Several transcription factors have been implicated in long-term synaptic plasticity and in memory formation, but CREB is perhaps the best studied. CREB is a family of transcription factors consisting of several functionally distinct isoforms. Some, known as activator isoforms, bind to DNA at CRE promoter regions and promote transcription. Others, known as repressor isoforms, compete with the binding of activator isoforms to DNA (Bartsch et al., 1995; Abel et al., 1998; Silva et al., 1998). CREB is an attractive candidate molecule for memory consolidation because it has direct interaction with the transcriptional machinery and also contains phosphorylation sites for the major protein kinase signaling pathways that are known to be involved in memory formation, including PKA, ERK/MAPK, and CaMKII (Silva et al., 1998).

The first evidence that suggested CREB might be involved in memory consolidation came from a study employing a Pavlovian conditioning task in *Drosophila.* Overexpression of a dominant negative (repressor) isoform of CREB in flies impaired LTM formation in a conditioned odor aversion task (Yin et al., 1994). Conversely, overexpression of an activator isoform of CREB facilitated LTM; that is, behavioral training that would normally produce only STM was effective at producing LTM (Yin et al., 1995).

CREB has also been implicated in fear conditioning. Mice lacking two critical isoforms of CREB, the α and δ, have impaired hippocampal L-LTP and memory consolidation for auditory and contextual fear conditioning; that is, LTM is impaired, while STM is intact (Bourtchuladze et al., 1994). Further, induced overexpression of a dominant negative isoform of CREB in the forebrain impairs LTM formation of fear conditioning (Kida et al., 2002). Conversely, overexpression of the transcription factor CREB in the LA facilitates fear memory formation (Josselyn et al., 2001). In the latter study, CREB was overexpressed locally in the LA, using viral transfection methods. Consistent with the role of CREB in long-term synaptic plasticity and memory formation, overexpression of CREB in the LA

facilitated LTM of fear conditioning, but had no effect on STM.

While CRE-mediated transcription clearly supports the development of long-term plasticity and memory, the downstream targets of CREB have remained largely unknown. However, a number of studies have shown that fear conditioning induces the expression of both IEGs (Beck and Fibiger, 1995; Rosen et al., 1998; Malkani and Rosen, 2000; Scicli et al., 2004) and downstream genes (Stork et al., 2001; Ressler et al., 2002; Rattiner et al., 2004) in the LA. While the specific contributions of many of these genes to fear conditioning is still unclear, it is widely believed that learning-induced gene expression ultimately contributes to changes in cell (especially synaptic) structure that stabilizes memory (Bailey and Kandel, 1993; Woolf, 1998; Rampon et al., 2000; Sweatt, 2004), presumably by altering the actin cytoskeleton underlying synaptic organization (van Rossum and Hanisch, 1999; Matus, 2000; Kasai et al., 2003). Such changes in synaptic structure have been well documented in invertebrates, where stimulation that promotes long-term synaptic plasticity has been shown to lead to an increase in new synaptic contacts (Bailey et al., 1992, 1994; Bailey and Kandel, 1993). Further, both learning and LTP result in a number of structural changes in the hippocampus and cortex, including an increase in spine head volume and widening and shortening of the spine neck (Van Harreveld and Fifkova, 1975; Fifkova and Van Harreveld, 1977; Fifkova and Anderson, 1981), spine perforation (Toni et al., 1999), and an increase in the total number of spines (Engert and Bonhoeffer, 1999; Leuner et al., 2003).

Recent studies have suggested that fear conditioning leads to alterations in cytoskeletal proteins and to new spine formation in the LA. Fear conditioning, for example, leads to the transcription of genes involved in cytoskeletal remodeling, including the CRE-mediated gene neurofilament-light chain (NF-l) (Ressler et al., 2002). Further, interference with molecular pathways known to be involved in structural plasticity during early development, such as the Rho GTPase (GTP: guanosine triphosphate) activating protein (Rho-GAP) signaling pathway, disrupts memory formation (Lamprecht et al., 2002), and fear conditioning drives actin cytoskeleton-regulatory proteins, such as profilin, into amygdala spines shortly after training (Lamprecht et al., 2006). Finally, a recent morphological study has suggested that fear conditioning leads to an increase in spinophilin-immunoreactive dendritic spines in the LA (Radley et al., 2006).

4.11.4.3 A Presynaptic Component to Fear Learning?

As outlined, most recent studies have focused on postsynaptic mechanisms and their role in amygdala LTP and memory formation (for review, see Schafe et al., 2001). There is growing evidence, however, that suggests that synaptic plasticity and memory formation in the LA involves a presynaptic process. McKernan and Shinnick-Gallagher (1997), for example, showed that auditory fear conditioning occludes paired-pulse facilitation (PPF) at cortical inputs to the LA, a type of short-term plasticity that is largely believed to be presynaptic. Similarly, Huang and Kandel (1998) observed that LTP at cortical inputs to the LA occludes PPF in this pathway. Further, bath application, but not postsynaptic injection, of a PKA inhibitor impairs LTP in LA neurons (Huang and Kandel, 1998). Conversely, bath application of forskolin, a PKA activator, in the presence of antagonists of postsynaptic NMDAR and AMPAR receptors, induces LTP and occludes PPF at cortical inputs (Huang and Kandel, 1998), suggesting that the presynaptic component of LTP in this pathway is PKA dependent. More recently, Tsvetkov et al. showed that auditory fear conditioning itself, in addition to LTP, occludes PPF at cortical inputs to LA (Tsvetkov et al., 2002). It is thus clear from the available evidence that a complete understanding of memory formation and synaptic plasticity in the LA will require attention to both sides of the synapse.

4.11.4.3.1 Nitric oxide signaling and fear learning

Recent evidence has suggested that nitric oxide (NO) signaling in the LA is critical to fear memory formation (Schafe et al., 2005) and may represent a mechanism whereby postsynaptic induction of plasticity induced by fear conditioning in LA neurons may engage accompanying presynaptic changes. NO is a highly soluble gas generated by the conversion of L-arginine to L-citrulline by the Ca^{2+}-regulated enzyme nitric oxide synthase (NOS) (Bredt and Snyder, 1992). In other memory systems, NO is thought to serve as a 'retrograde messenger' that engages aspects of presynaptic plasticity (Schuman and Madison, 1991; Zhuo et al., 1994; Arancio et al.,

1996; Doyle et al., 1996; Son et al., 1998; Ko and Kelly, 1999; Lu et al., 1999) and memory formation (Chapman et al., 1992; Bohme et al., 1993; Bernabeu et al., 1995; Holscher et al., 1996; Suzuki et al., 1996; Zou et al., 1998). One immediate downstream effector of NO, for example, is soluble guanylyl cyclase (Bredt and Snyder, 1992; Son et al., 1998; Denninger and Marletta, 1999; Arancio et al., 2001). This enzyme directly leads to the formation of cyclic guanosine monophosphate (cGMP) and in turn to the activation of the cGMP-dependent protein kinase (PKG). PKG, in turn, can have a number of effects, including targeting and mobilization of synaptic vesicles in the presynaptic cell, leading to enhanced transmitter release (Hawkins et al., 1993, 1998). In the hippocampus, pharmacological inhibition of NOS activation, guanylyl cyclase, or PKG impairs LTP in CA1 (Zhuo et al., 1994; Doyle et al., 1996; Son et al., 1998; Lu et al., 1999; Monfort et al., 2002). Conversely, bath application of exogenous NO or pharmacological activators of cGMP or PKG combined with weak tetanic stimulation, which would not produce LTP alone, induces long-lasting LTP (Zhuo et al., 1994; Son et al., 1998; Lu et al., 1999; Lu and Hawkins, 2002). Inhibition of NOS activity is equally effective at impairing LTP, whether the NOS inhibitor is injected directly into the postsynaptic cell or perfused over the entire slice, suggesting that the critical activation of NOS occurs postsynaptically (Schuman and Madison, 1991; Arancio et al., 1996; Ko and Kelly, 1999). However, NO is thought to act presynaptically, at least in part, because bath application of membrane-impermeable scavengers of NO also impairs LTP in CA1 (Schuman and Madison, 1991; Ko and Kelly, 1999). Collectively, this pattern of findings supports the notion that LTP in area CA1 is induced postsynaptically, but maintained or expressed presynaptically, at least in part, by an NO-dependent mechanism.

In our recent experiments, we showed that neuronal nitric oxide synthase (nNOS) is localized in LA spines (**Figure 5(a)**). Further, PPF was occluded by LTP at thalamic inputs to the LA, and bath application of either a NOS inhibitor or a membrane impermeable scavenger of NO impaired LTP at thalamo–LA synapses (**Figures 5(b)–5(d)**). Finally, intra-amygdala infusion of both compounds impaired fear memory consolidation; that is, LTM was impaired, while STM was intact (Schafe et al., 2005; **Figures 5(e)** and **5(f)**). While additional studies will be necessary, these are among the first to define a role for NO signaling in fear memory formation in the LA.

4.11.5 Is the Lateral Amygdala an Essential Locus of Fear Memory Storage?

In the previous sections, we have discussed the findings of lesion, neurophysiological, pharmacological, and biochemical studies. Collectively, these findings suggest that fear memory formation and consolidation involve alterations in synaptic transmission at LA synapses via an LTP-like mechanism. But is the LA really a site of fear memory storage? This question has proven extremely difficult to address experimentally. Recent findings, however, have provided a fresh look at this question and provide a new strategy for revealing the location of the fear engram.

4.11.5.1 An Alternative View of the Amygdala and Fear Conditioning

While an ever-increasing number of studies using lesion, neurophysiological, and most recently, pharmacological/biochemical techniques have suggested that the LA is an essential site of fear memory formation and storage, an alternative view has offered alternative interpretations of each of the aforementioned findings (Cahill et al., 1999). One obvious way to ask whether a brain structure might be involved in permanent storage of a memory is to lesion that structure at different time points after training (e.g., 1 day later, 1 week later, 1 year later). If memory is impaired as the result of the lesion at each of these time points it suggests that some type of permanent storage has occurred there, *provided that* the area of interest is not involved in some way in the expression of that type of learning. In support of the memory storage hypothesis, lesions of the amygdala impair fear learning even if given years after the initial training event (Gale et al., 2004). The conclusions drawn from these studies, however, have long been called into question due to the fact that the LA and its connections with the CE are also critical for fear expression (Cahill et al., 1999, 2001). Accordingly, lesion studies alone cannot unambiguously distinguish a role for the LA in fear acquisition from that of fear expression.

Neurophysiological and pharmacological studies supporting a role for the amygdala in memory storage have also been called into question. It has been pointed out, for example, that the LA is not unique but rather one of many regions of the wider fear network to exhibit training-related neurophysiological changes during and after fear conditioning.

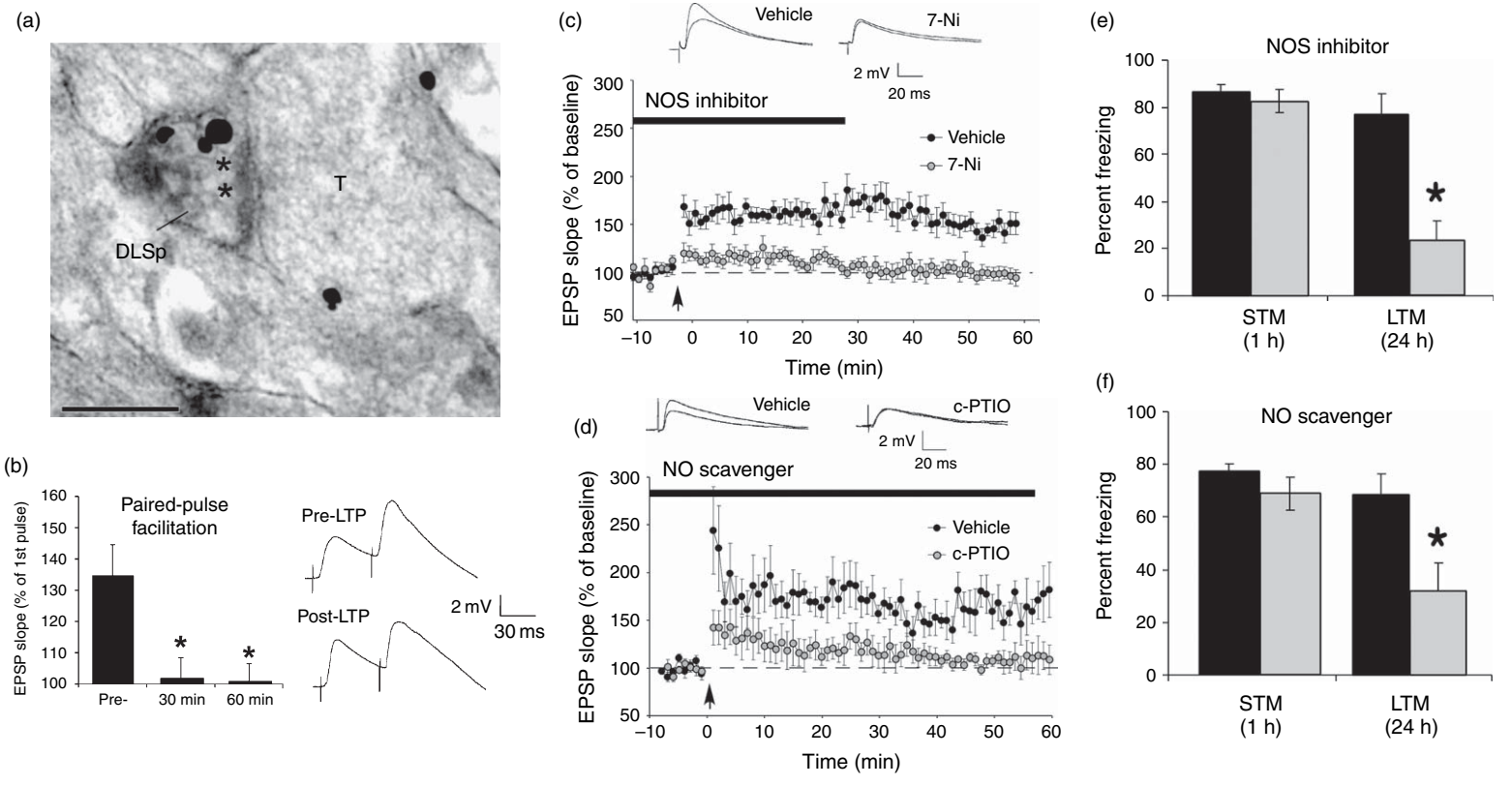


Figure 5 Nitric oxide (NO) signaling and fear memory formation. (a) Localization of neuronal NOS (nNOS) in LA spines. A terminal (T) forms an asymmetric synapse (asterisks) onto a nNOS- and αCaMKII-immunoreactivity dually labeled spine (DLSp) in the LA. The immunogold particles represent labeling of αCaMKII, while the peroxidase represents labeling of nNOS. (b) Paired-pulse facilitation at thalamic inputs to the LA before (Pre-) and 30 and 60 min after LTP induction. Each cell was given two stimulations that were spaced 50 ms apart, and the second pulse was expressed as a percentage of the first pulse. Representative traces can be seen at the right. (c–d) LTP at thalamic inputs to the LA is impaired by bath application of either (c) an inhibitor of nNOS (7-nitroindazole, 7-Ni) or (d) a membrane impermeable scavenger of NO (carboxy-PTIO, c-PTIO). (e–f) Fear memory consolidation is also impaired by 7-Ni and carboxy-PTIO. In both cases, STM is intact, while LTM is impaired. All figures adapted, with permission, from Schafe GE, Bauer EP, Rosis S, Farb CR, Rodrigues SM, and LeDoux JE (2005) Memory consolidation of Pavlovian fear conditioning requires nitric oxide signaling in the lateral amygdala. *Eur. J. Neurosci.* 22: 201–211. Copyright by Blackwell Publishing.

Auditory fear conditioning, for example, induces associative alterations in the activity of neurons not only in the LA, but also in the auditory cortex (Bakin and Weinberger, 1990; Edeline and Weinberger, 1993) and the auditory thalamus (Gabriel et al., 1975; Weinberger, 1993). Consequently, it has been difficult to argue with certainty that training-induced changes in the LA are of local origin rather than the result of a passive reflection of plasticity in these upstream regions (Cahill et al., 1999). This has been especially true of training-induced changes in the auditory thalamus (MGm/PIN), which are of short enough latency (e.g., 5–7 ms) to account for the shortest observed changes in the LA (e.g., 12–15 ms). Finally, rather than indicating that the LA is a site of storage of fear memories, it has been suggested that memory deficits observed after pharmacological manipulations may instead indicate that the LA is essential for triggering or modulating the strength of plasticity and memory storage in other regions of the wider fear network (Cahill et al., 1999). In support of this notion, recent studies have suggested that the acquisition of training-induced plasticity in the auditory thalamus is dependent on the amygdala (Maren et al., 2001; Poremba and Gabriel, 2001). Accordingly, one may argue that pharmacological manipulations of the LA that are aimed at disrupting synaptic plasticity may be doing so by modulating the strength of plasticity in regions of the wider fear network, such as the MGm/PIN, which is in turn reflected back to the LA.

4.11.5.2 A New Strategy for Tracking the Fear Engram

Recognizing that neurophysiological or pharmacological methods alone are unlikely to be able to answer the question of where fear memories are stored, a recent study from our lab has taken a new approach. In our studies, we combined simultaneous neurophysiological recordings from both LA and MGm/PIN with intra-LA infusion of the MAP kinase kinase (MEK) inhibitor U0126 (**Figure 6**). We reasoned that if local synaptic plasticity in the LA was necessary for fear memory formation and storage via an ERK/MAPK dependent mechanism, then local inhibition of MEK in the LA should selectively impair training-induced plasticity in the LA rather than the MGm/PIN. The findings showed that MEK inhibition in the LA impaired both memory consolidation of auditory fear conditioning (**Figures 6(a)** and **6(b)**), and also the consolidation of training-induced synaptic plasticity in the LA (**Figures 6(c)–6(e)**). That is, acquisition and short-term retention of fear learning and cellular changes were intact, whereas long-term retention was impaired. Intra-LA infusion of the MEK inhibitor had no effect, however, on training-induced neurophysiological changes in the MGm/PIN (**Figures 6(f)–6(h)**).

Together, these findings strongly indicate that ERK/MAPK-mediated signaling in the LA is required for memory consolidation of fear conditioning as well as for consolidation of conditioning-induced synaptic plasticity in the LA. Further, our findings rule out the possibility that MEK inhibition in the LA may be impairing to fear memory formation by influencing synaptic plasticity (either short- or long-term) in the MGm/PIN. Further, these findings suggest that conditioned enhancement of CS responses in the auditory thalamus is not sufficient to support memory storage of fear conditioning, whereas ERK-dependent conditioned enhancement of CS responses in LA is necessary, at least in part, for memory storage. Importantly, it should be emphasized that these recent findings do not diminish the potential importance of the auditory thalamus and other structures in the encoding of different components of the whole fear memory trace, nor do they suggest, as we will see later, that the amygdala plays no role in modulating certain types of memory storage. However, these recent findings provide strong support to the notion that long-term storage of an emotional memory trace relies, in part, on local synaptic plasticity in the LA.

4.11.6 Distributed Versus Local Plasticity in the Amygdala

While the LA clearly appears to be a critical locus of synaptic plasticity, fear memory acquisition, and storage, it should not be assumed that LA synapses are the only critical synapses in the amygdala which undergo changes that are essential to fear memory formation and/or consolidation. Several recent studies, for example, have suggested that a distributed, rather than localized, network of plasticity in the amygdala underlies fear learning (Medina et al., 2002; Paré et al., 2004).

4.11.6.1 Distributed Plasticity within the LA

The distributed view of plasticity underlying fear learning begins in the LA itself, where plasticity at two sets of synapses has been linked to fear learning,

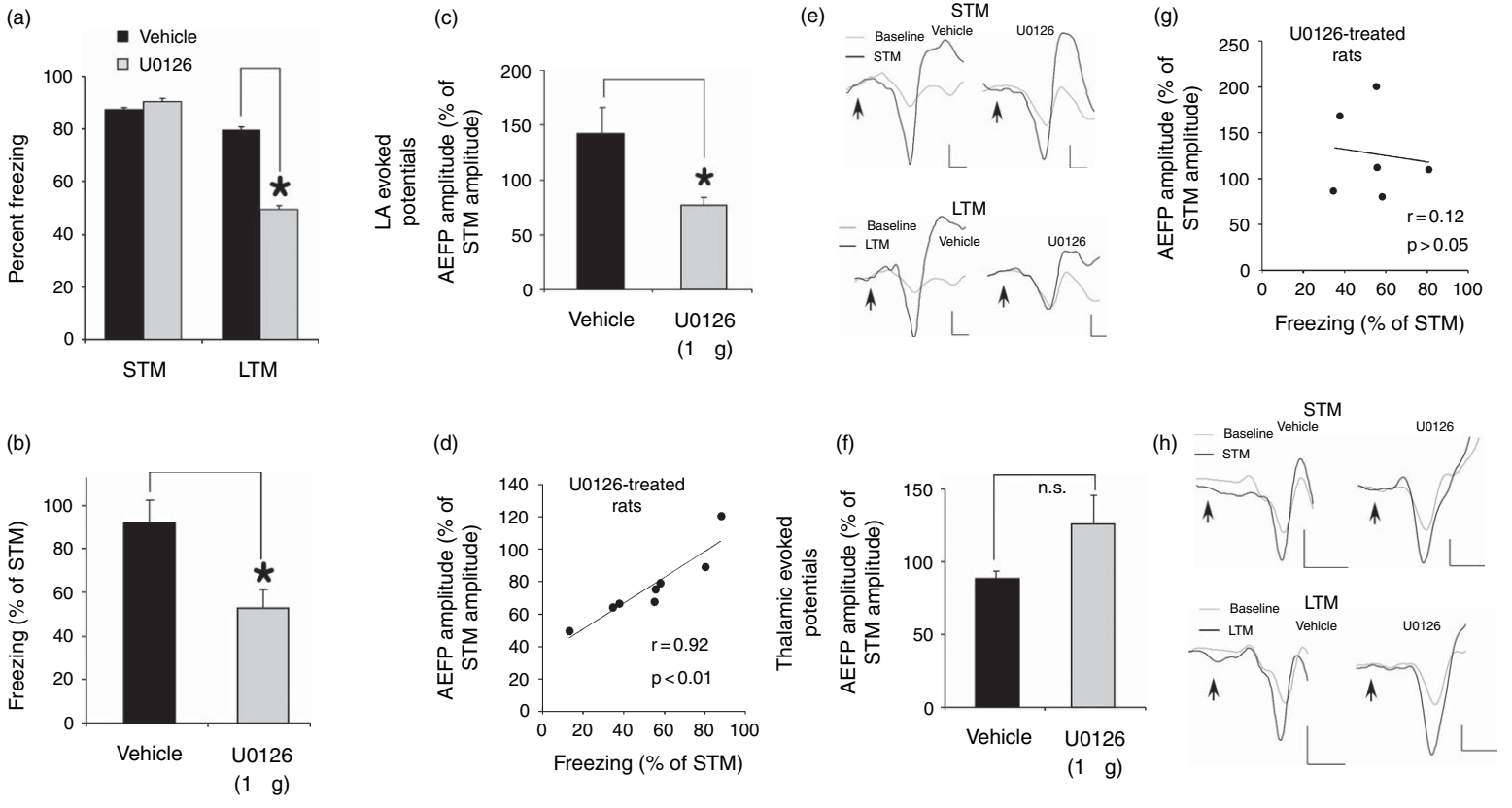


Figure 6 Local synaptic plasticity is required for fear memory consolidation in the lateral amygdala (LA). (a) Impaired fear memory in rats receiving intra-LA infusions of U0126 while neurophysiological recordings were made from both LA and the medial geniculate body and the posterior intralaminal nucleus (MGm/PIN). Mean (± SEM) percent freezing expressed during STM and LTM tests in rats treated with 50% dimethyl sulfoxide vehicle (black bars) or 1 μg U0126 (ray bars). (b) Mean (± SEM) retention of freezing in both groups; freezing during LTM is expressed as a percentage of that observed during the STM test. (c) Mean (± SEM) changes in the amplitude of LA auditory-evoked field potentials (AEFPs) during the LTM test, expressed as a percentage of that obtained during the STM test. (d) Correlation between freezing scores and LA AEFP amplitudes in U0126-treated rats (each expressed as a percentage of STM). (e) Representative AEFPs in the LA for each group (vehicle, U0126) during baseline, STM, and LTM tests. Scale = 20 μs, 5 ms. (f) Mean (± SEM) changes in the amplitude of MGm/PIN AEFPs during the LTM test, expressed as a percentage of that obtained during the STM test. (g) Correlation between freezing scores and LA AEFP amplitudes in U0126-treated rats (each expressed as a percentage of STM). (h) Representative AEFPs in the MGm/PIN for each group (vehicle, U0126) during baseline, STM, and LTM tests. Scale = 20 μs, 5 ms. Adapted from Schafe GE, Doyère V, LeDoux JE (2005b) Tracking the fear engram: The lateral amygdala is an essential locus of fear memory storage. *J. Neurosci.* 25: 10010–10015, with permission from the Society for Neuroscience.

and in unique ways. While most studies that have documented training-induced alterations in synaptic plasticity have focused on cells in the LAd (Quirk et al., 1995, 1997; Rogan et al., 1997; Maren, 2000; Blair et al., 2003), a relatively recent study has documented plastic changes in two populations of cells in the LA (Repa et al., 2001; **Figure 7**). The first is the traditionally studied dorsal population in the LAd that shows enhanced firing to the CS in the initial stages of training and testing and is sensitive to fear extinction. These so-called 'transiently plastic cells' exhibit short-latency changes (within 10–15 ms after tone onset) that are consistent with the involvement of rapid, monosynaptic thalamic input (**Figure 7(b)**). The second population of cells occupies a more ventral position in the LA. In contrast to the transiently plastic cells, these more ventral cells exhibit enhanced firing to the CS throughout training and testing and do not appear to be sensitive to extinction (**Figure 7(c)**). Further, these 'long-term plastic cells' exhibit longer latencies (within 30–40 ms after tone onset), indicative of a polysynaptic pathway. Thus, it has been hypothesized that a network of neurons within the LA is responsible for triggering and storing fear memories (Repa et al., 2001; Medina et al., 2002).

Interestingly, the cells that express activated ERK/MAPK after fear conditioning occupy a more ventral position in the LA, in the same anatomical location of cells that exhibit long-term plasticity during and after fear conditioning (Schafe et al., 2000; Repa et al., 2001; **Figure 7(d)**). In fact, very little activated ERK is observed in the dorsal region of the LA, the site of the majority of CS-US convergence and of cells that exhibit rapid, and transient, plastic changes during fear conditioning (Romanski et al., 1993; Repa et al., 2001). This pattern of findings is consistent with the hypothesis that fear conditioning induces long-term plastic change and memory formation in a ventral population of cells in the LA via the ERK/MAPK signaling cascade. It remains unknown whether this involves a rapid 'transfer' of plasticity between dorsal and ventral cells in the LA during fear conditioning, or an independent, parallel process.

4.11.6.2 Distributed Plasticity within Amygdala Nuclei

Recently, interest has also grown in the idea that distributed plasticity *between* amygdala nuclei may be critical for fear learning. This has been sparked, in part, by a recent study showing that the central nucleus of the amygdala may also be an important locus of fear memory acquisition and consolidation (Wilensky et al., 2006). In that study, functional inactivation restricted to *either* the LA or the CE impaired acquisition of auditory fear conditioning. Further, infusion of the protein synthesis inhibitor anisomycin into the CE impaired fear memory consolidation; that is, rats had intact STM but impaired LTM (Wilensky et al., 2006). These findings suggest that the CE plays an important role not only in fear expression, as has been previously thought, but also in the acquisition and consolidation of fear learning.

How might the CE participate in fear memory acquisition and consolidation? Since the CE, and particularly the medial division of the CE (CEm), also appears to be a recipient of somatosensory (Bernard and Besson, 1990; Jasmin et al., 1997) and possibly also auditory (LeDoux et al., 1987; Turner and Herkenham, 1991; Frankland et al., 1998; Linke et al., 2000) information, one possibility is that the CE encodes in parallel the same type of association that is encoded in the LA. In support of this possibility, a recent study showed that high-frequency stimulation of the auditory thalamus induces an NMDAR-dependent LTP in CEm neurons (Samson and Paré, 2005). If the CE were encoding memory in parallel to the LA, however, this would suggest that the CE should readily be capable of mediating fear learning when the LA is compromised, a finding which is not supported by the literature. Another possibility is that plasticity in the LA and the CE proceeds in a serial manner, such that plasticity and memory formation in the CE depends on plasticity in the LA. This view has been advocated in a recent model that proposes that plasticity in the LA enables CEm neurons to encode plasticity that is essential for fear conditioning, resulting in distributed plasticity and memory formation throughout the amygdala (Paré et al., 2004). The mechanism by which this distributed plasticity between the LA and the CE occurs is at present unknown, but likely involves projections from the LA to CEm neurons via the nearby intercalated cell masses which lie between the LA and the CE (Paré and Smith, 1993; Royer et al., 1999). Additional experiments employing single-unit recording techniques in both the LA and the CEm will be required to determine how these two regions influence one another during fear conditioning.

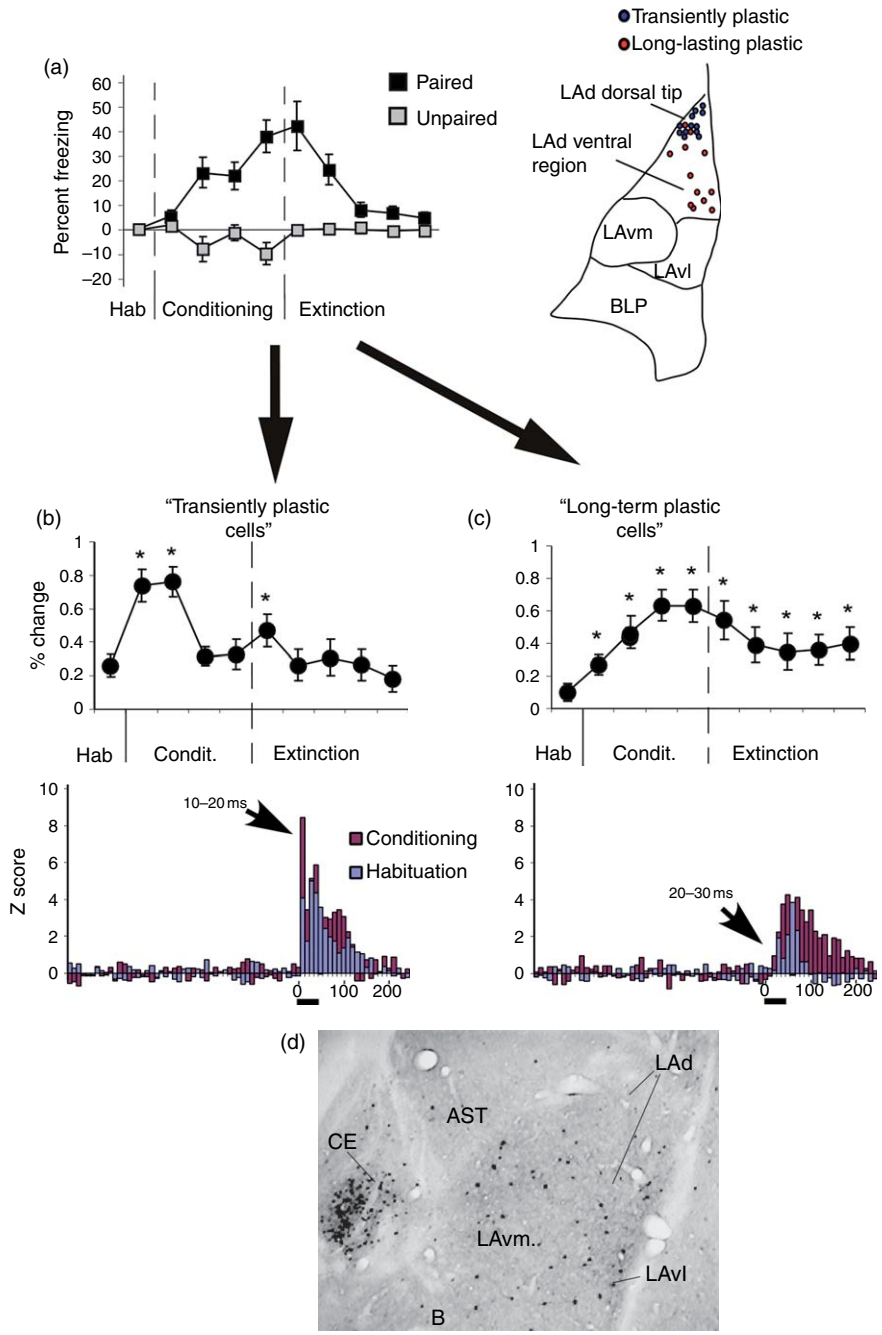


Figure 7 Distributed plasticity in the LA during fear conditioning. Pairing of CS and US during fear conditioning leads to changes in fear behavior (a) and also to changes in the responsiveness of single LA cells to auditory stimuli. During fear conditioning, there are two populations of cells that undergo plastic change. (b) 'Transiently plastic cells' are generally short latency and show enhanced firing shortly after training and during the initial phases of extinction, but not at other times. (c) 'Long-term plastic cells' are generally longer latency and show enhanced firing throughout training and extinction. INSET: 'Transiently plastic cells' are generally found in the dorsal tip of the LAd, where they may serve to trigger the initial stages of memory formation. 'Long-term plastic cells,' on the other hand, are found in the ventral regions of the LAd and may be important for long-term, extinction-resistant memory storage. (d) Location of cells expressing phospho-ERK following fear conditioning. Note the location of ERK-positive cells relative to 'long-term plastic cells.' CE, central nucleus of the amygdala; regions of the LA: LAd, dorsal; LAvm, ventromedial; LAvl, ventrolateral; AST, amygdala-striatal transition zone; B, basal nucleus of amygdala. (a–c) Adapted from Repa JC, Muller J, Apergis J, Desrochers TM, Zhou Y, and LeDoux JE (2001) Two different lateral amygdala cell populations contribute to the initiation and storage of memory. *Nat. Neurosci.* 4: 724–731, with permission from Nature Publishing Group. (d) Adapted from Schafe GE, Atkins CM, Swank MW, Bauer EP, Sweatt JD, and LeDoux JE (2000) Activation of ERK/MAP kinase in the amygdala is required for memory consolidation of Pavlovian fear conditioning. *J. Neurosci.* 20: 8177–8187, with permission from the Society for Neuroscience.

4.11.7 Summary: A Model of Fear Memory Acquisition and Consolidation in the Amygdala

In summary, the converging evidence from a number of recent studies supports a model of fear conditioning in which CS and US inputs converge onto individual LA neurons and initiate changes in synaptic function and/or structure (Blair et al., 2001; **Figure 8**). The convergence of CS and US inputs onto LA principal cells during training leads to Ca^{2+} influx through both NMDARs (Miserendino et al., 1990; Kim et al., 1991; Campeau et al., 1992; Walker and Davis, 2000; Rodrigues et al., 2001) and also L-VGCCs (Bauer et al., 2002). The NMDAR-mediated increase in intracellular Ca^{2+}, together with mGluR5 (Rodrigues et al., 2002), leads to the activation of a variety of local protein kinases at the postsynaptic density (PSD), including αCaMKII (Rodrigues et al., 2004a) and likely PKC, that promote STM formation by targeting and modulating the conductance and trafficking of glutamate receptors at LA synapses (Barria et al., 1997; Benke et al., 1998; Rumpel et al., 2005). The combined entry of Ca^{2+} through both NMDARs and L-VGCCs, together with signaling via the BDNF-TrkB pathway, however, may promote the activation of PKA and ERK/MAPK (Schafe et al., 2000; Schafe and LeDoux, 2000). These kinases, and particularly ERK/MAPK, appear to be exclusively involved in the formation of LTM, possibly via translocation to the cell nucleus and activation of transcription factors such as CREB (Josselyn et al., 2001). The activation of CREB by ERK/MAPK promotes CRE-mediated gene transcription (Bailey et al., 1999; Ressler et al., 2002) and the synthesis of new proteins (Schafe and LeDoux, 2000), which likely promotes LTM formation by leading to alterations in the structure of LA synapses (Lamprecht et al., 2002; Ressler et al., 2002; Radley et al., 2006). Intracellular signaling in the postsynaptic neuron, alone, however, does not appear to be sufficient for fear memory formation (McKernan and Shinnick-Gallagher, 1997b; Huang and Kandel, 1998; Tsvetkov et al., 2002). Modifications in presynaptic signaling, possibly engaged by retrograde signaling in the LA via NO and its downstream targets, also appears to be critical

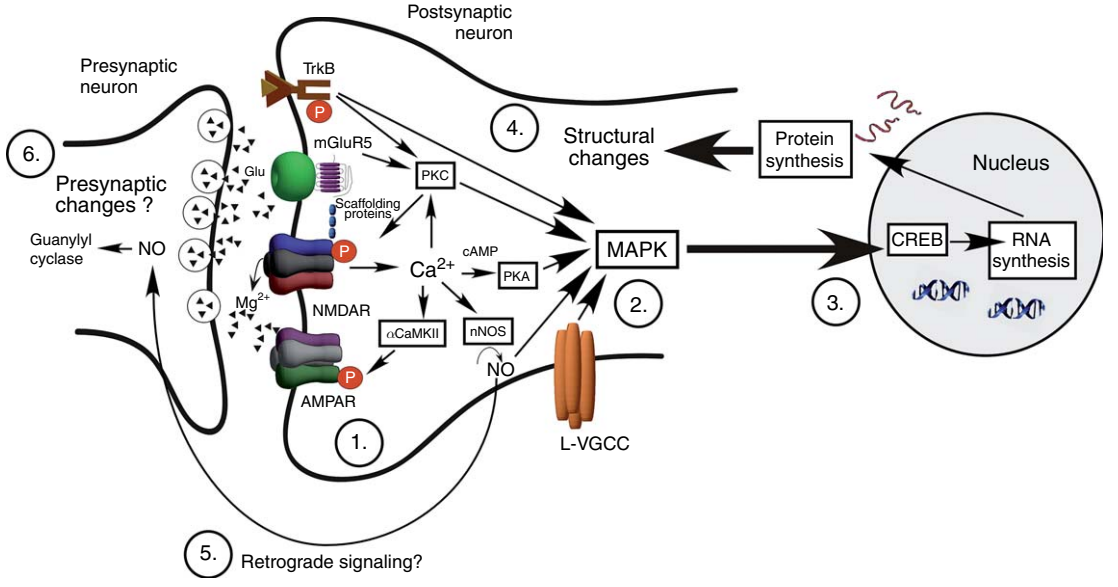


Figure 8 A model of fear memory consolidation in the amygdala. See text for details. (1) Acquisition and STM formation of fear conditioning requires events at the postsynaptic density, including activation of NMDARs, mGluR5, αCaMKII, and possibly PKC. Both αCaMKII and PKC may contribute to STM by influencing the conductance of NMDARs and AMPARs. (2) LTM formation of fear conditioning requires the activation of TrkB receptors, L-type VGCCs, and the cAMP-PKA signaling pathway. These pathways are thought to converge on ERK, which is thought to promote LTM and synaptic plasticity by translocating to the nucleus to influence gene expression. (3) CREB and CRE-mediated transcription are both required for LTM of fear conditioning. (4) The translation of CRE-mediated genes into proteins may lead to structural changes at LA spines that contribute to the permanence of LTM formation. (5) The activation of nNOS in LA neurons may promote retrograde signaling by NO and structural and/or functional changes on the presynaptic side of the synapse (6).

(Schafe et al., 2005). Finally, recent findings emphasizing a distributed network of plasticity in the amygdala have suggested that attention to synaptic plasticity at one synapse, or even one amygdala nucleus, will not be sufficient for a full understanding of how fear memories are acquired or consolidated (Repa et al., 2001; Paré et al., 2004). Accordingly, future experiments will need to consider not only how intracellular signaling mechanisms contribute to fear learning, but also how plasticity across amygdala synapses might be involved in fear memory formation.

4.11.8 Beyond 'Simple' Fear Conditioning

While great strides have been made in identifying the neural and molecular mechanisms that underlie auditory fear conditioning, we have also begun to learn a great deal about more complex aspects of fear learning. In this section, we will explore what is known regarding contextual fear learning, fear extinction, 'reconsolidation' of fear, instrumental fear learning, and memory modulation by the brain's fear system.

4.11.8.1 Contextual Fear Conditioning

In a typical auditory fear conditioning experiment, the animal not only learns to fear the tone that is paired with the foot shock, but also the context in which conditioning occurs. Contextual fear may also be induced by the presentation of foot shocks alone within a novel environment. In the laboratory, fear to the context is measured by returning the rat to the conditioning chamber on the test day and measuring freezing behavior (Blanchard et al., 1969; Fanselow, 1980).

In comparison to auditory fear conditioning, much less is known about the neural system underlying contextual fear. Much of the work examining the neuroanatomical substrates of contextual fear has relied exclusively on lesion methods, and, as in auditory fear conditioning the amygdala appears to play an essential role. For example, lesions of the amygdala, including the LA and basal nucleus, have been shown to disrupt both acquisition and expression of contextual fear conditioning (Phillips and LeDoux, 1992; Kim et al., 1993; Maren, 1998), as has reversible functional inactivation targeted to the LA (Muller et al., 1997). Contextual fear conditioning is also impaired by infusion of antagonists to NMDARs, mGluR5, and CaMKII into the LA, as well as inhibitors of PKA/PKC, RNA, and protein synthesis (Kim et al., 1991; Bailey et al., 1999; Goosens et al., 2000; Rodrigues et al., 2001, 2002, 2004a). Further, a recent study showed that memory consolidation for contextual fear is impaired by infusion of antisense oligonucleotides directed against EGR-1 (Malkani et al., 2004). Collectively, these findings suggest that essential aspects of the memory are encoded and stored in the amygdala via alterations in some of the same intracellular signaling mechanisms that underlie acquisition and consolidation of auditory fear conditioning. At this time, however, there are few data that allow us to distinguish between the involvement of different amygdala subnuclei in contextual fear, although recent lesion evidence suggests that the LA and anterior basal nuclei are critical, but not the posterior basal nucleus (Goosens and Maren, 2001). The CE is, of course, essential for the expression of contextual fear, as it is for auditory fear conditioning (Goosens and Maren, 2001). It remains unknown, however, whether the CE is also required for the acquisition and/or consolidation of contextual fear, or whether distributed plasticity in the LA underlies contextual fear learning.

The hippocampus has also been implicated in contextual fear conditioning, although its exact role has been difficult to define. A number of studies have shown that electrolytic and neurotoxic lesions of the hippocampus disrupt contextual, but not auditory, fear conditioning (Kim and Fanselow, 1992; Phillips and LeDoux, 1992; Kim et al., 1993; Maren et al., 1997). Posttraining lesions appear to be the most effective; pretraining lesions of the hippocampus have occasionally been shown to be without effect (Maren et al., 1997). This is presumably because the animal uses a nonhippocampal strategy to acquire fear to the contextual cues of the environment in the absence of an intact hippocampus during training. Posttraining hippocampal lesions, however, are only effective at impairing contextual fear if given shortly after training. If rats are given hippocampal lesions 28 days after training, there is no memory impairment (Kim and Fanselow, 1992). This 'retrograde gradient' of recall suggests that hippocampal-dependent memories are gradually transferred, over time, to other regions of the brain for permanent storage, an idea that is consistent with the findings of hippocampal-dependent episodic memory research in humans (Milner et al., 1998). The exact mechanism whereby these 'remote' contextual fear memories are consolidated remains unknown, but is thought to involve LTP-like changes in signaling between the

hippocampus and regions of the cortex that make up the individual elements of the contextual representation (Frankland et al., 2001).

What role does the hippocampus play in contextual fear? One prominent view is that it is necessary for forming a representation of the context in which conditioning occurs and for providing the amygdala with that information during training and CS-US integration (Phillips and LeDoux, 1992; Young et al., 1994; Frankland et al., 1998). In support of this view, the hippocampal formation has been shown to project to the basal nucleus of the amygdala (Canteras and Swanson, 1992). This pathway has been shown to exhibit LTP (Maren and Fanselow, 1995), thus providing a potential neuroanatomical substrate through which contextual fear associations can be formed (Maren and Fanselow, 1995). Further, it has recently been shown that intrahippocampal infusions of the protein synthesis inhibitor anisomycin impair the ability of the hippocampus to form a contextual representation, but not the ability of the animal to form a context-shock association (Barrientos et al., 2002). In these experiments, the 'immediate shock deficit' paradigm was used to tease apart the contribution of the hippocampus to learning about a context and learning to fear one. Normally, immediate shock (i.e., that which is given soon after introduction to the conditioning chamber) is not sufficient to support contextual fear conditioning, presumably because it takes time for the hippocampus to form a representation of the context in which the animal finds itself. However, if the animal is *preexposed* to the conditioning chamber briefly on the day before training, it can subsequently acquire contextual fear following immediate shock, presumably because the animal now enters the training situation with a contextual representation already intact (Fanselow, 1980). In the Barrientos et al. study (2002), rats were given an infusion of anisomycin or vehicle into the dorsal hippocampus immediately after exposure to a novel context on the day before they received immediate shock, or immediately after receiving immediate shock on the day after they received preexposure. The findings showed that intrahippocampal anisomycin resulted in impaired contextual learning only in the first group (Barrientos et al., 2002). This important finding suggests that the protein synthesis in the hippocampus is necessary for learning about contexts, but not for contextual fear conditioning. A similar finding has recently been reported by Frankland and colleagues using manipulations of NMDARs, CaMKII, and CREB in the hippocampus (Frankland et al., 2004).

It is clear, however, that the hippocampus undergoes plastic changes during fear conditioning, some of which may be necessary for memory formation of contextual fear. For example, intrahippocampal infusion of the NMDAR antagonist APV impairs contextual fear conditioning (Stiedl et al., 2000; Young and Wang, 2004), and contextual, but not auditory, fear conditioning is impaired in mice that lack the NR1 subunit of the NMDA receptor exclusively in area CA1 of the hippocampus (Rampon and Tsien, 2000). Further, fear conditioning leads to increases in the activation of αCaMKII, PKC, ERK/MAPK, and CRE-mediated gene expression in the hippocampus (Atkins et al., 1998; Impey et al., 1998b; Hall et al., 2000). These findings add support to the notion that NMDAR-dependent plastic changes in the hippocampus, in addition to the amygdala, are required for contextual fear conditioning. However, it should be emphasized that the exact contribution of this NMDAR-mediated signaling to contextual fear conditioning remains unclear. For example, most of these studies cannot distinguish between a role for NMDAR-mediated plasticity in formation of contextual representations as opposed to a role in fear memory acquisition and storage. Further, regulation of intracellular signaling cascades in the hippocampus by fear conditioning, while potentially indicative of some type of memory storage, does not necessarily indicate that these changes are related to the acquisition *fear* memories. They may be related to declarative or explicit memories of the training experience that are acquired at the same time as fearful memories (LeDoux, 2000). Indeed, a number of studies have shown that hippocampal cells undergo plastic changes during and after fear conditioning (Doyère et al., 1995; Moita et al., 2003), including auditory fear conditioning which is spared following hippocampal lesions (Kim and Fanselow, 1992). Clearly, more research is needed before a convincing picture of the role of the hippocampus in contextual fear conditioning emerges.

4.11.8.2 Fear Extinction

Extinction is a process whereby repeated presentations of the CS in the absence of the US leads to a weakening of the expression of conditioning responding. While extinction of conditioned fear has been well documented in the behavioral literature, until recently we learned comparatively little about its neurobiological substrates. Work in a

number of laboratories has recently implicated a number of structures, including the prefrontal cortex, amygdala, and hippocampus.

The medial prefrontal cortex (mPFC), and in particular the ventral mPFC, appears to play an important role in fear extinction. Early studies, for example, showed that selective lesions of the ventral mPFC retard the extinction of fear to an auditory CS while having no effect on initial fear acquisition (Morgan et al., 1993; Morgan and LeDoux, 1995). Further, neurons in the mPFC alter their response properties as the result of extinction (Garcia et al., 1999; Herry et al., 1999). Interestingly, studies by Quirk and colleagues suggest that the mPFC may not be necessary for fear extinction *per se,* but rather for the long-term recall of extinguished fear. For example, rats with mPFC lesions are able to extinguish within a session, but show impaired extinction between sessions (Quirk et al., 2000). Further, neurons in the mPFC fire strongly to a tone CS after behavioral extinction has occurred, and artificial stimulation of the mPFC that resembles responding in an extinguished rat is sufficient to inhibit behavioral expression of fear in nonextinguished rats (Milad and Quirk, 2002). Thus, it appears clear that the mPFC plays an essential role in long-term retention and/or expression of fear extinction. The question of whether the mPFC is a 'site of storage' of extinction or rather simply a region that is necessary for the long-term expression of extinguished memories has only begun to be explored. Recent studies, however, have shown that extinction training regulates the expression of the IEG cFos in regions of the mPFC (Santini et al., 2004). Further, intra-mPFC infusion of inhibitors to MEK or protein synthesis impairs long-term recall of fear extinction (Santini et al., 2004; Hugues et al., 2006), suggesting that essential aspects of the plasticity underlying extinction memory are localized in the mPFC.

The amygdala has also been shown to be an essential site of plasticity underlying fear extinction. Infusions of NMDAR antagonists or inhibitors of ERK/MAPK into the amygdala have been shown to impair fear extinction (Falls et al., 1992; Lu et al., 2001; Davis, 2002; Herry et al., 2006). Conversely, both systemic and intra-amygdala infusions of partial agonists of the NMDA receptor facilitate fear extinction (Walker et al., 2002). More recently, Ressler and colleagues showed that BDNF signaling in the amygdala was critical to the consolidation of fear extinction (Chhatwal et al., 2006). They showed, for example, that fear conditioning leads to an increase in BDNF expression in the LA and basal amygdala. Further, infusion of a viral vector encoding a dominant negative TrkB receptor into the amygdala impaired between-session, but not within-session, retention of fear extinction. These experiments suggest that some type of activity-dependent synaptic plasticity must take place in the amygdala during extinction learning, as it does during initial learning. After the memory of extinction is formed, the amygdala may then signal the mPFC to inhibit ongoing fear responses. Indeed, McDonald and colleagues have shown that the mPFC projects to GABAergic (GABA: gamma-aminobutyric acid) intercalated cells that are situated between the lateral and basal amygdala and the CE (McDonald et al., 1996), which may be important for regulating fear responses (Paré and Smith, 1993; Quirk and Gehlert, 2003; Quirk et al., 2003; Paré et al., 2004). In agreement with this hypothesis, a recent study has confirmed that stimulation of the mPFC neurons blunts the activity of CE neurons that are critical for the expression of fear responses (Quirk et al., 2003). Additional experiments will be necessary to define the exact contribution of connections between the mPFC and the amygdala in extinction processes, as well as the detailed biochemical mechanisms responsible for promoting fear extinction.

One of the more interesting facts about memories that have undergone extinction is that they are context specific. That is, an extinguished memory remains extinguished only in the context in which extinction has taken place, and responding returns or is subject to 'renewal' in a different context (Bouton and Bolles, 1979; Bouton and Ricker, 1994). This fact, along with the finding that fully extinguished memories are capable of 'reinstating' upon presentation of the US (Rescorla and Heth, 1975), has led to the long-held view that extinction does not result in the erasure of the original memory trace but is rather a new kind of learning that serves to inhibit expression of the old memory (Pavlov, 1927). Not surprisingly, recent studies have indicated that the hippocampus plays an important role in the contextual modulation of fear extinction. Maren and colleagues, for example, have shown that training-induced neurophysiological responses in the LA readily extinguish within a fear extinction session, but that this neural representation of extinction, like the behavior itself, is specific to the context in which extinction has taken place (Hobin et al., 2003). Further, functional inactivation of the hippocampus using the $GABA_A$ agonist muscimol can

impair the context-specific expression of fear extinction (Corcoran and Maren, 2001). While it remains unclear how the hippocampus might inhibit the expression of LA spike firing and fear behavior in a context-specific manner, it has been proposed that projections from the hippocampus to the mPFC may be critical (Hobin et al., 2003).

4.11.8.3 Retrieval and 'Reconsolidation' of Fear Memories

Fear extinction is not the only way to turn a fear memory off. Another, perhaps more clinically efficacious way, is to interfere with that fear memory's *reconsolidation*. The idea that memory undergoes a second phase of consolidation, or 'reconsolidation,' upon retrieval has been the subject of speculation for decades (Sara, 2000). Early studies showed that amnesic manipulations at or around the time of memory retrieval, rather than at the time of initial learning, resulted in loss of the memory on subsequent recall tests (Misanin et al., 1968; Lewis et al., 1972). These early findings suggested that the retrieval process could render a memory susceptible to disruption in a manner very similar to a newly formed memory.

Interest in the reconsolidation process has been rekindled in recent years, due in part to the progress that has been made in identifying the cellular and molecular mechanisms underlying long-term synaptic plasticity and the initial phases of memory consolidation (Milner et al., 1998). Accordingly, this has provided researchers with a set of tools and learning paradigms with which to study the reconsolidation process. Several years ago, for example, Nader and colleagues showed that infusion of the protein synthesis inhibitor anisomycin into the amygdala immediately after retrieval of auditory fear conditioning impaired memory recall on subsequent tests (Nader et al., 2000). This effect was clearly dependent on retrieval of the memory; that is, no memory deficit was observed if exposure to the CS was omitted. Further, the effect was observed not only when the initial recall test and drug infusion were given shortly after training (i.e., 1 day), but also if given 14 days later, suggesting that the effect could not be attributable to disruption of the late phases of protein synthesis necessary for the initial training episode. Thus, following active recall of a fear memory, that memory appears to undergo a second wave of consolidation that requires protein synthesis in the amygdala. More recent work has shown that this process does not appear to be attributable to rapid extinction of fear during the recall test, since fear memories that have failed to reconsolidate after intra-amygdala infusion of anisomycin fail to renew in a different context (Duvarci and Nader, 2004). Further, memories that fail to reconsolidate do not appear to be subject to reinstatement (Duvarci and Nader, 2004), a finding which suggests that manipulations of a fear memory at or around the time of retrieval may result in permanent impairment of the memory.

Reconsolidation does not appear to be unique to the amygdala; hippocampal-dependent contextual memories also appear to be sensitive to manipulation at the time of retrieval. In a recent study, Debiec et al. (2002) gave rats intrahippocampal infusions of anisomycin following recall of contextual fear conditioning and found that memory retrieval was impaired on subsequent tests. Interestingly, reconsolidation of contextual fear was impaired even when memory reactivation and intrahippocampal anisomycin treatment were given 45 days after the initial training session, a time when lesion studies have shown that contextual memories should no longer depend on the hippocampus (Kim and Fanselow, 1992). The initial experiments by Kim and Fanselow, however, used only a single recall test after training and hippocampal lesions; the ability of the animal to recall contextual fear on subsequent tests was not examined. Surprisingly, when Debiec et al. *reactivated* the contextual memory prior to making a lesion of the hippocampus, even as long as 45 days after training, subsequent recall was impaired (Debiec et al., 2002). Thus, hippocampal-dependent contextual memories appear to undergo both a cellular and a systems-level reconsolidation following memory retrieval. That is, recall of an older, hippocampal-independent contextual memory must return to the hippocampus during retrieval and undergo a protein synthesis–dependent process of reconsolidation to be maintained. As in most hippocampal studies, however, it remains unclear what information is being reconsolidated – the memory of the context or the contextual fear memory.

How might the reconsolidation process be accomplished at the cellular and molecular levels? Recent studies have shown that fear reconsolidation, like the initial phases of consolidation, requires both PKA and ERK/MAPK in the amygdala (Duvarci et al., 2005; Tronson et al., 2006). Further, transient overexpression of a dominant negative isoform of CREB in the forebrain at the time of memory retrieval impairs

reconsolidation of auditory and contextual fear conditioning (Kida et al., 2002). However, the reconsolidation process does not appear to be a mere recapitulation of the initial consolidation process; there have also been numerous reports of biochemical dissociations between consolidation and reconsolidation. These have included studies that have failed to find impairments in fear reconsolidation following inhibition of RNA synthesis (Parsons et al., 2006) or NO signaling (Schafe et al., 2005) in the amygdala. Further, reactivation of a contextual fear memory induces only a subset of genes in the hippocampus that are activated during the initial phases of memory consolidation (von Hertzen and Giese, 2005), and hippocampal-dependent reconsolidation of a contextual fear memory appears to be characterized by different classes of immediate early genes (Lee et al., 2004). Finally, a recent study has shown that blockade of β-adrenergic receptors in the LA impairs reconsolidation, but not consolidation, of fear conditioning (Debiec and Ledoux, 2004). Clearly, additional studies will be required for a full appreciation of how reconsolidation is accomplished at the cellular level.

4.11.8.4 Instrumental Fear Learning

In addition to its role in the rapid, reflexive learning that characterizes Pavlovian fear conditioning, the amygdala contributes to other fear-related aspects of behavior. Pavlovian fear conditioning, for example, is useful for learning to detect a dangerous object or situation, but the animal must also be able to use this information to guide ongoing behavior that is instrumental in avoiding that danger. In some experimental situations, the animal must learn to make a response (i.e., move away, press a bar, turn a wheel, etc.) that will allow it to avoid presentation of a shock or danger signal, a form of learning known as *active avoidance*. In other situations, the animal must learn *not* to respond, also known as *passive avoidance*. Both of these are examples of instrumental conditioning, and the amygdala plays a vital role in each.

Previously, we mentioned that only the LA and CE were critical for Pavlovian fear conditioning. However, we have recently begun to appreciate the significance of projections from the LA to the basal nucleus of the amygdala from studies that employ fear learning tasks that involve both classical and instrumental components (Killcross et al., 1997; Amorapanth et al., 2000). Amorapanth et al. (2000), for example, first trained rats to associate a tone with foot shock (the Pavlovian component). Next, rats learned to move from one side of a two-compartment box to the other to avoid presentation of the tone (the instrumental component), a so-called 'escape-from-fear' task. Findings showed that, while lesions of the LA impaired both types of learning, lesions of the CE impair only the Pavlovian component (i.e., the tone-shock association; **Figure 9(a)**). Conversely, lesions of the basal nucleus impaired only the instrumental component (learning to move to the second compartment; **Figure 9(b)**). Thus, different outputs of the LA appear to mediate Pavlovian and instrumental behaviors elicited by a fear-arousing stimulus (Amorapanth et al., 2000; **Figure 9(c)**). It is important to note, however, that these findings do not indicate that the basal nucleus is a site of motor control or a locus of memory storage for instrumental learning. Rather, the basal amygdala likely guides fear-related behavior and reinforcement learning via its projections to nearby striatal regions that are known to be necessary for instrumental learning and reward processes (Everitt et al., 1989, 1999; Robbins et al., 1989).

4.11.8.5 Memory Modulation by the Amygdala

Pavlovian fear conditioning is an implicit form of learning and memory. However, during most emotional experiences, including fear conditioning, explicit or declarative memories are also formed (LeDoux, 2000). These occur through the operation of the medial temporal lobe memory system involving the hippocampus and related cortical areas (Milner et al., 1998; Eichenbaum, 2000). The role of the hippocampus in the explicit memory of an emotional experience is much the same as its role in other kinds of experiences, with one important exception. During fearful or emotionally arousing experiences, the amygdala activates neuromodulatory systems in the brain and hormonal systems in the body via its projections to the hypothalamus, which can drive the hypothalamic-pituitary-adrenal (HPA) axis. Neurohormones released by these systems can, in turn, feed back to modulate the function of forebrain structures such as the hippocampus and serve to enhance the storage of the memory in these regions (McGaugh, 2000). The primary support for this model in animals comes from studies of *inhibitory avoidance learning*, a type of passive avoidance learning where the animal must learn not to enter a chamber in which it has previously received shock. In this paradigm, various pharmacological manipulations of the amygdala that affect neurotransmitter or

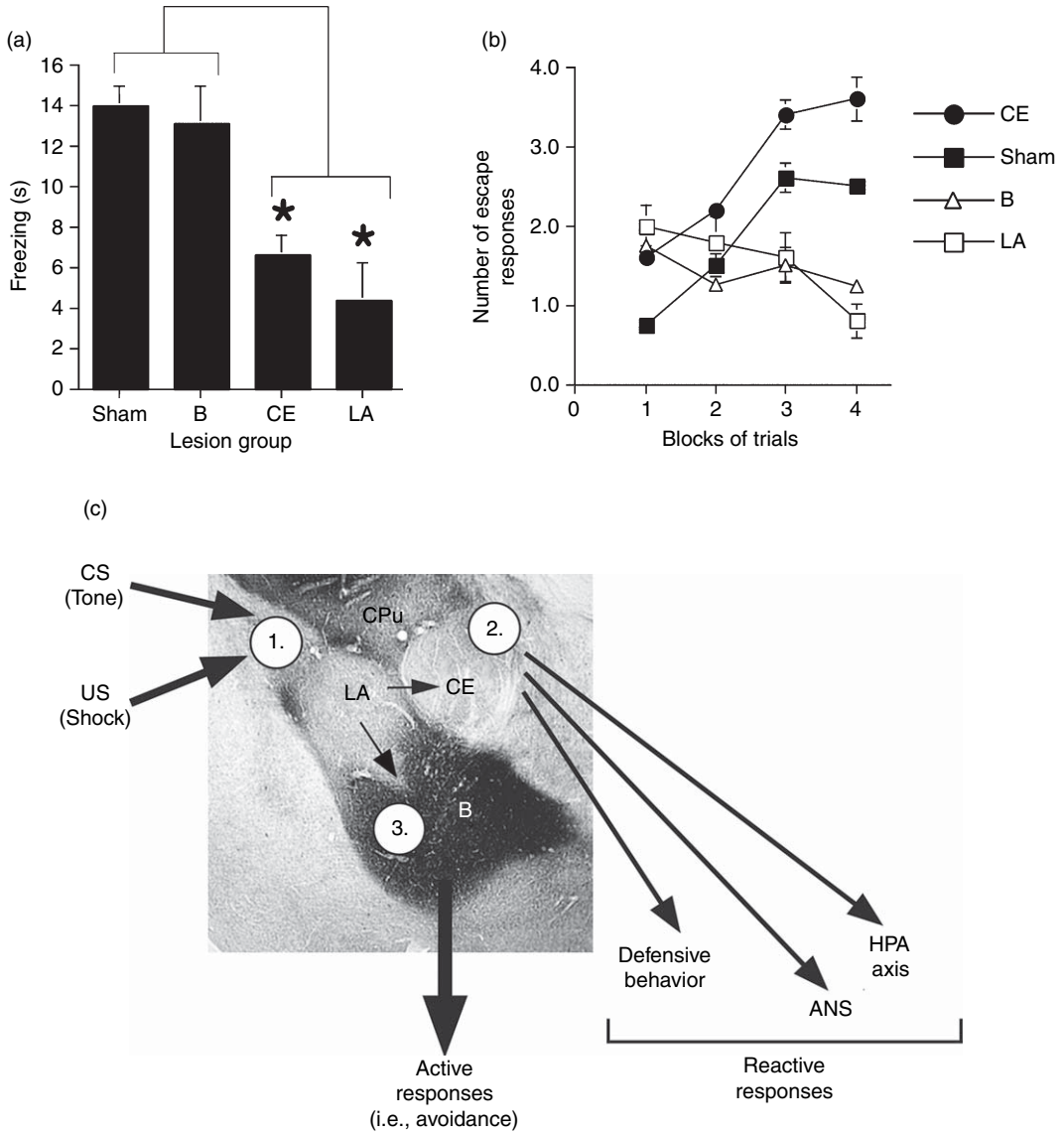


Figure 9 Active versus reactive fear. (a) Percent freezing in rats given auditory fear conditioning after receiving selective amygdala lesions. Auditory fear conditioning is impaired by lesions of the CE and LA, but spared by basal nucleus of the amygdala (B) lesions. (b) Number of escape responses across blocks of five trials during training in a one-way active avoidance task. Lesions of both LA and B impair this task, while lesions of CE do not. (c) The data are consistent with a model in which projections between LA and CE are sufficient for Pavlovian fear conditioning (reactive responses), while projections between LA and B are necessary for instrumental avoidance learning (active responses). HPA, hypothalamic-pituitary-adrenal; ANS, autonomic nervous system. Adapted from Amorapanth P, LeDoux JE, Nader K (2000) Different lateral amygdala outputs mediate reactions and actions elicited by a fear-arousing stimulus. *Nat Neurosci* 3: 74–79, with permission from Nature Publishing Group.

neurohormonal systems modulate the strength of the memory. For example, immediate posttraining blockade of adrenergic or glucocorticoid receptors in the amygdala impairs memory retention of inhibitory avoidance, while facilitation of these systems in the amygdala enhances acquisition and memory storage (McGaugh et al., 1993; McGaugh, 2000). The exact subnuclei in the amygdala that are critical for memory modulation remain unknown, as are the areas of the brain where these amygdala projections influence memory storage. Candidate areas include the hippocampus and entorhinal and parietal cortices (Izquierdo et al., 1997). Indeed, it would be interesting to know whether the changes in unit

activity or the activation of intracellular signaling cascades in the hippocampus during and after fear conditioning, as discussed earlier, might be related to formation of such explicit memories, and how regulation of these signals depends on the integrity of the amygdala and its neuromodulators. Interestingly, a recent study has shown that stimulation of the basal nucleus of the amygdala can modulate the persistence of LTP in the hippocampus (Frey et al., 2001), which provides a potential mechanism whereby the amygdala can modulate hippocampal-dependent memories.

4.11.9 Fear Learning in Humans

Within the last 10 years, considerable progress has been made in understanding how the human fear learning system is organized and what features it shares with the fear learning system of lower vertebrates. In this final section, we will briefly summarize these findings. For a more comprehensive look at this topic, see Phelps and LeDoux (2005).

4.11.9.1 The Human Fear Learning System – Lesion and fMRI Studies

It has long been known that amygdala damage in humans confers deficits in fear conditioning (Bechara et al., 1995; LaBar et al., 1995). In these studies, fear conditioning is typically accomplished by pairing the presentation of visual stimuli with either mild electric shock to the skin or an aversive high-amplitude (i.e., 100 dB or more) tone. Conditioned fear is then measured by changes in skin conductance upon presentation of the CS. Damage to the amygdala in humans produces deficits in conditioned emotional responding to a CS even though the knowledge of the CS-US contingency remains intact (Bechara et al., 1995). That is, a patient with amygdala damage will not respond fearfully to the CS after it has been paired with an aversive US, but is capable of stating that the CS was previously presented and followed by the US. Interestingly, patients with selective hippocampal damage exhibit the converse effect; they will respond fearfully to the CS but cannot tell you why (Bechara et al., 1995).

Fear conditioning in humans also leads to increases in amygdala activity, as measured by functional magnetic resonance imaging (fMRI) (Buchel et al., 1998; LaBar et al., 1998). These changes largely mirror what has been seen in neurophysiological studies of amygdala activity in rodents, namely, increases in CS-elicited amygdala activity during and after fear conditioning, a corresponding attenuation of CS-elicited amygdala activity, and an increase in CS-elicited activity in the mPFC with extinction of the behavioral response (LaBar et al., 1998; Phelps et al., 2004). Further, as suggested by the animal work, the human fear learning system appears preferentially suited to use subcortical 'low-road' information during fear learning. In a study by Morris and colleagues, CS-elicited increases in amygdala activity were observed even if the CS was presented too fast to be perceived consciously, a so-called 'unseen CS' (Morris et al., 1999). When the activity of the amygdala during fear conditioning is cross-correlated with the activity in other regions of the brain, the strongest correlations are seen with subcortical (thalamic and collicular) rather than cortical areas, further emphasizing the importance of the direct thalamo-amygdala pathway in the human brain (Morris et al., 1999).

4.11.9.2 Instructed Fear – Using the High Road

In humans, direct experience with an aversive US does not appear necessary for fear learning to occur. In a series of experiments, Phelps and colleagues have demonstrated that simply telling a human subject that presentation of a CS *might* lead to an aversive outcome is sufficient to induce a learned fear state, a phenomenon known as 'instructed fear' (Phelps et al., 2001).

Like fears that are learned from direct experience, instructed fears require the amygdala (Funayama et al., 2001). Interestingly, however, it is the left amygdala that appears to be the most critical in this type of fear learning. In fMRI studies, the left amygdala is preferentially active in a paradigm utilizing instructed fear (Phelps et al., 2001), and amygdala lesions confined to the left hemisphere are most effective at impairing this type of fear learning (Phelps et al., 2001). In general, this stands in contrast to studies that have examined amygdala activation to fears that have been acquired through experience, especially those involving an 'unseen' CS. In those studies, amygdala activity is typically observed to be lateralized to the right amygdala (Morris et al., 1999). It has been hypothesized that this left lateralization in the instructed fear paradigm is the result of a linguistic/cognitive fear representation acquired through

language, which, like other verbally mediated tasks, is mediated in the majority of individuals in the left hemisphere (Funayama et al., 2001).

4.11.9.3 Declarative Memory Formation and the Amygdala

It has long been recognized that memories formed during emotionally arousing situations are more vividly remembered than those formed under neutral circumstances. Earlier in this chapter, we reviewed evidence from the animal literature which provides a potential neural mechanism for this phenomenon, namely, that the amygdala and its various neurotransmitter systems modulate the strength of explicit or declarative memory formation by influencing the longevity of cellular processes such as LTP in the hippocampus (Frey et al., 2001). Does the human amygdala play a similar role in declarative memory formation? Evidence suggests that it does. For example, administration of the β-adrenergic antagonist propranolol to human subjects impairs long-term recall of an emotionally arousing short story (Cahill et al., 1994), while administration of the $\alpha 2$-adrenergic antagonist yohimbine, which is known to be anxiogenic, enhances recall (O'Carroll et al., 1999). A similar picture emerges in patients with bilateral amygdala damage; they cannot recall the details of an emotionally charged story to the extent that intact controls can (Cahill et al., 1995). Further, amygdala activity appears to correlate with the extent to which an emotionally arousing story is remembered. In one study, subjects in a positron emission tomography scanner were shown either emotionally arousing or emotionally neutral stories and tested for recall at a later time. The findings revealed that right amygdala blood flow during the emotionally arousing, but not neutral, stories correlated highly with the extent to which details of that story could be recalled at later test (Cahill et al., 1996). More recently, Dolan and colleagues studied amygdala-hippocampal activations and recall of emotionally arousing and neutral words in patients with varying degrees of hippocampal and amygdala damage. The findings revealed that left amygdala damage was inversely correlated with memory for the emotional words and also activity in the left hippocampus. Memory for neutral words, in contrast, was only related to the degree of hippocampal damage (Richardson et al., 2004). These findings parallel those found in the animal literature and suggest that interactions between the amygdala and hippocampal formation influence the strength of declarative memory in the human brain.

4.11.10 Conclusions

In this chapter, we have provided a comprehensive view of the neural system underlying fear learning, including the key synaptic events and downstream cellular cascades that are responsible for the acquisition and consolidation of fear memories in the amygdala. These findings provide a foundation for the continued study of the neural basis of emotional learning and memory at the cellular level, and also for bridging the gap between studies of memory formation and synaptic plasticity in the mammalian brain. These studies also provide us with a set of tools to continue our analysis of more complex and clinically relevant aspects of fear learning, including contextual control of learned fear, fear extinction, and reconsolidation. Finally, recent studies translating and extending what we have learned from laboratory rats to the human brain suggest that similar mechanisms and neural pathways are conserved across species.

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4.12 The Molecular Mechanisms of Reward

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4.12.1 Introduction

This article considers the molecular mechanisms which have been implicated in aspects of reward processing and reward-related learning. A brief description of the nature of reward and the animal learning theory associated with its assessment is provided. The neural circuitry involved in implicating these psychological processes is then described, with emphasis placed on the nucleus accumbens (NAc), amygdala, and frontal cortex. Dopaminergic regulation of these structures has been shown to play a pivotal role in mediating reward-related behavior, and the intracellular signaling cascades affected by dopamine release have provided us with novel insight into the molecular basis of reward processing. In particular, data are considered from research into both drug addiction and depression, with a focus on the transcription factors cyclic adenosine monophosphate response element binding protein (CREB) and ΔFosB as well as some of their downstream targets. Dissociable roles are identified for different molecules in the regulation of reward. Furthermore, in parallel to data from neurochemical investigations, the behavioral effects of manipulating molecular pathways depend on both the region targeted and the time course of action. Greater understanding of reward processing at the molecular level is being achieved through combining expertise developed within the fields of psychology and molecular biology. Such an approach can further our knowledge of the

detrimental changes in brain function which are inherent within addiction and affective disorders and which are associated with maladaptive assessment of reward and motivation.

4.12.2 Researching Reward Processes: What Do We Mean by Reward and How Do We Measure It?

Before we consider its molecular basis, we need to establish the nature of the psychological processes covered by the term 'reward.' This topic is dealt with in more detail elsewhere in this reference work (*See* Chapter 1.36); therefore, only a brief summary will be included here to enable discussion of subsequent experimental work. In its most simple terms, a reward is a positive stimulus, i.e., something which the individual values and enjoys. Rewards carry emotional significance, and individuals are motivated to expend effort to attain them. The study of how reward and reward-related stimuli inform behavior contributes a significant amount to our knowledge of learning and memory processes.

In terms of animal learning theory, an innately rewarding stimulus, such as food, is known as an unconditioned stimulus (US). This elicits an unconditioned, automatic response (UR), for example, salivation. If the US is repeatedly paired with a previously neutral stimulus, such as a light, an association will be learned between presentation of the light and food reward, such that illumination of the light alone will cause the animal to salivate. The light is now regarded as a conditioned stimulus (CS), and the response it elicits is termed a conditioned response (CR). This process is known as Pavlovian conditioning and forms the basis of associative learning. Although conceptually quite simple, the effects that a CS can exert over behavior are far-reaching and can influence goal-directed behavior. For example, a rat can learn to press a lever to earn a food pellet through instrumental conditioning processes (see **Figure 1**). If delivery of that food pellet is repeatedly paired with presentation of a light CS, the CS will acquire some of the appetitive value of the food and become rewarding in its own right such that the rat will press the lever to turn the light on. The CS is then called a conditioned reinforcer (CRf).

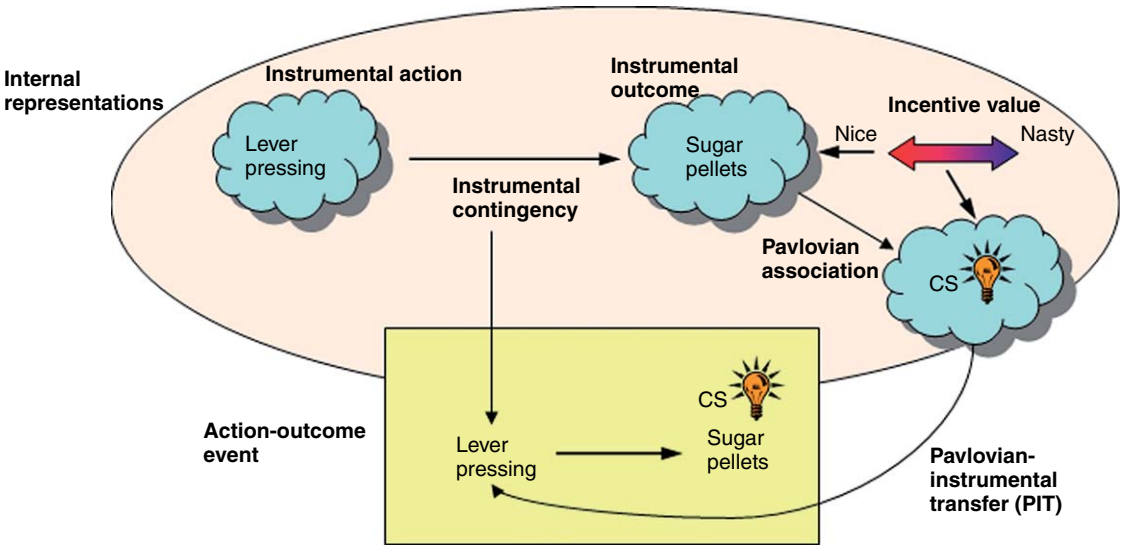


Figure 1 A schematic of some factors which affect instrumental learning. An action such as pressing a lever leads to the delivery of sugar pellets, accompanied by the onset of a stimulus light (the conditioned stimulus: CS). This action-outcome event (contained within the tan box) is detected and represented internally. Degrading the encoding of this contingency will have a direct effect on instrumental performance. The value of the outcome is determined by incentive learning (i.e., how nice the sugar pellets are). This can be affected by the motivational state of the animal. The attribution of incentive value affects the representation of the instrumental outcome, such that changing this value has an impact upon truly goal-directed instrumental behavior (see the section titled 'The prelimbic cortex'). The CS is also associated with the incentive value of the instrumental outcome through Pavlovian conditioning. Presentation of the CS can invigorate responding for the outcome through Pavlovian to instrumental transfer (PIT). Based on Cardinal RN, Parkinson JA, Hall J, and Everitt BJ (2002) Emotion and motivation: The role of the amygdala, ventral striatum, and prefrontal cortex. *Neurosci. Biobehav. Rev.* 26: 321–352; figure 2, p. 327; used with permission from Elsevier Science.

The noncontingent presentation of a CS can also influence ongoing instrumental responding in a process known as Pavlovian to instrumental transfer (PIT). For example, presentation of a tone associated with sucrose delivery will increase lever-pressing for sucrose (conditioned motivation). Conversely, if a light has been paired with a painful stimulus such as footshock, presentation of the light CS will decrease lever-pressing for reward (conditioned suppression).

These concepts inform more than just animal learning theory: experiments designed using these psychological constructs provide valuable insight into the processing of reward in psychopathology. The inability to find stimuli rewarding, as well as an excessive desire for certain rewards, are symptoms of several mental disorders, including depression, attention-deficit/hyperactivity disorder and drug addiction, among others. Research into the mechanisms of reward-related learning has therefore contributed to our understanding of these conditions. Just as a CS paired with food stimulates lever-pressing for reward in rats, stimuli paired with drug reward can lead to craving for drug and relapse to drug-seeking in both rats and human addicts (de Wit and Stewart, 1981; Childress et al., 1988). Failure to alter behavior when the incentive value of a reward changes also has obvious implications for substance abuse disorders, where addicts continue to use drugs despite increasingly negative consequences and reductions in the reward experienced. In rats, food reward can be devalued by pairing food delivery with an injection of lithium chloride, which induces nausea, or by feeding animals to satiation. Such devaluation procedures subsequently alter the way in which animals respond to food presentation or to a CS paired with that reward, making it possible to investigate the biological basis of this aspect of reward processing.

4.12.3 The Neural Circuitry of Reward

Considerable evidence has been amassed concerning the neural circuitry underpinning reward-related learning (see Cardinal, 2001, for review). The processing of reward occurs in a distributed network of structures comprising both cortical and subcortical areas, the majority of which are connected within the limbic or affective corticostriatal loop (**Figure 2**) (Alexander et al., 1986). Within this framework, structures involved in higher-order cognitive function such as the prefrontal cortex (PFC) interact with areas of the limbic system heavily implicated in emotional processing and memory, such as the amygdala and hippocampal formation. These structures are interconnected with the NAc, often described as the reward center of the brain. This circuit influences motor output and motivation via the ventral pallidum and mediodorsal thalamus, which also project back to the PFC. We will focus on three of the most studied areas: the NAc, the amygdala, and regions of the PFC. Given that the focus of this chapter is to discuss the molecular basis of reward, the majority of research of which has been undertaken in rodents, we will focus on data supporting a role for these regions in the reward system of the rat, although these areas and their homologues have been heavily implicated in reward processing in both monkeys and humans.

4.12.3.1 The Nucleus Accumbens

The NAc is probably the most widely studied region in terms of regulating reward-related learning. This region has been labeled the 'limbic-motor interface' due to its extensive connections with limbic structures, such as the amygdala, hippocampus, and PFC, in addition to its projections to motor output areas. The NAc is therefore thought to be a key node in the limbic corticostriatal loop, wherein diverse types of information from both cortical and subcortical structures are integrated and key signals generated to enable the implementation of behavioral change relevant to goal-seeking. The NAc can be divided into the core (NAc-C) and shell (NAc-Sh) subregions, which differ in both structure and function (Groenewegen et al., 1987; Voorn et al., 1989; Berendse et al., 1992). Whereas the NAc-C projects predominantly to the ventral pallidum, the shell also projects to subcortical structures, including the lateral hypothalamus and periaqueductal grey. Damage to the NAc does not prevent animals from making a response to earn food reward, or from adjusting their responding when the value of that reward changes, i.e., animals are still capable of goal-directed behavior (see the section titled 'The prefrontal cortex'). However, the ability of a CS to regulate behavior is profoundly affected by NAc lesions. Damage to the NAc-C disrupts PIT and the acquisition of autoshaping, a Pavlovian conditioning paradigm where presentation of a CS with food delivery leads animals to approach the CS (Parkinson et al., 1999; Hall et al., 2001). Damage to the NAc also impairs conditioned place preference, where animals learn to associate a specific context or place with reward delivery and therefore spend more time in this location. Neither

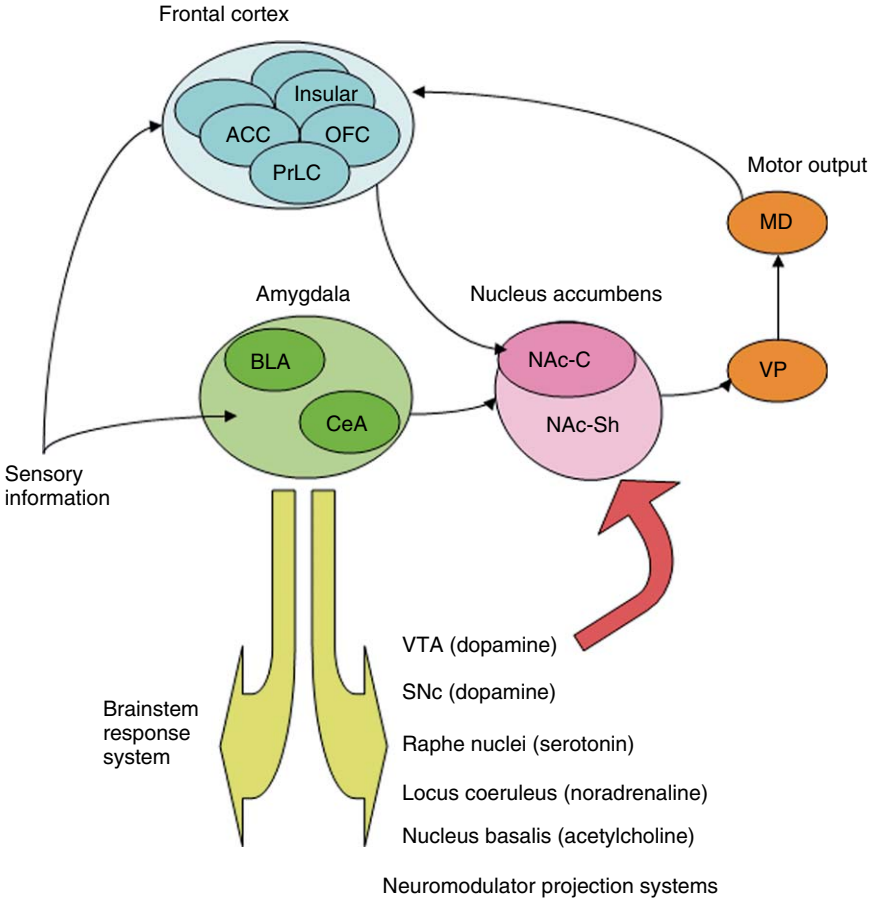


Figure 2 Simplified diagram of the limbic corticostriatal loop. (Abbreviations: ACC, anterior cingulate cortex, OFC, orbitofrontal cortex; PrLC, prelimbic cortex; BLA, basolateral amygdala; CeA, central amygdala; VTA, ventral tegmental area; SNc, substantia nigra pars compacta; NAc-C, nucleus accumbens core; NAc-Sh, nucleus accumbens shell; VP, ventral pallidum; MD, mediodorsal thalamus). The frontal cortex is functionally heterogeneous, and several frontal regions are involved in different aspects of instrumental responding. As discussed in the text, the PrLC, part of the medial prefrontal cortex, is involved in detecting the instrumental action-outcome contingency and is essential for the maintenance of goal-directed behavior. The functions of the ACC are complex and are not described in detail here, but involve resolving response conflict and error detection, whereas the insular cortex, containing the primary gustatory cortex, encodes the primary sensory qualities of specific foods. The OFC plays a role in integrating changes in the incentive value of a reward with representations of the expected outcome, a function which is thought to depend on its connections with the BLA. The BLA is one of the primary structures involved in encoding CS-US associations and is necessary for the presentation of a CS to trigger retrieval of the motivational value of its associated US. It can work in concert with the CeA to influence brainstem function, arousal, and neurotransmitter release. As the 'limbic-motor interface,' the NAc combines information from both frontal and amygdalar systems, as well as from other inputs, to generate motivational drive. The NAc-Sh signals the motivational properties of unconditioned (primary) reinforcers, whereas the NAc-C has a more pronounced role in mediating the motivational impact which Pavlovian conditioned stimuli have on behavior. Adapted from Cardinal RN, Parkinson JA, Hall J, and Everitt BJ (2002) Emotion and motivation: The role of the amygdala, ventral striatum, and prefrontal cortex. *Neurosci. Biobehav. Rev.* 26: 321–352; figure 3, p. 329; used with permission from Elsevier Science.

damage to the core or shell abolishes CRf *per se*, but manipulations of the dopaminergic innervation of the NAc can alter responding for CRf (see the section titled 'Dopamine and reward'). As well, damage to the NAc results in decreased tolerance to the delay of reward, such that animals will choose a small immediate reward over a larger but more delayed one, a concept which has direct relevance to models of impulsive behavior (Cardinal et al., 2001). In contrast, selective lesions of the NAc-Sh do not appear to have such a pronounced effect on the conditioned responses to rewards, i.e., reward-related learning, but this region plays an important role in the unconditioned response to primary reinforcers. In particular, the NAc-Sh appears to alter

the motivational impact of rewards, such that inhibition of NAc-Sh activity induces overeating, an effect attributable to its connections with the lateral hypothalamus (Stratford and Kelley, 1999). Inhibition of neuronal firing is also observed when animals engage in a sequence of reward-seeking and consumption (Taha and Fields, 2006), which results in disinhibition of activity in target brain regions such as the hypothalamus. Activity within the NAc may therefore act to gate appetitive behavior through its influence over reward-related brain circuitry.

4.12.3.2 The Amygdala

The first indication that the amygdala was one of the most important brain regions for the processing of affective stimuli was the discovery that damage localized to this area produced marked deficits in emotional display and apparent fearlessness in monkeys (Kluver and Bucy, 1939). Humans with damage to the amygdala show a variety of impairments in emotional perception and expression, and can unwittingly endanger themselves through failing to process danger or risk. The amygdala can be divided into multiple nuclei based on cytoarchitectonic distinctions (Pitkanen, 2000). Functional dissociations have also been observed between the different units. In particular, the central nucleus (CeA) and basolateral nucleus (BLA) of the amygdala have been implicated in divergent forms of affective processing (Everitt et al., 2000). Both the BLA and CeA receive sensory input, yet the BLA has more prominent connections with the frontal cortices and ventral striatum, while the CeA shares more numerous connections with areas within the hypothalamus and brainstem.

It is generally thought that the BLA plays an integral role in Pavlovian conditioning involving both appetitive and aversive stimuli (*See* Chapter 4.11) (Davis, 1998; LeDoux, 2000). One of the most commonly used paradigms to measure emotional learning is fear conditioning, in which animals form an association between a painful footshock and a particular stimulus such as a light or tone. Animals rapidly learn this association and freeze during subsequent presentations of the CS, indicative of a state of fear, yet this freezing is much less evident following BLA or CeA lesions. More sophisticated analysis of Pavlovian conditioning procedures suggests that the BLA is necessary in order for presentation of the CS to trigger retrieval of the value of the US with which it was paired. Although BLA-lesioned animals show evidence of learning simple CS-US associations, changing the value of a reward does not alter the way in which the animal responds to the associated CS. For example, BLA-lesioned rats show aversion to a devalued food, but still approach the food magazine when the CS paired with the devalued food is presented (Hatfield et al., 1996). Likewise, although BLA-lesioned monkeys show preference for different foods (i.e., can still make value judgments), they are unable to alter their choice preference when the value of a particular food has been changed through devaluation. This idea that the BLA is involved in processing the incentive value of rewards and reward-related stimuli associated with them has been very influential and is thought to depend on its connections with the orbitofrontal cortex (OFC) (Baxter et al., 2000; see the section titled 'The orbitofrontal cortex'). Given its proximity to the hippocampus and other limbic structures heavily implicated in memory processing and storage, the BLA is ideally positioned to mediate the effect of emotional arousal on memory.

4.12.3.3 The Prefrontal Cortex

The PFC is involved in numerous higher-order cognitive functions, such as decision-making, attention, problem solving, strategy development, and working memory (*See* Chapter 3.14). Such processes exert powerful control over goal-directed behavior. The PFC is both structurally and functionally heterogeneous, and a discussion of the role of each subregion is beyond the scope of this article. We will focus on two areas which appear to be particularly important in reward processing: the medial PFC (mPFC) as exemplified by the prelimbic and anterior cingulate regions, and the ventral PFC encompassing the orbitofrontal and agranular insular cortices.

4.12.3.3.1 The prelimbic cortex

This region of the rat mPFC is involved in the acquisition of goal-directed behavior. In order for action to be considered goal-directed, it must fulfill two criteria: (1) the animal must be aware of the *causal* link between the instrumental action and its outcome (i.e., a rat pressing a lever for food knows that pressing the lever results in delivery of food reward, and (2) the outcome for which the animal is responding must be considered a goal by the animal (i.e., the rat *wants* the food). Instrumental responding ceases to be goal-directed once it becomes habitual, that is, the animal is insensitive to changes in the incentive value of the reward or to the presence of an instrumental contingency. Behavior is instead controlled by simple stimulus-response (S-R)

associations in which stimuli or outcomes become directly associated with a motor response, so that the rat responds on the lever regardless of its motivational state. Damage to the prelimbic cortex (PrLC) not only retards acquisition of instrumental responding, but also disrupts the detection of instrumental contingencies (Balleine and Dickinson, 1998; Corbit and Balleine, 2003). These data indicate that rats with PrLC damage may acquire instrumental responses based on S-R associations and are no longer truly capable of goal-directed learning. The transition from goal-directed to habitual S-R responding can also happen naturally over time with repeated training and has some advantage in that it is thought to use fewer cognitive resources. However, habits are less flexible than goal-directed actions and can lead to maladaptive behavior, such as that commonly associated with addiction, where environmental stimuli trigger engagement in drug-taking even though drug intake itself is no longer rewarding.

In keeping with these data indicating that the mPFC is involved in maintaining cognitive flexibility, the PrLC is also thought to play an important role in extinction processes. Extinction refers to the decline in responding when that response no longer leads to the associated outcome. The role of the PrLC has been extensively studied in the extinction of conditioned fear (see Sotres-Bayon et al., 2004, for review). Repeated presentation of the CS in the absence of the associated shock reduces the ability of the CS to elicit fear-related responses such as freezing as the animal learns that the CS is no longer a reliable predictor of the US. This ability to update knowledge about what is, and what is not, an accurate predictor of a dangerous event is clearly important for adaptation and survival. Lesions to the mPFC encompassing the PrLC impair extinction of conditioned fear. The deficits in extinction observed in the absence of the PrLC may relate to the well-documented role of the frontal cortex in mediating behavioral inhibition and perseveration. Disconnection of the mPFC and the BLA also attenuates extinction of conditioned fear, suggesting that activity within the mPFC may act to inhibit the representations of the emotional value of the CS generated by the amygdala, highlighting the importance of prefrontal regulation of amygdala function in reward-related learning.

4.12.3.3.2 The orbitofrontal cortex

Perhaps more than any other region of the frontal cortex, the OFC has been heavily associated with the processing of rewarding or emotional stimuli and events. In humans, damage to the OFC produces a pattern of aberrant social behavior and maladaptive decision-making which is often described as impulsive. This behavior can be exemplified by performance of these patients on laboratory-based gambling tasks where subjects choose between different options to earn points. The optimal strategy is to choose options associated with small immediate gains but also low and infrequent losses, an approach which healthy volunteers learn. Persistent selection of options leading to large immediate gain but heavy losses in the long term is thought to reflect risky decision-making and is observed both in pathological gamblers (Cavedini et al., 2002) and substance abusers (Bechara et al., 2001) and in patients with damage to the OFC or BLA (Bechara et al., 1999). In the monkey, neurons within the OFC have been shown to fire preferentially to different food rewards and to decrease their firing rate specifically to a devalued reward (Critchley and Rolls, 1996). Similar to the BLA, the OFC therefore appears to be involved in creating representations of the incentive value of reward. The reciprocal connections between these two regions are well documented, and disconnection of the OFC and BLA prevents devaluation of reward from altering choice behavior in monkeys (Baxter et al., 2000). However, electrophysiology recordings in the rat suggest that the OFC may have a more sophisticated role to play in using this information. In a series of elegant studies, Schoenbaum and colleagues have developed the hypothesis that the OFC supports representations of outcome expectancy, that is, how rewarding the outcome of a certain action is anticipated as being (Schoenbaum et al., 2006). The BLA generates important information about the incentive value of reward-associated stimuli, which the OFC then uses to generate representations of the anticipated outcome predicted by those CS. Such outcome expectancy is then used to inform choice behavior.

Lesions to the OFC also affect aspects of impulsive and compulsive behavior in animals. As in humans, damage to the rodent or monkey OFC increases perseverative behavior and decreases cognitive flexibility. For example, in reversal learning paradigms, OFC-lesioned rats perseverate in responding to the previously rewarded stimulus (Schoenbaum et al., 2002; Chudasama and Robbins, 2003). In delay-to-reinforcement paradigms where rats choose between a small immediate versus a larger increasingly delayed reward, OFC-lesioned rats do not show such a strong aversion to the delay compared to their sham controls (Winstanley et al.,

2003). This deficit may arise from both perseverative tendencies as well as an inability to integrate the consequences of making a response with the incentive value of the reward, i.e., the delay does not sufficiently devalue the reward.

4.12.4 Dopamine and Reward

Converging evidence from numerous studies has implicated dopamine as the single most important neurotransmitter involved in the signaling of reward. Using intracranial self-stimulation techniques, where animals respond for electrical stimulation into a particular region of the brain, it has been shown repeatedly that animals will work for stimulation of their dopamine system (Wise and Rompre, 1989). Likewise, the addictive properties of drugs of abuse can be attributed in part to their ability to potentiate dopaminergic transmission. In particular, dopaminergic regulation of the NAc is critically involved in this process. The ventral tegmental area (VTA) sends dopaminergic projections to numerous regions within the brain including the NAc, PFC, and other parts of the limbic system. Both natural and drug rewards increase dopamine efflux in the NAc-Sh, whereas CS associated with such reward increase dopamine efflux in the NAc-C. Although animals are still capable of finding things rewarding or pleasurable in the absence of dopamine, they are no longer motivated to earn reward (Salamone et al., 2003), i.e., they are no longer capable of goal-directed behavior. Dopaminergic depletion of the NAc significantly decreases the amount of effort rats are willing to expend to earn reward, whereas manipulations which increase NAc dopamine function enhance goal-seeking.

Several groups have recorded from dopaminergic cells within the VTA in monkeys during Pavlovian conditioning paradigms. Using this methodology, it has been found that the firing of dopamine neurons may signal error prediction, i.e., they are particularly active when an unexpected reward is delivered, and firing is suppressed when an expected reward does not appear (Montague et al., 2004; Schultz, 2006). The potentiation of dopamine function caused by drugs of abuse may therefore generate a powerful signal that the reward was larger or better than expected regardless of the actual experience created by the drug (Hyman et al., 2006). Not only are psychostimulant drugs like amphetamine rewarding in their own right, they also enhance the effects of conditioned reinforcers, an effect which can be induced by direct application of amphetamine or dopamine agonists into the NAc, and which is blocked by dopaminergic lesions of the NAc and by ablation of the NAc-Sh (see Cardinal et al., 2002). Similarly, PIT can be enhanced by intra-NAc amphetamine and is abolished by dopamine receptor antagonists. This general potentiation of reward-related learning and reward-seeking likely plays an important role in the generation and maintenance of addiction.

Dopamine also regulates reward-related processing within the PFC. Data from both *in vivo* observations and computational modeling has led to the suggestion that phasic dopamine release acts as a gating mechanism, signaling when internal representations of reward and related stimuli need to be updated (Cohen et al., 2002). Damage to dopaminergic innervation of the PFC alters reward-related learning in a manner consistent with this theory. For example, lesions to dopaminergic inputs to the mPFC cause a deficit in fear extinction (Morrow et al., 1999), and ablation of dopaminergic terminals within the OFC leads to persistent choice of a larger but delayed reward, similar to excitotoxic lesions of these structures. Pharmacological manipulations also suggest that there is an optimum level of dopamine function within the PFC, and that too much as well as too little can have a negative impact on a range of cognitive behaviors (Arnsten, 1997). Long-term drug use leads to cognitive deficits that have been largely attributed to dysfunction of the frontal cortex (Rogers and Robbins, 2001), and hypofunction of the OFC has been observed in recently abstinent cocaine abusers (Volkow and Fowler, 2000). In rats, repeated exposure to addictive drugs has been shown to alter reward-related learning in tasks known to be dependent on the integrity of the OFC (Schoenbaum et al., 2004). Given the importance of the dopamine system in facilitating reward-related learning and the ability of addictive drugs to modulate this system, it seems likely that dysregulation of the dopaminergic input to frontal regions is responsible for these cognitive impairments.

4.12.5 Cellular and Molecular Targets of the Dopamine-Reward System: Insights from Drug Addiction

Given that the dopaminergic system has been heavily implicated in mediating the highly rewarding nature of addictive drugs, and that addictive behavior appears to arise from the hijacking of normal reward systems, a significant proportion of the data concerning the

molecular basis of reward have been obtained through studying the effects of drugs of abuse. The intracellular changes that occur following acute administration of an appetitive substance like cocaine can provide valuable information about the signaling cascades activated by such rewarding substances. However, the changes seen after repeated administration are of more relevance in determining the molecular basis of the alterations in reward-related learning underpinning the addicted state. Drug addiction is a chronic and often relapsing disorder, with human addicts remaining at risk of relapse even after years of abstinence. The fact that chronic drug intake produces such durable changes in brain function and behavior has led to the suggestion that long-lasting changes in gene transcription may play a prominent role (Nestler et al., 1993). This has led to a search for relatively stable markers of altered transcriptional regulation whose persistence matches the time course of aspects of addictive behavior. The contrasting effects of acute versus chronic administration of addictive drugs on intracellular signaling pathways can therefore provide information about different aspects of reward processing. The role played by these molecular mechanisms in the processing of natural rewards has also been studied in learning and memory paradigms, and also in animal models of depression.

The binding of dopamine, or any neurotransmitter or signaling molecule, by its membrane-bound receptors triggers the initiation of several intracellular signaling cascades which often culminate in the regulation of transcription factors (TFs), including those encoded by immediate early genes (**Figure 3**). In terms of our understanding of reward processing and addiction, a considerable amount of data is now available concerning the particular TF families activated by dopaminergic agents. In this section, we will focus on some specific examples of this aspect of gene regulation and consider the role of these different TFs in the development of addiction as well as in the response to natural rewards. In terms of reward-related learning, we will largely restrict our discussion to key areas within the affective corticostriatal loop highlighted in the previous section. However, it should be noted that many of these intracellular signaling pathways have been implicated in the emotional memory processes

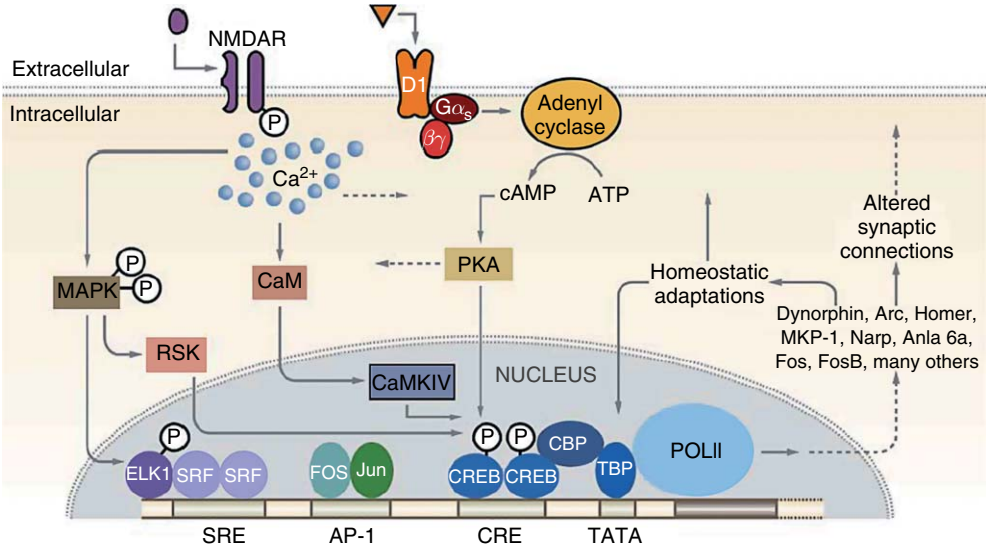


Figure 3 Regulation of gene expression within the striatum by dopamine and glutamate. Stimulation of dopaminergic D_1 receptors and glutamate receptors activates intracellular second messenger signaling cascades which result in changes in gene expression within the cell nucleus. Shown here are examples of DNA binding sites within the cFos promoter, including a serum response element (SRE), an activator protein-1 element (AP-1), and a cyclic adenosine monophosphate (cAMP) response element (CRE). Numerous other genes are also activated, including Homer, Arc, FosB, etc. Abbreviations: CBP, CREB binding protein; CREB, cAMP response element binding protein; MAPK, mitogen-activated protein kinase; NMDAR, *N*-methyl-D-aspartate glutamate receptor; PKA, protein kinase A; TBP, TATA binding protein; RSK, ribosomal S6 kinase; CaMKIV, Ca^{2+}/calmodulin-dependent kinase IV; ELK1, Ets-like transcription factor; SRF, serum response factor; POLII, RNA polymerase II; TATA describes a short sequence of base pairs that is rich in adenine (A) and thymidine (T) residues. Reprinted from Hyman SE, Malenka RC, and Nestler EJ (2006) Neural mechanisms of addiction: The role of reward-related learning and memory. *Annu. Rev. Neurosci.* 29: 565–598; used with permission from the Annual Reviews Permission Department.

involved in fear conditioning through their actions in the hippocampus (HPC).

Several techniques have been combined to determine whether these changes in TF regulation alter addiction-related behavior. Mice lacking these TFs have been developed, as have transgenic mice which either overexpress certain TFs or express mutant or dominant negative proteins which inhibit their effects. Reporter lines have also been developed, where the promoters regulated by a particular TF (e.g., cAMP response elements (CREs)) drive expression of a reporter gene expressing an easily visualized marker, such as β-galactosidase or green fluorescent protein (GFP). Such mice enable the consequences of different environmental manipulations on that TF to be determined. As well, viral vectors designed to express these proteins can be infused into specific regions using standard stereotaxic surgical techniques, which localizes the effects of changes in gene transcription to particular areas of interest. A small number of studies have infused antisense oligonucleotides into a certain brain area, although concerns remain about the toxicity and specificity of this approach. In addition to monitoring changes in cellular excitability and synaptic plasticity caused by these manipulations, development of these tools has made it possible to investigate the role these TFs play in reward-related learning. The majority of these studies have used conditioned place preference to assess the hedonic impact of substances of abuse given the relatively high throughput of this method. Instrumental responding for drug reward can also be assessed using self-administration paradigms, where animals learn to press a lever to obtain a drug infusion delivered into an indwelling intravenous catheter.

As mentioned before, one of the factors central to understanding addiction is the changes in behavior and brain function which occur following repeated rather than acute administration. In terms of behavioral output, one of the most widely studied phenomena is that of locomotor sensitization, whereby repeated administration of virtually any abused drug leads to a potentiation of the hyperlocomotor response seen after an acute drug injection in rodents. This increased sensitivity to the motor stimulating properties of addictive drugs is long-lasting, indicating that it could be mediated by some of the long-term changes in gene transcription and brain function which characterize the persistent nature of addiction. However, human addicts do not show sensitization to the arousing effects of drugs like cocaine following repeated use, with most users reporting tolerance of the drugs' stimulant effects. Nevertheless, it is hoped that understanding the changes in neuronal activity that accompany the development of locomotor sensitization may provide valuable insight into the changes in brain function caused by long-term drug use. The behavioral phenotype is very robust and easy to study, thereby facilitating the investigation of its underlying neurobiology. Furthermore, there is evidence to suggest that sensitization to psychostimulants enhances their ability to potentiate the impact of CS on behavior (Taylor and Horger, 1999). Some of the mechanisms underlying behavioral sensitization could also be involved in mediating the powerful ability of addictive drugs to influence goal-directed behavior and stimulate drug-seeking.

Given that addictive drugs heavily stimulate the dopamine system to cause long-term behavioral changes, it is likely that the regulatory mechanisms controlling neuronal plasticity within reward circuitry are targets of drugs of abuse. Repeated activation of neurotransmitter receptors leads to a change in the physiological state of neurons, rendering them more or less sensitive to subsequent stimulation. This could be mediated via changes in the effective strength of synaptic connections (referred to as synaptic plasticity), or via changes in the overall excitability of the affected neurons (referred to as whole-cell plasticity). The former in particular have been implicated in learning and memory processes. In keeping with this hypothesis, chronic administration of several, but not all, addictive drugs increases dendritic spine formation within the NAc (Robinson and Kolb, 2004). This increase in synaptic plasticity is proposed to underlie the locomotor sensitization discussed earlier. Considering the importance of the NAc in mediating the rewarding properties of addictive drugs, the majority of molecular studies have focused on manipulating gene transcription in this region.

4.12.5.1 The CREB and Fos Families of TFs

Ligand-binding at the dopamine D_1 receptor activates the cAMP second messenger signaling cascade, leading to the phosphorylation of protein kinase A (PKA) which can in turn phosphorylate downstream protein targets. Among the prominent targets of this signaling cascade is CREB. This transcription factor is constitutively expressed at fairly high levels throughout the brain, but needs to be phosphorylated for full transcriptional activity. In addition to PKA, CaM kinases such as Ca^{2+}/calmodulin kinase type IV (CaMKIV) and growth factor-associated kinases are all capable of

performing this phosphorylation event, indicating that CREB activation is a point of functional convergence for several signaling pathways. Dimers of CREB bind to CRE sites located within the promoter regions of certain genes and alter the rate at which they are transcribed. More information about this pathway can be found in other chapters in this reference work (*See* Chapters 4.21, 4.27).

Acute administration of psychostimulant drugs stimulates phosphorylation of CREB through D_1 receptor-dependent mechanisms at several nodes within the reward circuitry, including the VTA, amygdala, PFC, and NAc. Increased expression of cFos, a product of an immediate early gene, is observed in similar locations, and its induction may be partly dependent on CREB activation, at least for amphetamine (Konradi et al., 1994). This occurs through CRE sites present in the promoter region of the cFos gene. cFos expression is also induced by several other intracellular signaling cascades, in particular, serum response factor (SRF) acting on serum response elements (SREs) within the cFos promoter. cFos is a member of the Fos family of transcription factors, which includes FosB, Fra1, and Fra2. These proteins heterodimerize with members of the Jun protein family to form the activator protein 1 (AP-1) transcription factor complex, which then binds to AP-1 sites within gene promoter regions. Increases in the activation of both cFos and CREB are rapid and transient, returning to basal levels within hours of the acute stimulus. In fact, repeated administration of drug causes the induction of cFos to desensitize, so that subsequent drug exposure no longer induces the robust elevation seen following first administration (Nestler et al., 2001). A similar pattern is observed in the expression of FosB. In contrast, the activation of CREB appears to become greater and more persistent with repeated drug exposures, an effect most firmly established within the NAc (Shaw-Lutchman et al., 2003). This pattern of expression suggests a more pronounced role for CREB-mediated gene transcription in aspects of addiction.

However, in contrast to all the TFs mentioned so far, isoforms of a truncated splice variant of FosB, known as ΔFosB, is only induced at high levels within the same reward-related areas following chronic, *but not acute*, administration of addictive drugs (**Figure 4**) (Nestler et al., 2001). The 35–37 kDa isoforms of ΔFosB dimerize predominantly with JunD to form an active AP-1 complex. These isoforms of ΔFosB also have an unusually long half-life due to their resistance to degradation by the proteosome, a property conferred at least in part by its truncated C-terminus and by a casein kinase 2–mediated phosphorylation (McClung et al., 2004). Once induced, levels of ΔFosB have been detected up to 2 months after cessation of drug treatment. Such accumulation has been observed following treatment with virtually any addictive substance, including cocaine, *d*-amphetamine, morphine, nicotine, alcohol, and phencyclidine (PCP). This relatively unique pattern of induction and stability has lead to the suggestion that ΔFosB may be a particularly important mediator of long-term changes in gene regulation associated with addiction.

Activation of PKA within the NAc reduces the rewarding effects of cocaine in self-administration and relapse assays, whereas inhibition of PKA has the opposite effect (Self et al., 1998). Likewise, overexpression of CREB in the NAc, through viral mediated gene transfer, decreases place conditioning

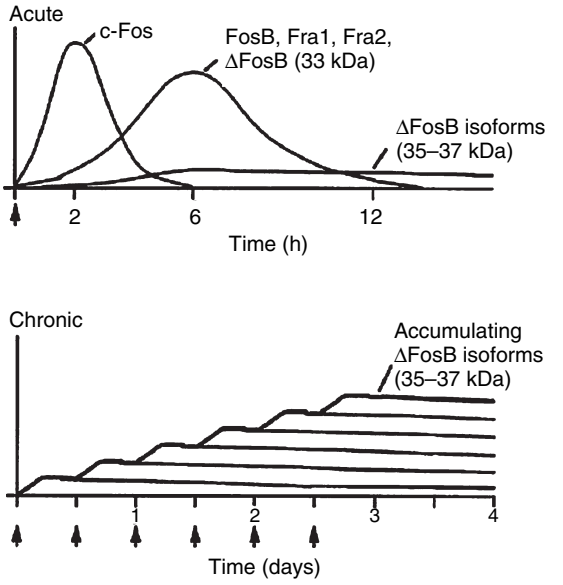


Figure 4 Diagrammatic representation of the induction of the Fos family of transcription factors (TFs) by acute and chronic drugs of abuse. The top panel charts the response to acute stimulation. cFos is rapidly induced, followed by FosB, Fra-1, and Fra-2. Levels of these TFs return to baseline between 6 and 12 hours poststimulation. Only a small increase is observed in levels of stabilized isoforms of ΔFosB. As shown in the bottom panel, this induction persists in the brain for a much longer time course and accumulates with chronic drug administration. Taken from Nestler EJ, Barrott M, and Self DW (2001) ΔFosB: A molecular switch for addiction. *Proc. Natl. Acad. Sci. USA* 98: 11042–11046; used with permission from National Academy of Sciences, USA.

to cocaine and to morphine, whereas overexpression of a dominant negative mutant form of CREB (mCREB), which cannot be phosphorylated due to a point mutation (Ser133 to Ala), potentiates the rewarding effects of both drugs (Carlezon et al., 1998; Barrot et al., 2002). Similar results are seen in transgenic mice that inducibly overexpress CREB or mCREB in the NAc and dorsal striatum. These data suggest that inhibition of CREB in the NAc enhances the hedonic value of cocaine and morphine; thereby animals 'like' the drug more, or alternatively could be facilitating the formation of the Pavlovian CS-US association between context and drug exposure. The viral infusions targeted the NAc-Sh, which is associated with encoding the rewarding properties of primary reinforcers rather than Pavlovian conditioning processes linking those rewards to environmental stimuli (see earlier). Therefore, it is likely that the behavioral changes observed arise from enhancing the rewarding effects of the drug. This interpretation is consistent with preliminary findings that mCREB decreases brain stimulation reward thresholds, while CREB has the opposite effect (Carlezon et al., 2005). Nevertheless, analysis of tissue from animals killed shortly after completion of the place conditioning test demonstrated increases in the phosphorylated form of CREB in the NAc core but not shell. Blocking this induction disrupted both the retrieval and consolidation of the CS-US association, indicating that CREB in the NAc-C is critically involved in this aspect of reward-related learning (Miller and Marshall, 2005). Thus, activation of CREB may play distinct roles in these two subregions of the NAc. CREB has been heavily implicated in multiple memory processes, particularly those underpinning long-term memory, which may reflect the known role for CREB in mediating certain changes in synaptic plasticity.

Transgenic mice have been developed which selectively overexpress ΔFosB within the NAc and dorsal striatum (Kelz et al., 1999). Furthermore, this overexpression is inducible (it occurs in adult animals) and is cell-type specific in that it is only observed in medium spiny neurons containing dynorphin/substance P (as opposed to those which contain enkephalin). The behavioral phenotype of these mice resembles animals treated chronically with drugs in several ways. The mice are more responsive to cocaine-induced hyperactivity, both acutely and after repeated administration, suggesting that ΔFosB expression may be involved in the development of locomotor sensitization. They also show enhanced place conditioning for cocaine and morphine, indicative of increased sensitivity to the rewarding properties of drugs or (as discussed earlier for CREB) of potential enhancement of the ability to form CS-US associations (Kelz et al., 1999; Zachariou et al., 2006b). Mice overexpressing ΔFosB self-administer lower doses of cocaine than wild-type controls and are more motivated to work for cocaine reward, as indicated by their elevated breakpoints in progressive ratio schedules (Colby et al., 2003). In contrast, mice overexpressing ΔcJun, a truncated form of cJun which acts as a dominant negative antagonist of all AP-1 mediated transcription, show reduced place conditioning to cocaine and morphine (Peakman et al., 2003). Together, these data may reflect more generalized increases in incentive motivation for reward.

One important molecular target of the dopamine system is dopamine and adenosine 3′5′-monophosphate-regulated phosphoprotein (32 kDa), or DARPP-32 as it is commonly known (Fienberg et al., 1998). DARPP-32 has been shown to be a potent modulator of both CREB and ΔFosB as well as many other facets of dopaminergic transmission. As with CREB, D_1-receptor-mediated activation of PKA induces the phosphorylation of DARPP-32 at threonine 34 (Thr34), which then acts as a potent inhibitor of protein phosphatase-1 (PP-1). In contrast, dopaminergic activation through D_2 receptors inhibits PKA signaling, through G-protein-coupled inhibition of adenylyl cyclase, leading to a decrease in phosphorylation of DARPP-32. Through its inhibition of PP-1, DARPP-32 regulates the phosphorylation of numerous proteins. With respect to TFs, mice lacking DARPP-32 show reduced phosphorylation of CREB in response to stimulant drugs of abuse, as well as reduced induction of cFos, in striatal regions (Fienberg et al., 1998). The mice also show reduced induction of ΔFosB after chronic stimulant administration. Consistent with these deficits in biochemical responses to drugs of abuse, DARPP-32 mutant mice show reduced responses to acute drug administration, including reduced locomotor activation and place conditioning (Zachariou et al., 2002). However, paradoxically, the mice show enhanced locomotor sensitization to chronic cocaine. The molecular basis of this latter abnormality is hard to explain on the basis of available data and requires more investigation (Hiroi et al., 1999). Mice in which Thr34 of DARPP-32 is mutated to Ala exhibit virtually the same biochemical and behavioral phenotype as DARPP-32 knockout mice, which demonstrates the importance of DARPP-32's phosphorylation by PKA in regulating its function (Zachariou et al., 2006a).

4.12.5.2 Clock

TFs traditionally associated with other roles of the dopamine system have also been recently implicated in reward-processing and the response to addictive drugs. For example, the dopaminergic system exerts an important influence over the entrainment of circadian rhythms during fetal development and in response to food and other stimuli, and disruption of the sleep cycle is observed in patients treated with dopaminergic drugs, such as *d*-amphetamine and L-DOPA. Abnormal circadian rhythms are also commonly found in substance abuse disorders. The most studied focus of circadian rhythms in brain is the suprachiasmatic nucleus (SCN) of the hypothalamus. This nucleus is particularly important for entraining the body's circadian rhythms to environmental lighting. The molecular basis of the circadian clock is now well established. The transcription factor Clock dimerizes with Bmal1 to form a transcription factor complex essential for accurate circadian rhythmicity (Vitaterna et al., 1994). The complex activates expression of Period and other proteins, which feed back and suppress their own expression in addition to regulating many other cellular targets. Increasing evidence indicates that this molecular clock operates in all tissues, which raises the interesting notion that many circadian rhythms are driven outside the SCN. For example, recent findings have shown that Clock is highly expressed within dopaminergic neurons of the VTA (McClung et al., 2005). Mice lacking functional Clock protein are hyperactive under baseline conditions, an effect which is most pronounced during the transition from the light to dark phases of their diurnal light cycle. Despite this hyperactivity, these mice show still greater activity following administration of cocaine as compared to littermate controls, and also show increased place conditioning to cocaine as well as decreased brain stimulation reward thresholds. These findings suggest that Clock, at the level of the VTA, may serve to dampen dopaminergic function and suppress reward, and that this may contribute to circadian rhythms in reward and motivation that have been well documented over the years.

4.12.6 The Role of CREB and ΔFosB in Response to Natural Rewards and Stress

Understanding the response to natural rewards has implications for research into depression due to the obvious relationship between value judgments and anhedonia. Pharmacologically, most currently used antidepressant treatments inhibit the reuptake of the monoamines serotonin and noradrenaline, or inhibit monoamine oxidase (a major catabolic enzyme for monoamine neurotransmitters). Although the dopamine system is critically associated with reward judgments, it is less well studied in the context of depressive etiology. However, changes in some of the same intracellular signaling mechanisms identified in drug addiction research are also affected in animal models of depression and within areas associated with reward-related learning such as the NAc, PFC, and BLA, as well as in areas more strongly associated with memory storage and retrieval such as the HPC (Nestler and Carlezon, 2006). Animal models of depression have generally focused on the response to stress, such as the forced swim test (FST), where antidepressants have been shown to increase the latency to immobility and decrease the total time rodents spend immobile when confined to a water-filled container. Similarly, antidepressants increase the time an animal struggles when suspended by its tail. A lack of struggling in these models is regarded as indicative of a state of behavioral despair. Likewise, animals exposed repeatedly to inescapable stressors, such as shocks, show an increased latency to escape when subsequently given the opportunity.

CREB activity in the NAc appears to play an important role in gating an individual's response to both rewarding and aversive stimuli (Barrot et al., 2002). Increased CRE-mediated transcription in the NAc has been observed following several stressors, including inescapable foot shocks, restraint stress, and the more natural stress of introducing an animal into a novel social group. Increased CREB expression reduces both the nociceptive reaction to painful stimuli and conditioned place aversion to naloxone withdrawal in morphine-dependent rats, whereas mCREB potentiates the response to these aversive stimuli. A similar pattern is observed in anxiety tests, where intra-NAc infusion of herpes simplex virus (HSV)-CREB appears to be anxiolytic and HSV-mCREB anxiogenic. In addition to modulating place conditioning for drug rewards, CREB in the NAc also alters preference for sucrose as assessed by a simple two-bottle choice test. Overexpression of CREB decreases sucrose preference, whereas mCREB increases sucrose preference. Conversely, levels of CRE-mediated transcription in the NAc decrease following protracted social isolation, a manipulation which increases anxiety and impairs initiation of sexual behavior. This

phenotype can be rescued by overexpressing CREB within the NAc using viral-mediated gene transfer. Likewise, overexpressing mCREB in this region mimics these effects of isolation in nonisolated rats, an effect that can be reversed with the anxiolytic diazepam (Barrot et al., 2005).

In summary, CREB appears to reduce the impact of emotionally significant stimuli, whereas inhibition of CRE-mediated transcription enhances emotional responsivity. Although mCREB is not a naturally occurring protein, the endogenous inhibitor of CREB function, inducible cAMP early repressor (ICER), is capable of mediating the same functions as mCREB *in vivo* and is induced by both stress and amphetamine (Green et al., 2006). In keeping with this hypothesis that CREB in the NAc numbs the emotional response to stimuli, overexpression of CREB in the NAc induces depressive-like behavior in the FST and learned helplessness test, whereas inhibition of CREB function in this region, through overexpression of either mCREB and ICER, induces antidepressant-like behavior in these tests (Pliakas et al., 2001; Green et al., 2006).

The proposal that CREB gates the response to emotional stimuli within the NAc is consistent with recent elecrophysiological findings, where CREB was shown to increase the electrical excitability of NAc neurons and mCREB to cause the opposite effect (Dong et al., 2006). Moreover, direct inhibition of NAc neurons, via viral-mediated overexpression of a K^+ channel, which would mimic the mCREB effect, increased an animal's behavioral response to cocaine. These findings are interesting in light of work, cited earlier, where inhibition of NAc neurons has been linked with increases in goal-directed behavior.

The effect of CREB in the NAc contrasts with its established role in the HPC where, as noted earlier, CREB is thought to mediate long-term memory formation. Virally mediated overexpression of CREB in the HPC also produces antidepressant-like behavior in rats, an effect potentially mediated in part by CREB-induced elevations of brain-derived neurotrophic factor (BDNF; see section 'Brain-derived neurotrophic factor'). Changing CRE-mediated gene transcription within different brain areas, therefore, has very different effects (Carlezon et al., 2005). Such functional dissociations are not uncommon when considering the effects of neurotransmitters such as dopamine or serotonin. Therefore, it is not surprising that the same intracellular signaling mechanisms activated by these neurotransmitters likewise produce region-specific changes in behavior.

In comparison to CREB, less is known regarding the role of ΔFosB in regulating the response to natural rewards or stressors. ΔFosB upregulation is seen after chronic wheel-running behavior, an activity which rodents are thought to find pleasurable but potentially compulsive or 'addictive,' and mice overexpressing ΔFosB in striatal regions exhibit greater compulsive wheel-running than their wild-type littermates (Werme et al., 2002). Moreover, overexpression of ΔFosB in striatal regions, either by viral vectors or in inducible transgenic mice, increases motivation for food in progressive ratio and instrumental learning tests (Olausson et al., 2006). These findings support the hypothesis, mentioned earlier, that ΔFosB in this neural pathway promotes reward.

4.12.7 Target Genes of CREB and ΔFosB

This section will primarily focus on examples of the downstream targets of CREB and ΔFosB associated with reward processing, addiction, and depression-like behavior at the level of the brain's reward pathways. A broad survey of CREB and ΔFosB targets in the NAc has been published recently (McClung and Nestler, 2003). However, changes in targets upstream of the TFs considered here have also been associated with the response to rewarding stimuli, including the enzymes responsible for the synthesis and degradation of cAMP, namely adenylyl cyclase and cyclic nucleotide phosphodiesterases, respectively. Kinases such as PKA and extracellular signaling kinases (ERKs) have been implicated in the effects of addictive drugs as well as in numerous facets of learning and memory. These aspects of the intracellular response to reward are considered in other chapters (*See* Chapters 4.21, 4.22, 4.25).

4.12.7.1 Dynorphin in the VTA-NAc Pathway

One of the primary mechanisms by which CREB is thought to affect reward-related learning and addiction is through induction of dynorphin within the NAc (**Figure 5**). Dopaminergic neurons in the VTA innervate GABAergic neurons in the NAc, which express dynorphin and in which activation of CREB has been observed after chronic treatment with addictive drugs. Dynorphin acts on κ opioid receptors expressed on the terminals of these

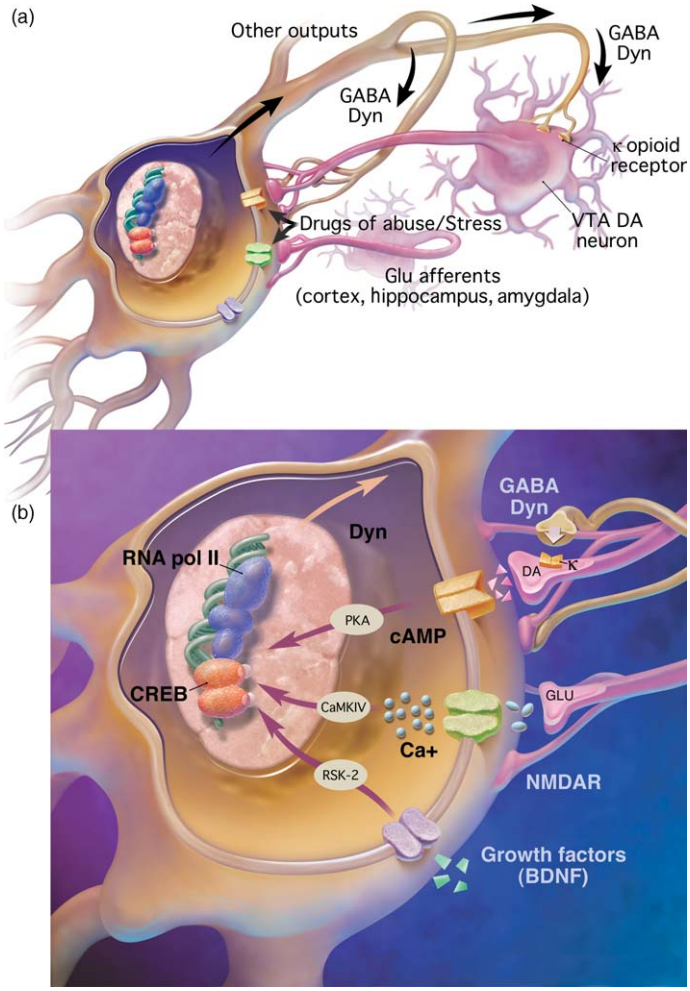


Figure 5 Regulation of NAc function by CREB and dynorphin (Dyn). The figure shows a dopaminergic neuron from the VTA innervating a medium spiny neuron within the NAc which expresses dynorphin. Glutamatergic input from other areas such as the PFC and amygdala, as well as BDNF (released from glutamatergic or dopaminergic projections) are also shown. Dynorphin acts as a negative feedback signal: when released, it binds to κ opioid receptors on dopaminergic neurons and inhibits their function. Drugs of abuse and stress increase CREB activity and induce dynorphin expression, upregulating this feedback loop. Activation of CREB could be caused by some of the mechanisms shown in the figure, all of which lead to its phosphorylation at Ser 133. Abbreviations: GABA, gamma-aminobutyric acid; NMDAR, *N*-methyl-D-aspartate receptor; PKA, protein kinase A; CaMKIV, Ca^{2+}/calmodulin-dependent protein kinase type IV; RSK-2, ribosomal S6 kinase-type 2; RNA pol II, RNA polymerase II complex. Taken from Nestler EJ, Barrot M, DiLeone RJ, Eisch AJ, Gold SJ, and Monteggia LM (2002) Neurobiology of depression. *Neuron* 34: 13–25; used with permission from Cell Press.

dopaminergic projections and inhibits their function, thereby forming a negative feedback loop to minimize the effects of dopaminergic stimulation. This dampening of the dopamine signal could contribute to the depressant-like effects of overexpressing CREB within the NAc and the reduction in place conditioning to addictive drugs (Carlezon et al., 2005). Increased ΔFosB is also primarily observed in dynorphin-containing cells within the NAc, but acts to decrease expression of dynorphin (Zachariou et al., 2006b) and to thereby potentiate dopaminergic signaling. Such reciprocal regulation of dynorphin by CREB and ΔFosB could explain some of the reciprocal behavioral changes observed following upregulation of these TFs. It could also account for the changes in reward processing that occur during different timepoints of withdrawal from addictive drugs. Drug-induced activation of CREB is relatively short-lived, yet increasing drug use potentiates CREB expression. The anhedonia and negative emotional symptoms which predominate during acute withdrawal could therefore arise partly from the ability of

CREB to downregulate dopaminergic signaling. In contrast, the sensitization to the rewarding effects of addictive drugs and the incubation of craving which predominate at later timepoints could be mediated in part by the prolonged expression of ΔFosB.

4.12.7.2 Cyclin-Dependent Kinase 5

Cyclin-dependent kinase 5 (Cdk5) was identified as a downstream target of ΔFosB within the NAc through microarray analysis (Bibb et al., 2001). Activation of Cdk5 alters dopaminergic signaling through phosphorylation of DARPP-32 at a different site from PKA, namely threonine 75 (Thr75) (see Benavides and Bibb, 2004). This converts DARPP-32 from an inhibitor of PP-1 to an inhibitor of PKA. The fact that DARPP-32 can function as either a protein phosphatase inhibitor or a protein kinase inhibitor, depending on the site at which it is phosphorylated, may be unique, and this high level of phosphorylation-site-specific regulation further highlights the importance of this molecule in intracellular signaling cascades. Furthermore, PKA activation can decrease phospho-Thr75 DARPP-32 through activation of protein phosphatase 2A (PP-2A). Acute administration of cocaine can increase phosphorylation of DARPP-32 at Thr34 and reduce it at Thr75 via activation of PKA and inhibition of the PP-2A pathway, respectively. However, chronic cocaine administration has the opposite effect, increasing phosphorylation of Thr75, and reducing the ability of D_1 receptor stimulation to activate PKA. Cdk5 is upregulated by chronic cocaine administration, and this effect appears to be mediated by ΔFosB: overexpression of ΔFosB induces Cdk5 expression, while expression of the dominant negative ΔcJun prevents the ability of cocaine to induce the enzyme (Bibb et al., 2001; Peakman et al., 2003).

The behavioral contribution of Cdk5 induction is complex. Intra-NAc infusion of the Cdk5 inhibitor roscovitine has been shown to potentiate the hyperlocomotor response to cocaine seen following chronic drug administration (Bibb et al., 2001). These behavioral data suggest that cocaine-induced upregulation of Cdk5 activity may be an attempt to compensate for overstimulation of the dopaminergic system. However, intra-NAc infusions of roscovitine also block the increase in dendritic spine proliferation seen in this region with chronic cocaine administration, which is correlated with the development of locomotor sensitization (Norrholm et al., 2003). As discussed earlier, these neuroplastic changes are one potential mechanism by which repeated drug administration perpetuates changes in learning and memory processes integral to the sensitized and addicted state. Chronic cocaine exposure, via ΔFosB, may therefore trigger an adaptive homeostatic response involving increased Cdk5 activity that ultimately commits the affected neurons to a maladaptive process of cytoarchitectural changes.

4.12.7.3 Nuclear Factor Kappa B

Nuclear factor kappa B (NFκB) is a transcription factor induced in many tissues by inflammation and immune responsiveness (see Chen and Greene, 2004). It is composed of two subunits, most commonly p50 and p65. Under basal conditions, it remains sequestered in the cytoplasm by inhibitory kappa B (IKB) protein. Upon phosphorylation by I kappa kinase (IKK), IKB releases an inactive dimer of p50 and p65, which can then be phosphorylated and transported to the nucleus where it can initiate gene transcription (*See* Chapter 4.28). This TF is more commonly associated with the field of immunology than neuroscience. However, in parallel to Cdk5, both ΔFosB overexpression and chronic cocaine treatment upregulate NFκB-related proteins such as p65, the precursor of p50 (p103), and IKB within the NAc (Ang et al., 2001). NFκB has been implicated in regulating cell survival and neuroplasticity and has been associated with long-term potentiation (LTP) and long-term depression (LTD), responses implicated in learning and memory processes. In terms of reward processing, intra-amygdala infusions of κB decoy DNA impaired fear-potentiated startle responses, suggesting that this molecule may play a role in the intracellular signaling pathway underpinning emotional CS-US learning (Yeh et al., 2002). Preliminary data also indicate that potentiating NFκB signaling within the NAc through overexpression of a constitutively active form of IKK increases place conditioning to cocaine and also increases local dendritic spine formation.

4.12.7.4 Brain-Derived Neurotrophic Factor

Neurotrophic factors facilitate neural growth and differentiation during development and also have a critical role to play in mediating neuronal survival and plasticity in adulthood. BDNF has been identified as an important downstream target of CREB and is implicated in numerous processes related to learning and memory, particularly within the HPC.

BDNF also modulates emotional learning within the amygdala, where increases in BDNF mRNA have been reported following fear conditioning. In addition, overexpression of a mutated dominant negative form of the tyrosine kinase B (TrkB) receptor within the amygdala blocks the acquisition of fear conditioning (Rattiner et al., 2004), indicating that the ability of BDNF to mediate changes in synaptic plasticity could be of particular import in the encoding of emotionally significant events in this region.

4.12.7.4.1 The neurotrophic hypothesis of depression

The neurotrophic hypothesis of depression suggests that a deficiency in neurotrophic support may contribute to the observed hippocampal pathology associated with depression (e.g., reduced hippocampal volume in depressed patients, decreases in dendritic arborization, decreased adult hippocampal neurogenesis), and that antidepressants relieve the symptoms of depression through increasing neurotrophic action. BDNF has been widely studied within the context of this hypothesis. Chronic administration of numerous antidepressant drugs increases expression of BDNF within the HPC despite their diverse pharmacological actions (Nibuya et al., 1995). Both acute and chronic stress decreases BDNF expression in hippocampal regions, effects which may contribute to the etiology of depression and which can be blocked by antidepressant treatment. Direct infusion of BDNF into the hippocampus also produces antidepressant-like effects on the FST and learned helplessness paradigms (Shirayama et al., 2002), while mice lacking BDNF do not show antidepressant behavioral responses (Monteggia et al., 2004, 2007), further indicating that BDNF may be important in mediating depressive symptoms.

Observations that both intra-cerebral infusions of BDNF (Pencea et al., 2001) and chronic administration of antidepressants (Malberg et al., 2000) increase adult neurogenesis has led to the suggestion that this may be one mechanism underlying the therapeutic action of antidepressants. However, a direct, causal relationship between neurogenesis, BDNF, and antidepressant action has proved difficult to demonstrate conclusively. Although X-ray irradiation of the brain blocks cell proliferation and also prevented the chronic effects of antidepressants in a novelty-suppressed feeding assay (Santarelli et al., 2003), irradiation also disrupts numerous intracellular signaling cascades, which may confound interpretation of these findings (Silasi et al., 2004).

Although it is clear that antidepressants can increase CREB and that CREB activity can increase BDNF expression, it is currently unclear as to whether CREB-mediated activation of BDNF is the critical pathway for the antidepressant actions of BDNF. Thus, the increase in BDNF caused by antidepressant administration is blocked in CREB-deficient mice (Conti et al., 2002), yet these mice still respond to antidepressant drugs in tests such as the FST. Although CREB phosphorylation is thought to have pro-survival properties in newly formed hippocampal neurons, the atypical antidepressant tianeptine increases hippocampal neurogenesis but does not activate the cAMP signaling cascade (Czeh et al., 2001). The role of CREB in the antidepressant effects of BDNF clearly merits further study (Malberg and Blendy, 2005).

4.12.7.4.2 BDNF within the VTA-NAc: Reward processing and addiction

In addition to its roles in neuroplastic responses, BDNF is critically involved in the regulation of dopaminergic neurotransmission. Through binding at TrkB receptors on dopaminergic terminals within the NAc, BDNF is capable of potentiating dopamine release in this region. In addition, BDNF acts directly on TrkB receptors expressed by NAc neurons. Hence, it is not surprising that this molecule has been implicated in addiction and reward-related learning. Direct administration of BDNF into the NAc or VTA increases cocaine-induced hyperactivity, whereas BDNF heterozygous knockout mice show reduced locomotor activity and reduced place conditioning to cocaine (Hall et al., 2003). Intra-NAc BDNF also increases responding for CRf (Horger et al., 1999), suggesting that induction of BDNF may contribute to the increases in incentive motivation for drugs that are associated with addiction. In support of this hypothesis, increases in BDNF have been observed in the NAc, BLA, and VTA following withdrawal from cocaine, and these increases appear to track the incubation (potentiation over time) of craving for cocaine as measured by drug-seeking behavior following presentation of a drug-paired CS (Grimm et al., 2003). A direct infusion of BDNF into the VTA can also potentiate such drug-seeking behavior (Lu et al., 2004).

However, despite these increases in incentive motivation for drugs and the potentiation of local dopaminergic transmission, increases in BDNF within the NAc and VTA appear to induce a pro-depressant phenotype. Intra-VTA infusions of BDNF decreased

the latency to immobility in the FST, whereas overexpression of a mutant TrkB receptor within the NAc (which inhibits BDNF signaling) produces an antidepressant-like effect in the same task (Eisch et al., 2003). Recent data using the social defeat model of stress in mice provide further insight into the role of BDNF in affective processing. In this paradigm, an animal is defeated by a larger, dominant, and aggressive mouse and is then housed in close confinement with the aggressor (although the animals can no longer fight). Animals defeated chronically in this way develop a behavioral syndrome characterized by numerous indices of anhedonia which may reflect aspects of human depression, such as decreased preference for natural rewards such as sucrose, sex, and social interaction, as well as a general decrease in locomotor activity. Some of these changes are particularly long-lasting and are reversed by chronic antidepressant treatment (Berton et al., 2006). Knocking out BDNF within the VTA selectively, by use of a viral vector expressing the Cre recombinase, prevents the development of this depressive-like syndrome following social defeat. These data suggest that BDNF within the VTA-NAc pathway plays an important role in reward-related learning, and that overstimulation of this signaling pathway by repeated drug intake or chronic stress could lead to potentiated and maladaptive learning, both to rewarding and aversive stimuli. As with CREB, the effects of BDNF in the HPC versus the VTA-NAc appear to be diametrically opposed. Indeed, the observation that stress decreases BDNF in the HPC, yet increases it in the VTA-NAc, indicates that these areas mediate very different aspects of an animal's behavioral repertoire in response to stress.

4.12.7.5 Glutamate Receptors

In addition to the changes observed in dopaminergic signaling, repeated administration of addictive drugs increases the expression of both alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA) glutamate receptor (GluR) subunits within the VTA. The GluR1 subunit of the AMPA receptor has received particular attention (Carlezon et al., 2002). Increases in the number of GluR1 subunits present in an AMPA receptor increase its overall conductance as well as its permeability to calcium (Ca^{2+}) ions. Ca^{2+} is involved in numerous intracellular signaling pathways, and changes in levels of intracellular Ca^{2+} can alter the regulation of gene expression. Given that sensitizing regimes of drug administration increase the electrophysiological responsiveness of dopaminergic cells within the VTA to AMPA receptor agonists (Thomas and Malenka, 2003), and that the increase in GluR1 subunits is most prominent in animals showing behavioral signs of sensitization (Churchill et al., 1999), it has been suggested that upregulation of the GluR1 subunit may be one molecular mechanism underlying the potentiated response to chronic drug treatment. In support of this hypothesis, increasing GluR1 expression within the VTA increases both conditioned place preference and hyperlocomotion caused by morphine (Carlezon et al., 1997). Recent evidence indicates that drug-induced upregulation of GluR1 in the VTA may be mediated via drug induction of CREB in this brain region (Olson et al., 2005).

In contrast, increases in GluR1 subunit expression within the NAc-Sh can facilitate the extinction of cocaine-seeking (Sutton et al., 2003), suggesting that increased glutamatergic action within this region can reverse some of the detrimental adaptations caused by chronic drug intake. Chronic cocaine treatment has been shown to reduce the electrophysiological sensitivity of NAc neurons to AMPA agonists (Thomas and Malenka, 2003). This effect may be accounted for by increased levels of GluR2 subunits within the NAc, which decrease the conductance of AMPA receptors and reduce their permeability to Ca^{2+}. Overexpression of ΔFosB increases GluR2 levels within the NAc, while ΔcJun prevents the ability of cocaine to induce the protein (Kelz et al., 1999; Peakman et al., 2003). Moreover, viral-mediated overexpression of GluR2 within the NAc mimics the effects of increased ΔFosB, in that both manipulations enhance place conditioning to cocaine (Kelz et al., 1999). Drug-induced adaptations in several postsynaptic density proteins, which modulate GluR function, have also been observed in the NAc (Yao et al., 2004). It would, therefore, appear that drug-induced changes in the expression of different GluR subunits within different reward-related regions may have opposing actions on cellular excitability, yet may both contribute to the addicted phenotype.

4.12.8 Molecular Changes within the PFC

The majority of work to date has focused on changes in transcriptional regulation within the subcortical regions of reward-related circuitry, with an understandable emphasis on the NAc and VTA. However,

changes in gene expression within the PFC have also been observed in models of addiction and depression, although less is known about their functional consequences (Kalivas, 2004). For example, a recent study examined patterns of cFos expression within the PrLC, NAc, and BLA following reexposure to a cocaine-associated context, as assessed by place conditioning. The authors observed a selective increase in GABAergic cells expressing cFos within the PrLC (Miller and Marshall, 2004). Similarly, a decrease in protein kinase C (PKC) has been observed in this region during retrieval of a discrete CS paired with cocaine during self-administration training (Thomas and Everitt, 2001). These data suggest that output from this region is reduced in response to cue exposure, a finding which may be of relevance to cue-elicited drug craving.

Alterations in G-protein-coupled receptor signaling pathways within the mPFC have recently been identified which may underlie potentiated responding to drug versus natural rewards and relapse to drug-seeking (Kalivas et al., 2005). The activator of G-protein signaling 3 (AGS3) is increased in the mPFC following withdrawal from cocaine self-administration, and reinstatement of cocaine seeking can be blocked by decreasing levels of AGS3. AGS3 sequesters the alpha subunit of inhibitory G proteins (Giα) and reduces signaling through Giα-coupled receptors such as D_2 dopamine receptors. It is thought that this reduction in D_2 receptor signaling leads to increased inhibition of PFC output to the NAc which can only be overcome by relatively strong inputs, such as drug reward. This hypothesis needs further investigation, but potentially provides a molecular mechanism to explain the increased control over goal-directed behavior exerted by drugs of abuse.

Increases in ΔFosB have also been reported in regions of the frontal cortex following chronic exposure to both addictive drugs and stressful manipulations (Perrotti et al., 2004). Recent evidence suggests that increased expression of ΔFosB within this region increases preference for sucrose, potentially indicative of an increased sensitivity to rewarding stimuli. Increased ΔFosB in this region also appears to sensitize animals to the locomotor stimulant actions of cocaine, yet produces tolerance to the disruptive effects of the psychostimulant on operant behavioral measures of motivation and impulsivity. These changes closely parallel those observed after chronic cocaine treatment. Further work aimed at understanding the changes in cognition caused by long-term drug use, and their underlying molecular basis, is clearly warranted.

4.12.9 Beyond Corticolimbic Circuitry: A Role for Hypothalamic Feeding Peptides in Reward-Related Learning?

The hypothalamus is one of the most important regions of the brain in terms of regulating more physiological aspects of reward such as the homeostatic control of hunger and thirst. Animals will preferentially self-stimulate the lateral hypothalamus (LH), a finding partially explained by the fact that dopaminergic fibers from the VTA to the NAc pass through this structure. Intriguingly, the threshold for LH self-stimulation in the perifornical region increases with weight loss, suggesting a relationship between the physiological homeostatic drive for natural rewards and the sensitivity of the brain to rewarding stimuli in general (see Shizgal et al., 2001). Although this region has not been associated with the more cognitive process of mediating goal-directed behavior, modulation of hypothalamic activity forms a critical part of the output pathway of the corticolimbic circuitry discussed earlier. The maintenance of energy balance depends on the allocation of behavior between feeding and competing activities; therefore, the signals of hunger or satiety generated by the hypothalamus have significant impact on the motivation for food reward and, therefore, potentially on numerous models of goal-seeking discussed previously. Whether modulation of this signal is also involved in assessment of the rewarding properties of addictive drugs is currently under investigation. The hypothalamic–pituitary adrenal axis is also one of the most prominent mechanisms by which the brain reacts to stress. Neurons in the paraventricular nucleus of the hypothalamus secrete corticotropin-releasing factor, which stimulates the release of adrenocorticotropin from the anterior pituitary. This in turn leads to production of glucocorticoids (cortisol in humans and corticosterone in rodents) within the adrenal cortex, which can have profound effects on behavior and brain function in numerous regions, as well as affecting general metabolism. Given that stress contributes to the development of affective psychiatric disorders such as depression and can trigger relapse to drug-seeking, a growing number of studies are addressing the role of signaling peptides within this region in reward-related learning and emotional processing. Some examples are considered in the following, though this is by no means an exhaustive list.

Melanin-concentrating hormone (MCH) is an orexigenic (pro-appetite) protein expressed within

the lateral hypothalamus. The MCH_1 receptor is highly expressed within the NAc, and intra-NAc infusions of MCH increase food intake, whereas antagonists of the MCH_1 receptor have the opposite effect. MCH_1 receptor antagonists acting within the NAc also exert antidepressant effects within the FST, an effect which is also observed in MCH knockout mice (Georgescu et al., 2005) and with systemic administration of MCH antagonists (Borowsky et al., 2002). These data suggest that molecules primarily thought to control the regulation of food intake can also have an effect on mood through their influence on NAc function.

Orexin (hypocretin) may have a similar role to play. Expressed within the lateral hypothalamus, orexin increases food intake by promoting a state of wakefulness and arousal, and deficits in orexin are known to cause the sleep disorder narcolepsy (Mignot, 2001). This debilitating condition, characterized by daytime sleepiness, cataplexy, and other sleep abnormalities, is frequently associated with depression, and some of the sleep-related symptoms are treated with antidepressants (Daniels et al., 2001). Narcolepsy and depression are both associated with alterations in circadian rhythms, and a dampening in the naturally occurring diurnal variation in orexin levels has been observed in depressed patients (e.g., Salomon et al., 2003). One mechanism by which hypothalamic peptides may influence reward processing may be via their modulation of the dopaminergic system. For example, orexin neurons project prominently to the dopaminergic cells of the VTA, where orexin binds to orexin 1 (OX_1) receptors to stimulate the neurons. Administration of an OX_1 receptor antagonist blocks the development of locomotor sensitization to cocaine (Borgland et al., 2006), whereas orexin precipitates relapse to drug-seeking in animals withdrawn from cocaine self-administration through induction of a stress-like state (Boutrel et al., 2005). Orexin knockout mice also show reduced physical dependence on morphine as indicated by a reduction in the physical signs of naloxone-precipitated withdrawal symptoms (Georgescu et al., 2003).

Functional interactions between the dopaminergic system and another hypothalamic peptide family, the melanocortins, have been reported and have likewise been implicated in mediating drug reward. Mice lacking the melanocortin-4 (MC_4) receptor, which is highly expressed within the NAc, fail to develop locomotor sensitization, and direct intra-NAc infusions of an MC_4 antagonist peptide, SHU-9119, reduces cocaine self-administration and place conditioning. As with BDNF, this peptide also prevents cocaine from potentiating the response to CRf (Hsu et al., 2005).

Neuropeptide Y (NPY) is perhaps best known for its ability to antagonize the behavioral consequences of stress within the central nervous system (CNS) (see Heilig, 2004). Administration of NPY is anxiolytic in numerous animal models, which is thought to result in part from its actions at Y_1 receptors within the amygdala. Acute stress decreases NPY expression, whereas chronic stress exposure, which leads to behavioral habituation, reverses this effect so that NPY is upregulated. The hypothesis that increased NPY expression could mediate coping responses is supported by the observation that NPY transgenic rats are less sensitive to stressful manipulations (Thorsell et al., 2000). In keeping with the view that stress promotes depression, at least in vulnerable individuals, antidepressant treatments also increase NPY within the frontal cortex, providing another mechanism by which antidepressant drugs may confer their therapeutic benefit. Dysregulation of NPY regulation has also been implicated in drug addiction, particularly in relation to alcoholism, where it is thought to mediate the anxiolytic properties of alcohol, thereby increasing motivation to consume the drug (see Valdez and Koob, 2004).

Whether induction of these hypothalamic feeding peptides is regulated by the same TFs as other proteins implicated in reward and addiction has yet to be determined. However, it is known that NPY is a downstream target of CREB, and whether the behavioral effects of NPY expression likewise vary depending on its locus of action remains a possibility.

4.12.10 Overview

Within this article, we have briefly considered the psychological processes involved in signaling rewarding events, and the roles played by different regions within the corticostriatal loop associated with reward-related learning. Through analysis of the intracellular signaling cascades affected by the dopaminergic system, specific molecules involved in mediating aspects of reward processing have been highlighted, and data pertaining to their influence over reward-related behavior have been discussed. In parallel to much of what is known regarding the neurochemical basis of reward signaling, it is clear that different molecules can have very different effects on behavior depending on their

locus of action. Likewise, the time course of molecular changes, whether transient or long-term, can have a profound influence on their behavioral consequences. The molecular tools have now been developed to directly manipulate intracellular signaling pathways and gene transcription at the level of different transcription factors and their downstream targets within highly circumscribed brain nuclei. As these advances in molecular biological techniques become more accessible, a wider array of behavioral and genetic studies will become possible. Although significant progress has been made in determining the role of such molecular events in reward-related learning, further integration between the fields of psychology and molecular biology will enable greater understanding of the biological basis of goal-directed behavior.

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4.13 Conditioned Taste Aversion and Taste Learning: Molecular Mechanisms

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4.13.1 Introduction

The sense of taste, together with that of odor, belongs to the family of chemical senses, and it is an important defensive sense, evolved to guide food intake and to aid in avoiding poisons. Moreover, the very existence of an organism is dependent on its ability to maintain intrinsic homeostasis in a continuously changing world. The guarding gate, both for the intake of energy and other metabolites necessary for the organism's survival and for the avoidance of poisonous substrates, is the sense of taste. In order to maintain homeostasis, the organism must recognize its bodily needs, identify the relevant substances that contain the means to satisfy these needs, and ensure that the process of ingestion maintains the balance between the needs and the intake. In addition and in parallel, the organism must avoid substances that will make it sick.

In order to consume the necessary and beneficial substances and to avoid the damaging ones that may cause malaise, the organism mainly uses taste and tags the substances as pleasant, indifferent, or unpleasant. However, the reaction of the organism to a specific substance is determined by a combination of automatic responses that developed during evolution and learning mechanisms that are plastic and can modify the perceived food value according to individual experience. An ethological view may account for the predisposition for different tastes according to the theory that the animals are well adapted to conditions that may occur with high probability and/or have critical survival value.

Learning the value of new tastes may be in line with or in contrast to this evolution-dependent genetic programming. There are just five different taste categories: Sweet, salt, bitter, sour, and the less well known umami (manifested in monosodium glutamate). However, combinations of different concentrations within these five categories and the additional information related to texture and temperature enable the characterization and identification of thousands of different tastes. Animals, including humans, can react to the various tastes by using two main strategies: Genetic programming (like sweet, dislike bitter), and complex learning mechanisms that involve the participation of several forebrain structures.

Taste learning has been found and studied in vertebrates and invertebrates and seems to be universal throughout the animal kingdom. However, most of the research into the biological mechanisms underlying taste learning has involved mice and rats, and the present chapter deals mainly with these studies. A simple sense stimulus such as taste can be very well defined in terms of a number of molecules (e.g., known molarities in a known volume). However,

even simple unimodal taste input includes not only the chemical properties of a substance, but also other physical dimensions, such as temperature and texture, and association with other cues and modalities.

This chapter does not address taste recognition on the receptor level, nor taste reactivity as it is defined genetically. The aim of this chapter is first to describe taste behavior in the context of laboratory attempts to identify molecular and cellular mechanisms of learning and memory, to present the various learning paradigms used in the laboratory, and the relevant neuroanatomy. Later, I will review and discuss in detail the molecular and cellular mechanisms of taste learning in the gustatory cortex, which reside in the insular cortex. I focus this chapter on recent publications, because the earlier development of the subject is covered in a seminal book by Jan Bures, Federico Bermudez-Rattoni, and Takashi Yamamoto (Bures et al., 1998). Finally, I present the current working model of taste memory formation, consolidation, and retention and suggest future research directions.

4.13.2 Measuring Taste Learning, Memory, and Consolidation: The Behavioral Paradigms

Learning is conventionally classified from the behavioral point of view into nonassociative (habituation and sensitization), associative (relationships between amounts and events), and incidental learning (learning in the absence of an explicit external reinforcer). In addition, memory can be classified according to the temporal phases of short- and long-term memories. The use of several different behavioral paradigms enables the different classification and temporal phases to be analyzed. Taste learning and conditioned taste aversion are considered to be implicit learning paradigms and they can result in short-term memory (hours) or lifelong memory (Bures et al., 1998; Houpt and Berlin, 1999). In animals, as in humans, a subject can prefer one taste over another without recognizing either (Adolphs et al., 2005).

The most familiar taste-learning paradigm is an association between taste and malaise: The process of conditioned taste aversion (CTA). In CTA learning, an animal learns to avoid a novel food associated with delayed poisoning (Garcia et al., 1955; Bures et al., 1988). CTA can be explained as an associative learning paradigm: The novel taste is the conditioned stimulus (CS), the malaise-inducing agent is the unconditioned stimulus (UCS), and the learned avoidance of the taste is the conditioned response (CR). However, it was clear from the first time that CTA was reported scientifically (Garcia et al., 1955) that CTA has very special and unique features.

The most prominent characteristic of CTA learning is the long delay between the novel food that serves as the CS and the toxic substance that serves as the UCS. This time frame of association is measured in hours (1–12 h) (Bures et al., 1998), which is in strong contrast to other forms of association, which tolerate time frames of a few seconds. The long delay between the CS and the UCS can be explained on the sensory level or in terms of the slow release of a substance from the stomach. Moreover, short time frames or backward conditioning do not yield good association and learning (Schafe et al., 1995). Thus, the long delay between the CS and the UCS in CTA should be explained in terms of the neuronal system subserving the CTA learning. Theoretically, the internal representation of the novel taste is kept in an on-hold position for many hours, ready for the UCS to produce the association. It is not clear how the internal representation of taste is stored, but it is hypothesized that it underlies ongoing activity that is dependent in part on the gustatory cortex (Katz et al., 2002; Bahar et al., 2003; Berman et al., 2003). CTA has other special features such as one-trial learning that produces strong and stable long-term memory; it can produce aversion to odor by odor potentiation taste aversion (Schneider and Pinnow, 1994), and, in contrast to the strong association between taste and malaise, there is no or very weak association between other sensory stimuli (e.g., sound, light) and malaise or discomfort. Similarly, there is hardly any association between taste and noninternal stresses, such as pain.

CTA can be affected if the CS is experienced either before the CTA, i.e., latent inhibition of CTA (LI-CTA), or after it, i.e., CTA extinction, as can other learning paradigms, but with specific characteristics for taste learning. In LI-CTA, if a taste stimulus was learned with no negative consequence there will be decreased aversion for the same taste following CTA (Rosenblum et al., 1993). This modulation in behavior can be attributed either to reduced strength of the association at the time of the CTA or to competition during the retrieval phase (Lubow, 1989). LI-CTA can be used successfully in a method

to study incidental taste learning, i.e., learning with no external reinforcer or association. One exposure to the novel taste has a significant effect on reducing the aversion elicited by CTA. However, a critical parameter is the amount of the novel taste consumed at the preexposure (Belelovsky et al., 2005), e.g., consuming 5 ml or less of a novel taste will produce no significant latent inhibition (LI) in rats (Belelovsky et al., 2005).

Experiencing a given taste after its association with malaise will reduce the aversion responses, i.e., the experience will cause an extinction of the learned CTA. The extinction of the association is dependent on the strength of the association, the number of extinction trials, and the amount of novel taste consumed during the session (Dudai, 2006). Extinction can be viewed as imparting an evolutionary advantage when the food supply is restricted and there is a constant need to test the negative or positive effects of a given food.

Two other behavioral phenomena that are important with respect to taste learning are attenuation of neophobia and taste interference. Neophobia to food, as to other stimuli, is manifested in the careful consumption of a novel food/taste. If a few exposures to the novel taste lead to no gastric consequences, consumption of the given food will increase and thus will reduce the primary neophobic response. In a similar way to extinction, attenuation of neophobia will mark a given taste as safe and will increase its consumption in response to other needs of the animal.

An interaction between different familiar and unfamiliar tastes can lead to overshadowing or blocking. However, in interference one taste can interfere with the learning of another taste (Merhav et al., 2006). Specifically, it was shown that when two novel tastes are given before an associated malaise, only the second taste will acquire the association (Best and Meachum, 1986), Moreover, by using the latent inhibition paradigm, it was shown that consumption of a novel taste after another taste will eliminate completely the effect of the first taste (Merhav et al., 2006). This interference was inversely correlated with the time between the two tastes, and could be established only if the second taste was novel but not if it was familiar.

The above behavioral paradigms were established mainly with rats and mice. In all methods, mild water restriction is needed to stimulate the animals to consume the taste in a specific time. However, harsh water deprivation can modulate the behavior, and the behavioral method itself determines the biological mechanisms underlying taste learning (Berkowitz et al., 1988; Bernstein et al., 1996a,b; El-Gabalawy et al., 1997; Koh et al., 2003; Wilkins and Bernstein, 2006). The following are the main methods used in these experiments.

1. Single pipette: Animals are presented with single pipette with a given taste. The amount of intake can be compared with the amount of water consumed on the previous day, following one of the behavioral manipulations described.
2. Multiple pipettes: A main problem with the single bottle setup is conflict between the animal's urges to drink, because it is water-deprived, and not to drink, because it underwent CTA. The most common behavioral test is to allow the animals to choose, during the retention phase, from a series of pipettes containing water or the taste that is under investigation. Usually, an aversion index is calculated (water/water + studied taste). The more aversive the animal is to the conditioned taste, the higher the aversion index will be.
3. Taste reactivity test: The amount of drinking measured in session with one or multiple pipettes does not necessarily mirror the attractiveness of a given taste. This can be measured directly according to several characteristic responses to palatable or unpalatable tastes, in a taste reactivity test (Grill and Norgren, 1978), or via the licking behavior (Halpern and Tapper, 1971).
4. Learning without ingestion: In some behavioral studies, it is possible or necessary, sometimes because of limitations of the experimental setup, e.g., when studying electrophysiology in the anesthetized animal, to test taste learning without ingestion or consumption of the novel food. In such cases, the test is performed by intraoral infusion of the food/taste onto the tongue to elicit the response of the taste buds. CTA and other taste learning can be acquired through this passive experience. However, it seems that learning with or without ingestion could involve different learning mechanisms (Bernstein et al., 1996a).

4.13.3 Neuroanatomy of Taste and Conditioned Taste Aversion Learning

The sensation of taste involves, similarly in principle to the other senses, chemical recognition but, in addition, the physical features are always associated with hedonic aspects of the sensory input. Indeed, functional analysis of the taste neuroanatomic

pathway reveals a strong association with the reward and feeding centers in the brain, including the ventral tegmental area (VTA), the nucleus accumbens (NAcb), the ventral palladium (VP), and the lateral hypothalamus (LH). The central gustatory pathway has been studied extensively in humans, monkeys, and rodents. **Figure 1** shows a schematic depiction of the rat's main taste pathway. Following activation of the taste buds, three cranial nerves (VII, IX, X) convey the taste input to the rostral part of the nucleus of the solitary tract (NTS), the first relay nucleus. In addition, the NTS receives input both from the area postrema (AP), which is sensitive to blood-transported toxins, and from the vestibular system, which is sensitive to nausea caused by motion. Lesioning this part of the NTS induces severe impairment of taste preference, but CTA can be still learned (Shimura et al., 1997). Taste information is that transduced from the NTS to the parabrachial nucleus (PBN) in the pons. The main taste-responsive neurons in the NTS project to medial subnuclei of the PBN, and the PBN projects both to the parvocellular part of the ventralis postmedial thalamic nucleus (VPMpc) and to other forebrain structures, including the amygdala, the lateral hypothalamus, the substati innominata, and the bed nucleus of the stria terminalis.

Yamamoto et al. (1995) studied the effects of lesions to various forebrain structures including the PBN, the hippocampus, the VPMpc, the gustatory

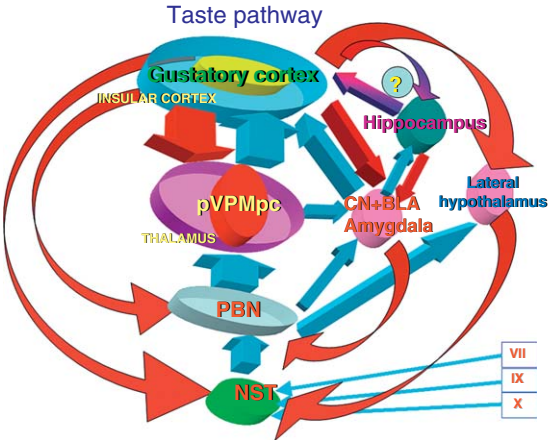


Figure 1 The neuroanatomy of the taste system. The processing of gustatory information begins with transduction of chemical stimuli which reach the oral cavity. Taste can be divided among five primary sensations: salty, sour, sweet, bitter, and umami. Typically, taste cells are broadly tuned and respond to several taste stimuli. The sensitivity to taste quality is not uniformly distributed throughout the oral cavity, and the same chemotopic arrangement is preserved to some degree at the gustatory relay. CN, central nucleus; BLA, basolateral amygdala.

Taste cells are innervated by cranial nerves VII, IX, X, which project to the primary gustatory nucleus in the brainstem (nucleus of solitary tract, NST). The NST sends information to three different systems:

1. The reflex system. This comprises medullary and reticular formation neurons which innervate the cranial motor nuclei (trigeminal, facial, hypoglossal).
2. The lemniscal system. The gustatory portion of the NST projects to the secondary nucleus situated in the dorsal pons (parabrachial nucleus, PBN). The PBN sends axons to the parvocellular part of the ventralis postmedial thalamic nucleus of the thalamus (VPMpc), which, in turn, relays gustatory information to the anterior part of the insular cortex (gustatory cortex, GC). The transition from the somatosensory lingual representation to the gustatory representation corresponds to the transition from the granular to the agranular insular cortex. The GC is thus situated dorsally to the intersection of middle cerebral artery and the rhinal sulcus and can be identified easily using these two markers. Although rodents have only a primary taste cortex, humans also have a second one.
3. The visceral-limbic system. The central gustatory pathway involves a collateral network of connections to the hypothalamus and limbic areas in the forebrain. The PBN is connected to the amygdala, the hypothalamus, and the bed-nucleus of the stria terminalis. All the limbic gustatory targets are interconnected with each other as well as with the PBN and the gustatory cortex. The GC and the thalamocortical system are required for acquisition and retention of taste information. The amygdala is required for learning the negative and possibly positive values of a taste. The prefrontal cortex is involved in CTA extinction. It is not clear what is the specific role of the hippocampus in taste learning, though it is hypothesized that it takes part in novel taste learning.

and enthorinal cortices, the amygdala, and the lateral and ventromedial hypothalamic nuclei, and reported that lesions to the PBN impaired both acquisition and retention of CTA. Other studies suggested that basic integration between taste and visceral inputs indeed took place at the level of the PBN.

The PBN projects to the VPMpc (Hamilton and Norgren, 1984), and from the relay station in the thalamus the taste information is transuded to the gustatory cortex (GC), which resides within the anterior portion of the insular cortex. Small lesions to the VPMpc did not affect CTA learning nor retrieval (Reilly and Pritchard, 1996), but a combination of lesions to the VPMpc and the GC eliminated CTA learning (Yamamoto, 1995).

Humans and monkeys have an additional, secondary area of taste, and it has been suggested that subdivisions within the insular cortex might serve as a secondary taste area in the rat brain. Similarly to the way the subcortical areas convert taste information, as described, the insular cortex also processes both taste information (in its anterior part) and visceral information (caudodorsally to the GC).

The first indication of the role of the GC in processing taste information was provided by Braun et al. (1972). Later, many experimental techniques, based on lesions, electrophysiology, imaging, correlative biochemistry, pharmacology, and, recently, direct imaging studies, were proven to be useful in analyzing the role of the GC in taste learning. It is clear that the GC plays a pivotal role in CTA acquisition and retention. Reversible inactivation of the amygdala and the insular cortex by microinjection of tetrodotoxin (TTX) to these two brain structures at different intervals before taste learning suggested that the insular cortex is pivotal for taste learning, whereas the amygdala is crucial for CTA formation (Gallo et al., 1992). The insular cortex and its gustatory portion can be anatomically divided into granular (normal neocortex), dysgranular, and agranular cortices (i.e., the gradual disappearance of the fourth layer). In rodents, most of the neurons that are responsive to taste stimuli reside within the dysgranular insular cortex. However, the input from the VPMpc terminates in both the granular and the dysgranular insular cortices. A topographical spatial organization of the GC in relation to the various taste stimuli was suggested recently by means of direct imaging of the GC *in vivo* (Accolla et al., 2007).

The experience of taste has other dimensions than the chemical input itself, including temperature and structure. These dimensions were hypothesized to be processed by the adjacent cortex, but also by the granular insular cortex itself (Simon et al., 2006).

The hippocampus, a forebrain structure known to be involved in many forms of learning, has been investigated also in relation to its role in taste learning. The role of the hippocampus in CTA is controversial; however, its involvement in neophobic responses to taste has been reported in several experiments. A temporal correlative response was found in the hippocampus and the GC. However, different molecular pathways were activated in the hippocampus and in the insular cortex (Yefet et al., 2006).

4.13.4 Long-Term Potentiation in the Insular Cortex

Long-term potentiation (LTP) is an attractive model for learning and memory: Activity-dependent, sustained increases in synaptic efficacy have been suggested to be the cellular manifestation of the learning process (Bliss and Collingridge, 1993; *See* Chapter 4.16). LTP was first described in the hippocampus but has been investigated in other brain structures, including the cortex; recently it was studied in the pathway from the basolateral amygdala to the insular cortex (Escobar et al., 1998) and in correlation with taste learning. High-frequency stimulation to the basolateral amygdala induced *N*-methyl-D-aspartate (NMDA)-dependent but metabotropic glutamate receptor (mGluR)-independent LTP in the insular cortex (Escobar et al., 1998, 2002). A pharmacological administration of the neurotrophin brain-derived neurotrophic factor (BDNF) locally to the insular cortex induced LTP that inclined slowly in a similar way to BDNF-induced LTP in the hippocampus (Escobar et al., 2003). Analysis of the molecular mechanisms of basolateral amygdala-insular cortex (IC) LTP identified correlative induction of extracellular signal-regulated protein kinase (ERK) activation and muscarine-dependent induction of several immediate early genes, including Zif268, Fos, Arc, and Homer (Jones et al., 1999). In a similar way to novel taste learning and to LTP in other brain structures, ERK was both correlative and necessary for LTP expression in the IC (Jones et al., 1999). It is clear from other studies that LTP and taste learning share molecular mechanisms in the IC. However, very little is known about the hypothesized possibility that LTP-like mechanisms in the IC subserve taste learning. A study that examined the possible interaction

between the two found that LTP in the insular cortex enhanced CTA retention (Escobar and Bermudez Rattoni, 2000). However, much more investigation is needed to achieve better identification of the relevant circuit within the IC, and to prove that LTP-like processes underlie taste learning in the GC.

4.13.5 Processing of Taste in the Gustatory Cortex

Neuronal responses in the GC are driven by somatosensory and chemosensory inputs received from the oral cavity (Yamamoto et al., 1989; Ogawa et al., 1992a,b). Neurons in the GC are responsive to both the quality of a given taste, i.e., chemical identification, and its hedonic value, i.e., attractive, palatable, or repulsive (Yamamoto et al., 1989), and it is difficult to dissociate the two from one another. This may represent a unique feature of the taste sensory information, whereby a given stimulus is always tagged as pleasant, indifferent, or repulsive, i.e., this value represents one of the dimensions of gustatory processing, including the chemical and physical dimensions. Relatively very few cells (<10%) are responsive to a specific taste (Yasoshima and Yamamoto, 1998; Bahar et al., 2003), and most of the others are broadly tuned. Recently, however, direct imaging of the insular cortex was used to reveal some spatial distribution of the various taste stimuli within the GC (Accolla et al., 2007). An interesting correlation between neuronal activity in the GC and novel taste input is that the increased response to a novel taste was not detected until 1 day following learning (Bahar et al., 2003). Currently, there are no good models of taste coding in the taste system. The two main models of taste coding in the neuronal system are labeled line and cross-fiber patterns. Both models are static and noninteractive and are insufficient to describe taste learning (Katz et al., 2002). Another hypothesis is that the anatomical divisions of the insular cortex, i.e., granular, dysgranular, and agranular, serve as primary, secondary, and tertiary sensory cortices, respectively.

A recent review on the subject suggested that the gustatory pathways, including the GC, use distributed, ensemble codes of the various dimension of taste stimuli, i.e., chemical, thermal, and tactile, to produce an internal representation of a given taste (Simon et al., 2006). Other reviews summarized a vast amount of seminal research on taste, olfactory, food texture, and control of food intake (Rolls, 2004, 2006), but that research is not within the scope of the current chapter and mainly addressed monkeys.

4.13.6 Molecular Mechanisms of Taste Learning in the Taste Cortex

The previous sections introduced the behavioral, neuroanatomical, and cellular levels of analysis. The sections below discuss aspects of molecular mechanisms that subserve taste learning in the taste cortex, within the frameworks of the behavior and the neuroanatomy, in more detail. The working hypothesis underlying the research in the field is that during learning, physical or chemical information about the world is depicted in part by the organism's sensory system, i.e., in the present case, the taste system. This information is transformed into neuronal activity that uses neurotransmitters to create an internal representation of the received sensory information in the central nervous system (CNS). This neuronal creation or modification of an internal representation, i.e., sensory learning, comprises several different temporal phases: Acquisition, which immediately follows experiencing the sensory input, the imprinting of the internal representation in the CNS, a consolidation phase that is divided into two major processes: molecular consolidation, which is dependent on functional protein synthesis in the relevant brain area and is limited to several hours following learning; and system consolidation, which involves the transfer of information between brain areas and which has a temporal domain ranging from many days to months. The consolidation phase is defined in negative terms; it is the time window during which the memory trace is still fragile and can be disrupted by various interventions: behavioral (e.g., Merahv et al., 2006), pharmacological (Rosenblum et al., 1993), or others (Bures et al., 1998). In addition, it is highly likely that the consolidation phase contains subdivisions. For a given internal representation, the last phase is retrieval. However, any retrieval may have the dimension of relearning, as discussed in detail elsewhere (*See* Chapters 1.05, 2.16).

Taste learning and CTA are highly suitable learning paradigms for studying the molecular mechanisms of learning and memory. Their relevant features include one-trial learning; strong incidental learning; clear and short learning time; minimal behavioral manipulation, since the animals can learn in their home cage with very little interference from

other modalities; clear definition of the sensory input, which can be quantified in molecular terms; and clearly defined cortical area(s) subserving the learning. Within the framework of the present chapter, we will specifically discuss molecular mechanisms taking place in the GC which underlie the various phases of taste learning. It is important to note that two basic means of acquiring information are studies of the correlations between taste behavior and molecular modifications/processes and causality experiments, which show that a given biochemical pathway is necessary for a given phase in taste learning. In addition, it is highly likely that one signal transduction cascade can operate in one brain area and not another. For example, protein kinase B (PKB)/Akt phosphorylation is correlated with taste learning in the hippocampus but not in the GC (Yefet et al., 2006); also, ERK is activated in the GC but not in the hippocampus after the same length of time after learning (Yefet et al., 2006). These examples suggest that from the neuroanatomical point of view, positive correlation between molecular changes and learning is informative, whereas negative correlation can teach us very little. A summary of recent molecular observation related to taste learning and mainly to the taste cortex is presented in the following sections.

4.13.7 The Neurotransmitters in the Gustatory Cortex Involved in Taste Learning

Information on the physical and chemical properties of a given taste and also on its prominence reaches the GC via different neurotransmitters. Several different neurotransmitter systems are released in the GC, and the relevant receptors for these neurotransmitters are expressed in the GC. These include acetylcholine (ACh), dopamine, noradrenaline, gamma-aminobutyric acid (GABA), glutamate, and various neuropeptides. However, only the muscarinic and NMDA receptors have been studied extensively for their role in taste memory acquisition, consolidation, and retention. I will, therefore, focus mainly on these two neurotransmitter systems and their possible role in taste memory formation. The physical and chemical taste information is transferred from the oral cavity to the cortex via fast neurotransmission mediated by the neurotransmitter glutamate. This is consistent with other known modalities (*See* Chapter 4.30). Glutamate is the main excitatory neurotransmitter in the mammalian CNS, and it acts via both ionotropic and metabotropic effects. However, the prominence of a given taste is hypothesized to be mediated via activation of the neuromodulatory system (e.g., Kaphzan et al., 2006). It is thus conceivable that the interaction between the two systems produces a long-term taste memory trace and that it coincides on specific neurons and probably molecules that can serve as coincidence detectors of the sensory input and its meaning (Kaphzan et al., 2006). Glutamate can affect four types of receptors – alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), NMDA, kainate, and mGluRs – that can produce complex fast (ion influx) and slow (second messenger) changes in the neuron. These receptors are structurally and functionally multifaceted molecules (*See* Chapter 4.30). Microdialysis studies of the amygdala and the IC demonstrated enhanced glutamate release in both the amygdala and the IC, following induction of malaise (Miranda et al., 2002).

Application of the AMPA/kainate antagonist, NBQX, specifically to the insular cortex impaired both acquisition and retrieval of taste memory, which suggests that, in agreement with the general understanding, the AMPA receptor plays a major role in mediating the physical properties of the taste (Berman et al., 2000) and that normal activity of the AMPA receptor is needed to mediate taste properties in either learning or recognition. In contrast to the effects on the AMPA receptor antagonist, microinjection of the NMDA antagonist aminophosphonovaleric (APV) into the GC induced severe impairment of CTA and taste memory acquisition, but not of retrieval (Rosenblum et al., 1997; Berman et al., 2000). Thus, taste learning and CTA are dependent on the NMDA receptor in the insular cortex similarly to many other learning paradigms. Moreover, LTP in the insular cortex is NMDA-dependent, which suggests that similar molecular mechanisms subserve learning and LTP (Jones et al., 1999).

Application of the NMDA antagonist specifically to the insular cortex blocked taste memory formation and reduced the correlative activation of ERK (Rosenblum et al., 1997; Berman et al., 2000). It did not, however, reduce the basal level of ERK phosphorylation (Berman et al., 2000) as it did in hippocampal slices (Kaphzan et al., 2006). In addition, the NMDA antagonist, APV, did not affect the correlative increase in tyrosine phosphorylation of the NMDA receptor, suggesting that this increased phosphorylation is

dependent on neuromodulatory neurotransmitters, most probably ACh (Rosenblum et al., 1997). Interestingly, APV microinjection to the insular cortex impaired long-term memory but did not affect short-term memory (Ferreira et al., 2002). These results are in contrast to the dogma that the NMDA receptor is crucial for memory formation but not for its consolidation, and may hint at the existence of unique molecular mechanisms that subserve the CS in the CTA case and that remain active for many hours.

Posttranslation modification and, especially, phosphorylation of the glutamate receptors, was hypothesized to play a major role in learning and LTP induction (*See* Chapter 4.30). The dogma suggests that changes in the steady state of the phosphorylation site of a specific receptor, e.g., the AMPA receptor, caused by modulation of the activity of kinases or phosphatases, alter the function of the receptor so as to increase or decrease synapse efficacy and thus to imprint a new cellular structure in the brain, which is manifested in the formation of an internal representation. However, on the assumption that the kinase/phosphatase modulation of activity is reversible, the memory of such a change is limited to the time span of the receptor that underwent phosphorylation. Different proteins are known to have different half-lives but, in any case, this cannot account for structural stability lasting more than a few days. In addition, the very rapid turnover of the receptors between different subcellular compartments shortens the time during which the receptor is expressed in the brain. Phosphorylation occurs on serine, threonine and, to a much smaller extent, tyrosine residues. However, tyrosine phosphorylation events have been found to be crucial in many cellular functions (Huang and Reichardt, 2003).

In the forebrain of mature animals, the NMDA receptor is composed of the NR1 subunits and a combination of NR2A and B subunits (*See* Chapter 4.20). The main tyrosine phosphorylated protein in the synapse is the 2B subunit of the NMDA receptor (Moon et al., 1994). However, it is clear that the 2A subunit is phosphorylated on tyrosine also, and in response to physiological inputs (Thornton et al., 2003). Tyrosine phosphorylation of both subunits induces modulation in the receptor function (Kalia et al., 2004), and it can be measured for a specific residue of a given protein, e.g., 1472 of the NR2B subunit, or as total tyrosine phosphorylation of a given protein after immunoprecipitation, or of a population of proteins. This measurement can be taken using antiphosphotyrosine antibodies for a population of proteins. Measuring tyrosine phosphorylation using general antiphosphotyrosine revealed that, compared with other tissues, the brain is highly phosphorylated on tyrosine residues in the absence of stimulation. In addition, following CTA or novel taste learning, tyrosine phosphorylation of a set of proteins was increased, but a familiar taste did not induce this correlative effect (Rosenblum et al., 1995). Following taste learning, the main molecular weights to be modulated in the insular cortex were 100, 115, and 180 kDa. At the same time, other proteins were unaffected by learning (Rosenblum et al., 1995). With regard to the findings of many other studies that identified correlations between posttranslation modifications and learning, the following questions can be raised in attempting to understand mechanistically the role of tyrosine phosphorylation in the GC during the formation of the internal representation of a taste:

- What is the identity of the tyrosine-phosphorylated proteins?
- What is the identity of the specific residue that is phosphorylated in a given protein?
- Is this phosphorylation event a mere correlation or a necessary step in memory formation?
- Can similar modifications be found in other learning paradigms or learning models?
- What is the time window and what is the physiological function of such phosphorylation?
- What are the upstream events that induce this phosphorylation in correlation with learning, and which neurotransmitters induce the modifications?
- What, and in which cortical layer, are the cells that are involved in these modifications? What is the localization, i.e., subcellular compartment, of such modification within the cells?

There are answers to some of these questions, whereas others are still subjects of ongoing research. It is clear that the NR2B is tyrosine phosphorylated in correlation with taste learning in the taste cortex (Rosenblum et al., 1997); a similar increase in NR2B tyrosine phosphorylation was detected in the hippocampus following LTP (Rosenblum et al., 1996; Rostas et al., 1996). As for the specific residue, the main tyrosine to be phosphorylated in the sequence of the NR2B is residue 1472. Indeed, a clear increase in pY1472 was correlated with taste learning in the GC. A recent study of genetic replacement of pY1472 in the mouse forebrain identified malfunction in amygdala-dependent learning, and suggested

that the localization of the NR was defective in these transgenic mice (Nakazawa et al., 2006). In any case, mapping of the phosphorylation sites specifically on the NR and generally in synaptic proteins, which might explain synaptic plasticity, is currently the subject of proteomic investigation (Schrattenholz and Soskic, 2006).

In order to test the hypothesis that induced tyrosine phosphorylation is necessary for taste learning, the general tyrosine kinase inhibitor, genistein, was injected locally into the GC during taste learning; it inhibited the induced tyrosine phosphorylation of the NR2B and attenuated taste learning, which suggests that tyrosine phosphorylation during learning is crucial for memory formation.

What might be the identity of the neurotransmitter that induces tyrosine phosphorylation of the NR2B? Surprisingly, local blocking of the NMDA receptor itself by microinjection of APV into the GC blocked taste learning but did not affect the induced level of its tyrosine phosphorylation correlated with learning (Rosenblum et al., 1997). Thus, it seems that the process is not Ca^{2+} dependent and is involved in cross-talking with other receptors. Indeed, pharmacological activation of the muscarinic ACh receptor (AChR) in the GC induced tyrosine phosphorylation of the NR2B (Rosenlum et al., 1996). This cross talk between the two receptors may partly account for the important role played by muscarinic AChRs in novel taste learning. An interesting point is that the time window of increased tyrosine phosphorylation correlated with taste learning. The induction was still clear several hours following learning, in clear contrast to the behavior of other phosphorylation events that can be monitored only within minutes following learning. It is therefore suggested that the synaptic tyrosine phosphorylation represents an intermediate phase of molecular mechanisms and that it is not involved in the acquisition phase but rather in the consolidation phase. Another possibility is that this prolonged increase in tyrosine phosphorylation following learning is unique to taste learning and plays a role in the ability of the taste to be on hold, ready for association with malaise.

The involvement of muscarinic AChRs in taste and CTA learning was studied mainly through the administration of antagonists during the various phases of taste learning, followed by microdialysis measurements of ACh in the GC. Indeed, local application of atropine or scopolamine to the GC disrupted both taste learning and CTA (Naor and Dudai, 1996; Gutierrez et al., 2003; Berman et al., 2000). It was suggested that microinjection of scopolamine but not APV to the insular cortex attenuates the acquisition of familiarity of a novel taste (Gutierrez et al., 2003), though APV clearly affected latent inhibition of CTA (Rosenblum et al., 1997). Another study suggested that APV treatment impaired only long-term but not short-term taste memory (Ferreria et al., 2002). In a similar way to the prolonged duration of NR2B tyrosine phosphorylation, this result implies that the NR is more involved in the consolidation than in the acquisition processes. Microdialysis experiments have shown that ACh is released in the GC following consumption of novel but not of familiar tastes (Shimura et al., 1995; Miranda et al., 2000) in a similar way to other modalities. It is yet to be determined whether the prolonged endogenous release of ACh is the physiological reason for tyrosine phosphorylation of the NR; what is the exact function of such interaction? Other biochemical interactions between these two receptors may occur and await further analysis.

The glutamatergic and cholinergic systems were studied in detailed in the GC following novel taste aversion. Fascinatingly, the correlated expression of different immediate early genes in the GC that followed LTP induction was dependent both on NMDA and muscarinic AChR activation. However, the involvement of other neurotransmitters is well documented. Local microinjection of antagonists for the $GABA_AR$, dopamine (D1, 5), mGluR, and β-adrenergic neurotransmitters into the GC impaired both novel taste learning and CTA acquisition. However, only application of the AMPA/kainate antagonist and the $GABA_AR$ impaired CTA retrieval (Berman et al., 2000). A recent report suggests that metabotropic GABA receptors are differentially involved in CTA learning. The $GABA_{B(1a)}$ receptor was found necessary for CTA learning, whereas the $GABA_{B(1b)}$ receptor was involved in extinction of CTA (Jacobson et al., 2006), but the brain locus involved in these manipulations could not be identified. Various pharmacological tools have been used to assay the correlative induction of ERK with taste learning. It was found that antagonists for AMPA/kainate, dopamine (D1, 5), mGluR, and agonist of the $GABA_AR$ reduced the basal level of ERK activation, whereas $GABA_AR$ antagonist induced the levels of ERK activation in the GC (Berman et al., 2000). However, antagonists of the muscarinic AChR, NMDAR, AMPA/kainate, dopamine (D1, 5), and mGluR inhibited the induced expression of ERK with learning (Berman et al., 2000). It is clear from these experiments

that cortical excitation is positively correlated with ERK activation and that antagonizing the muscarinic and the NMDA receptors is correlative with both taste and CTA learning, and with the correlative induction of ERK.

4.13.8 The Role of the MAPK/ERK Pathway in the Gustatory Cortex

ERK1 and ERK2 belong to the mitogen-activated protein kinase (MAPK) family of signal cascades. ERK is activated in and is necessary for the development of several forms of memory, such as fear conditioning, CTA memory, spatial memory, step-down inhibitory avoidance, and object recognition memory (*See* Chapter 4.25). Inhibition of MAP/ERK (MEK), the upstream kinase of ERK, affected both early and late phases of LTP in the hippocampus (Rosenblum et al., 2002). The role of ERK activation in the GC was first studied by means of radioactive kinase assays and, indeed, increased ERK activity was found in the GC, in correlation with novel taste learning. Later, most of the experiments in the field used phospho-specific antibodies that recognized the phosphorylated state of ERK as well as the activated state of these proteins in this unique case (*See* Chapter 4.25).

ERK activation was correlated with novel taste learning, whereas the actual amount of ERK protein was unchanged (Berman et al., 1998; Belelovsky et al., 2005). The time scale of the induced activation was a few minutes following consumption of a novel taste, and it could not be detected after more than 1 h. Microinjection of the MEK inhibitor into the GC prior to learning attenuated CTA memory; examination of other members of the MAPK family revealed that Jun N-terminal kinase 1/2 (JuNK1/2) was activated 1 h following novel taste learning, whereas p38 was not modified at any of the examined time points (Berman et al., 1998). A similar study in mice identified different temporal activation of ERK following novel taste learning (Swank and Sweatt, 2000). Further examination of the phosphorylation of the ERK substrate ELK-1 found a similar time scale for the relation between ERK activation and ELK-1 phosphorylation following novel taste learning as well as similarity in the neurotransmitters that induce ELK-1 phosphorylation in the GC in response to taste learning (Berman et al., 2003). Interestingly, the expression of LTP in the GC was found to be ERK dependent, and ERK was activated in correlation with LTP induction in the GC (Jones et al., 1999).

Similarly to other brain areas, ERK activation is correlated with learning, and possibly mainly with the consolidation phase of learning. Moreover, as discussed, the upstream mechanisms for ERK activation have been studied extensively in the GC and elsewhere. However, we know very little about the downstream targets of ERK or about the mechanisms in neurons that affect learning and synaptic plasticity. Much more proteomic research is needed to identify ERK substrates and to understand the effects of ERK on various neuronal processes, including trafficking, membrane properties, and nuclear targeting. One major role attributed to the ERK pathway is regulation of gene expression and, recently, translation regulation (Govindarajan et al., 2006).

4.13.9 The Role of Translation Regulation in Taste Memory Consolidation

Memory consolidation is defined biochemically by its dependence on functional protein synthesis within the relevant brain structures (Davis and Squire, 1984). Indeed, local application of the protein synthesis inhibitor anisomycin to the GC attenuated CTA and taste learning, as measured in the latent inhibition paradigm (Rosenblum et al., 1993). However, another study found that the same treatment had no effect on short-term memory, which suggests that short-term taste memory is independent of protein synthesis (Houpt and Berlin, 1999). Local application of anisomycin had a dose-dependent effect on CTA learning: Whereas 50- and 75-μg doses had no effect, 100- and 150-μg doses abolished CTA. This study (Rosenblum et al., 1993) is one of very few that measured the effect of anisomycin on protein synthesis *in vivo*. Local application of anisomycin inhibited more then 90% of protein synthesis in the GC for hours, but not in the hippocampus. Application of the same amount of anisomycin to the lateral ventricles did not affect either taste learning or CTA, and had a weaker and much faster effect on protein synthesis in the GC (Rosenblum et al., 1993; Meiri and Rosenblum, 1998). Usually, in order to achieve an effect on memory and LTP consolidation, one should apply the protein synthesis inhibitor(s) just before or immediately after learning, though some studies have suggested the existence of two or more sensitive periods for application of protein synthesis inhibitors. A recent study determined the temporal phase of sensitivity for

protein synthesis inhibitors, and found that up to 100 min following novel taste learning, local application of anisomycin attenuated taste learning, which suggests that the short period after learning, i.e., the beginning of consolidation, is not the only sensitive period, but so, also, is a more advanced phase of consolidation.

As discussed, sensitivity for protein synthesis is a negative definition of memory consolidation. One may assume that following learning there is increased expression of proteins in the relevant brain areas (*See* Chapters 4.22, 4.33), but it was only recently that scientists began to explore directly the possibility that the translation machinery is regulated during memory consolidation (Govindarajan et al., 2006). Translation comprises initiation, elongation, and termination phases. The initiation phase is the most highly regulated step and it is modulated via phosphorylation of initiation factors and ribosomal proteins (Proud, 2000). Analysis of taste learning in mice that lacked the translation repressor eukaryotic initiation factor 4E-binding protein (4E-BP2) revealed no difference in taste recognition but enhanced CTA learning (Banko et al., 2007). Similarly, mice with reduced phosphorylation of eukaryotic initiation factor 2 (eIF2α) exhibited enhanced taste learning, with no effect on taste recognition (Costa-Matiolli et al., 2006). In addition, it is clear that in these mice other forms of learning and plasticity are enhanced during the consolidation phase. Both the 4E-BP2 and eIF2α mice represent genetic modifications that enhanced translation initiation and thus induced better taste learning and memory. In contrast, knockout mice for both S6K1 and S6K2, which are characterized by reduced initiation rates, exhibited impaired taste learning.

Analysis of the initiation phase suggests a simple image: More initiation, better taste learning. However, the picture is more complex when the elongation phase of translation is analyzed.

The elongation phase requires activity of eukaryotic elongation factors (eEFs). Eukaryotic elongation factor 2 (eEF2) mediates ribosomal translocation (Ryazanov and Davydova, 1989) and is phosphorylated on Thr56 by a specific Ca^{+2}/calmodulin-dependent kinase. Phosphorylation of the kinase inhibits its activity and leads to general inhibition of protein synthesis (Nairn and Palfrey, 1987). Analysis of eEF2 phosphorylation in the GC, following novel taste learning, revealed that, in contrast to the simple hypothesis, the phosphorylation of eEF2 was increased and not decreased, which indicates an attenuation in translation elongation (Belelovsky et al., 2005). At the same time and among the same samples, the phosphorylation levels of S6K1 and ERK were increased, which suggests that the initiation levels were indeed increased (Belelovsky et al., 2005). On the assumption that the increased initiation and decreased elongation were taking place in the same neurons in the GC, one may suggest that this situation might lead to increased expression of mRNAs that are poorly initiated (see **Figure 2** for the proposed model). The suggested mechanisms could serve as a switch-like mechanism to express a specific set of mRNAs for a restricted time in a cellular microdomain such as the synapse.

It is clear that in the near future much more research will be aimed at understanding how regulation of the various phases of translation subserves consolidation processes. Other important proteins such as mammalian target of rapamycin (mTOR) and ERK are thought to be molecules that integrate the information delivered by the various neurotransmitters, in time and space, and translate it into a cellular decision that enables the consolidation of memories. In the near future, these very same molecules could serve as targets for possible cognitive enhancers in the consolidation phase.

4.13.10 Modulation of Specific Protein/mRNA Expression During Taste Learning and Consolidation

In many studies, a positive correlation was identified between enhanced expression of protein and/or mRNA, on the one hand, and learning in the relevant brain area(s), on the other hand (e.g., Guzowski, 2002). This correlation was assumed to be the positive aspect of the dependence of memory and synaptic plasticity on protein and mRNA inhibitors. As shown in **Figure 3** and discussed in detail earlier, many phosphorylation events are correlated with both novel taste learning and CTA learning. However, two conceptual observations can be derived from these findings. First, the posttranslation modulation that is correlated with novel taste learning is attributed to the meaning/prominence/novelty dimension and not to the physical/chemical dimension of the taste information (**Figure 3**); a familiar taste does not induce these correlations. One may suggest that the synaptic and neuronal activities underlying the formation of the taste memory *per se* are so small compared with the ongoing activity in the cortex that the biochemical methods used in these studies are not sensitive enough

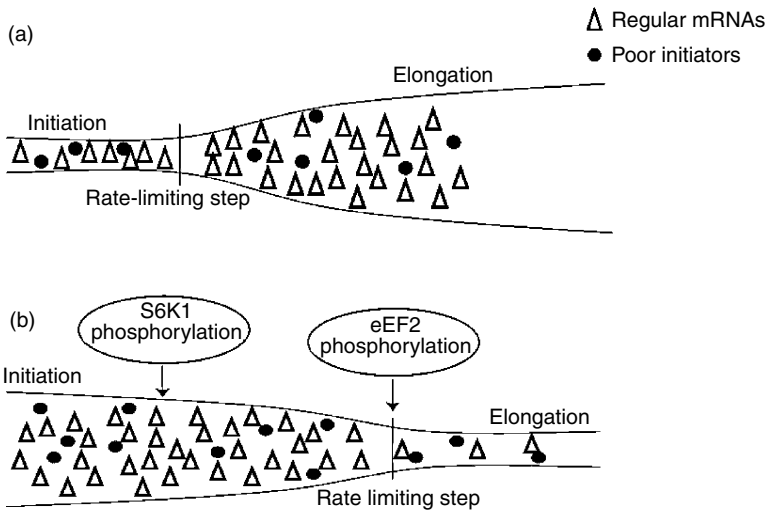


Figure 2 A proposal for the role of translation regulation underlying memory consolidation. (a) In a normal situation the rate-limiting step of protein synthesis is the initiation phase. The amount of protein synthesis is dependent on mRNA availability and the initiation rate as determined by ribosomal proteins and initiation factors. For a given amount of mRNA, regular mRNAs (triangles) are translated more than poor initiators (circles). A component of the intracellular signaling can alter the initiation rate, e.g., as in MAPK activation. (b) Taste memory consolidation is correlated with an increased initiation rate, which can be measured as increased ERK2 activation and decreased eIF2α phosphorylation and S6K1 phosphorylation. Within the same time frame, and possibly within the same cellular compartment, there is also a decrease in translation elongation, which can be detected in the increase in eEF2 phosphorylation. The end result of increasing initiation and decreasing elongation may be a shift in the rate-limiting step to the elongation phase. Together with the decrease in total protein synthesis, the synthesis of poorly initiated proteins such as α-Ca^{2+}/calmodulin-dependent protein kinase II (α-CaMKII) is probably increased. The mechanism described here can perform a switch-like function for various biological processes that shift protein expression patterns within a restricted time scale. In the cortex the mechanisms described can serve as molecular mechanisms that consolidate changes in synaptic strength over time. Adapted from Belelovsky K, Elkobi A, Kaphzan H, Nairn AC, and Rosenblum K (2005) A molecular switch for translational control in taste memory consolidation. *Eur. J. Neurosci.* 22: 2560–2568 with permission.

to detect the biochemical alterations subserving the physical properties of the taste. However, it is clear that some of the observed correlations (Rosenblum et al., 1997; Belelovsky et al., 2005) can be detected in the synaptosomal fraction. Another possibility lies in the strong link between taste and its perceived quality that is less inherent in other modalities. Taste is always perceived as good, bad, or indifferent, and thus the qualitative value of the physical input is a major building block in the memory of any taste.

The second observation is the difficulty of distinguishing between the biochemical correlations with the CTA or with the UCS itself, the malaise. In many experiments, the correlation between CTA and biochemical alterations can be detected following the UCS itself. In addition, the feeling of sickness is a strong input that can modulate several different parts of the brain.

What about mRNA or protein expression in the insular cortex that is correlated with taste learning or CTA?

The most common immediate early gene to be studied in correlation with learning is c-fos. Indeed, acute suppression, but not chronic genetic deficiency, of c-fos attenuated CTA learning (Yasoshima et al., 2006). Correlative studies for c-fos expression were carried out in Ilen Bernstein's laboratory (Wilkins and Bernstein, 2006; Koh et al., 2003; Koh and Bernstein, 2005) and elsewhere (Houpt et al., 1996; Ferriera et al., 2006). Exposure to a novel but not to a familiar taste induced c-fos expression in the central amygdala and the GC. The injection of the malaise-inducing agent LiCl induces c-fos in several brain structures, including the central amygdala and the IC. It is important to note that the posterior part of the IC processes visceral information and, therefore, it is possible that c-fos expression is not located in the same brain loci. The peak in c-fos expression can be detected about 1 h following novel taste learning and the malaise induced by the LiCl injection, and is degraded thereafter similarly to the processes in other brain areas subserving other learning paradigms (Wilkins and Bernstein, 2006).

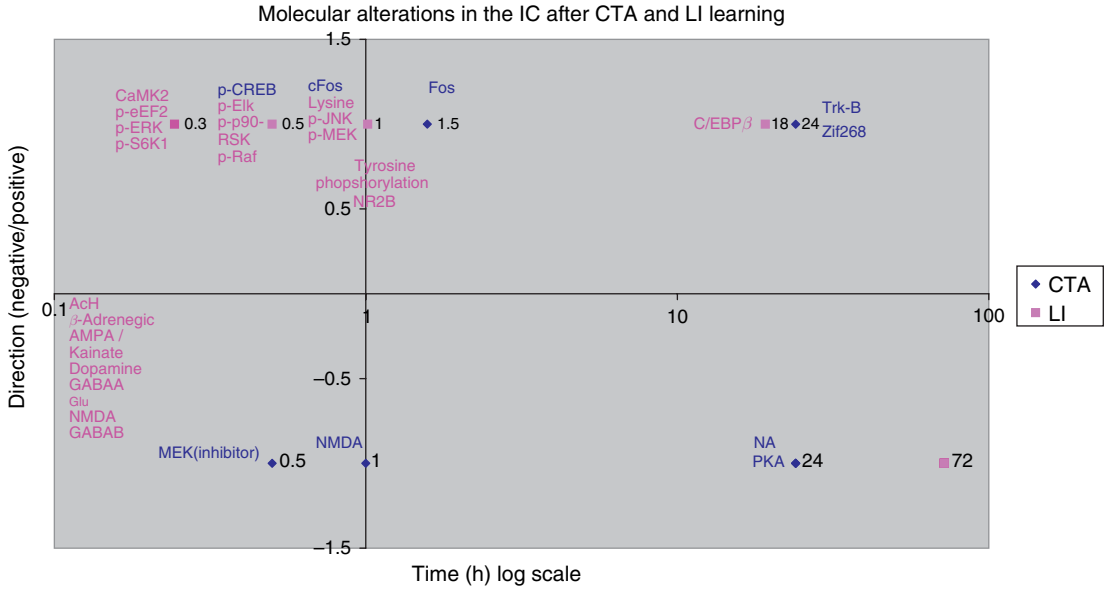


Figure 3 Summary of recent molecular analysis of taste learning in the rodent brain. The figure summarizes recent publications that examine the molecular mechanisms of taste learning; it shows whether the changes are correlative or are necessary for memory formation, and the direction of the changes. In addition, the figure indicates the method that was used and the temporal window of the changes. As can be seen, the research by Swank and Sweatt (2001) that used solid food with high metabolic value revealed a different time scale from that revealed by the other studies, which used liquid. In addition, the data in the figure can be divided into those related to CTA or novel taste learning; gene expression modulation, e.g., c-fos, or posttranslation regulation, e.g., MAPK activation; and manipulation affecting acquiring, retention, or both.

Another protein to be induced in correlation with taste learning in the GC is CCAAT enhancer binding protein β (C/EBPβ). However, the striking difference lies in the temporal phase of its induced expression. C/EBPβ is induced in both the hippocampus and the GC 18 h after taste learning (Yefet et al., 2006). Such late expression can be found following other learning paradigms (Taubenfeld et al., 2001). The correlative temporal increase in C/EBPβ 18 h following novel taste learning suggests that in a simple implicit learning task such as taste learning, both the hippocampus and the cortex are engaged in memory consolidation many hours after the acquisition phase. Moreover, another study demonstrated that this correlative induction of C/EBPβ can be deleted if another novel taste is consumed after the studied taste. A second novel taste induces memory interference that can be measured in behavior. However, at the same time, the second taste interferes with the correlative expression of C/EBPβ. Both the biochemical and behavioral interference suggest that a very long process of consolidation of taste information takes place, at least in part, in the GC (Merhav et al., 2006).

Careful analysis of the literature reveals very few correlations between mRNA-induced expression and taste learning. There are a few reports that identified induced expression of various mRNAs following CTA or LiCl injection (Lamprecht and Dudai, 1995, 1996). These investigations did not observe any modulation in mRNA expression in the insular cortex, following novel taste learning *per se*. Interestingly, LTP induced strong elevation in the expression of various mRNAs in the GC. These induced expressions were dependent on NMDA and muscarinic AChR (Jones et al., 1999). It is yet to be determined whether the major process that induces protein in the GC, in correlation with taste learning, is indeed translation and not transcription. Another possibility is that the temporal window for mRNA induction is prolonged and different from those in other learning paradigms, which would be consistent with the ability of the CS (novel taste) to maintain an on-hold position and to be associated with malaise hours later.

4.13.11 Temporal Phases in Taste Learning

The main feature of CTA learning is the long delay between the taste-related CS and the aversive internal symptoms (UCS). As a matter of fact, when first

published in the scientific community, this long delay put the behavioral results into question (e.g., Garcia et al., 1955). The delayed CS–UCS association cannot be explained as a phenomenon related to transfer of the taste input but as one related to the processing machinery itself within the CNS. In a way that is not clear, a better association is created with a time interval in the range of many minutes than with shorter time intervals (Sachfe et al., 1995). Thus, the system is well suited for prolonged delay between the CS and the UCS. It may be assumed that taste sampling creates a taste memory that remains ready for association, for many minutes. This taste memory trace is both short-lived for an association (usually a few hours) and also long-lived (weeks or more), as can be measured in the latent inhibition paradigm. The creation of taste memory is dependent on the functional taste cortex. One way to study the stability of the taste memory trace, which is dependent on GC activity, is to examine the interactions between taste inputs. Indeed, it was shown recently that taste memory is fragile and can be disrupted for hours by learning another novel taste input (Merhav et al., 2006). This intrinsic limitation of the taste system may be related to its capacity for prolonged association. Interestingly, the interference interaction is only for a second taste over the first one. The inverse interaction of the first novel taste on the second one induces a facilitation effect for the second taste. This facilitation effect has a time window of a few hours; it is shorter than the interference effect and it may be attributed to molecular interactions within the same neuron, i.e., a similar phenomenon to that in the tagging hypothesis model in LTP or to interactions among neuromodulators released within vast GC areas over a long period.

In any case, the unique ability of the taste system to form an association in such a delayed manner presents the researcher with the possibility of studying associative learning in a time frame that allows biochemical/molecular experiments, and not only electrophysiological manipulations and measurements.

4.13.12 Summary and New Directions

It was clear from the first scientific report of CTA that some of its features do not suit the mainstream learning models (Garcia et al., 1955). The two main differences, which may in fact be linked to one another, are the long delay between the CS (taste) and the malaise, and the built-in meaning of a given taste input. Thus, the chemical internal representation of a given taste always has a value: good, bad, or indifferent. It is indeed possible that any taste input is remembered by its association with its value. Definitely, the biochemical correlates of taste learning in the taste cortex – those that have been identified so far – are always correlated with the novelty of the taste input, which is a major factor in the saliency of any input. This means that, according to the current synaptic-Hebbian view of memory formation, the biochemical correlates cannot be attributed to encoding of the physical properties of the taste input. It is possible that these biochemical and cellular modifications are too small to be detected with the currently available technology.

One may argue that any long-term memory trace is associated with a value; however, it is clear that incidental taste learning (i.e., with no known external associations) is genetically strongly associated with the implication of the input for the animal, and it is clear that food consumption is a major factor in animal survival. Whereas the various senses can report to the brain regarding the potential of a given food for the animal (e.g., sound, color, smell, texture, and shape), the taste conveys the essence of the food for the animal. It is interesting that in Hebrew the word 'taste' is synonymous with meaning or reasoning, while 'vision' (I see) is analogous to understanding in English.

In any case, the perceived value of a given taste can be changed rapidly. The association with malaise is strong (CTA), but past experience (the behavioral paradigm of LI-CTA) and future experience (the behavioral paradigm of CTA extinction) can modify the taste value, and all three learning modes are dependent, at least in part, on the GC.

Taste learning offers unique opportunities to study the biological mechanisms underlying learning and memory. In the last decade, a number of laboratories have contributed to vast progress in this field. The development of new research tools (e.g., multielectrode arrays, genetic and molecular tools, and imaging techniques) enables us to ask new questions and to propose new hypotheses. Here are some of the major questions that will be addressed in the near future.

1. A major question in the neurobiology of learning and memory is whether the diverse experiences that create the internal representations are

modifications of a major internal representation or represent competition between different internal representations. It seems that the current knowledge of the taste system favors competition between different internal representations. However, further research is needed to obtain better evidence for one or the other of the options.

2. CTA learning is a hybrid between conscious learning, i.e., learning the taste information, and unconscious learning, i.e., visceral information. A major question concerns where this association takes place within the brain and neuronal circuit. The data so far indicate that the mainly subcortical structures, most probably the PBN, subserve the association. New tools and further analysis may enable us to answer this question.

3. Taste information is encoded, at least in part, by the GC, which resides within the insular cortex. However, very little is known about the circuit within the GC, and there are no good models that encompass the correlation between the electrophysiological and molecular information, on the one hand, and taste learning, on the other hand. A major line of research involves dissecting out the functional organization of the insular cortex on the circuit level, in order to better understand the encoding, consolidation, and retrieval processes, and it includes a number of questions. What types of cells produce the plasticity? Are they inhibitory interneurons, or excitatory cells? In which layer within the cortex do the correlative biochemical alterations take place? Does a given neuron contribute to the encoding of different tastes? Where within a given neuron do the modifications take place? The current experimental tools, which provide good spatial and temporal resolution, should provide answers to these basic questions in the near future.

4. Taste information can be kept on hold for many hours, waiting for an association, in contrast to other associations that have time windows measured in seconds. It is not clear if this unique ability represents distinct biological hardware, or if it is a prolongation of similar biological processes that underlie conventional association processes. A major question is how this associative potential is retained for many hours. If the mechanisms are similar to those in other learning paradigms, this unique ability of CS, which derives from processing mechanisms within the CNS, could be exploited to study association mechanisms by means of research tools with resolution times of minutes to hours, which could not be used in other learning paradigms, i.e., most molecular/biochemical tools.

5. The time frame of molecular modifications in the GC that are correlated with taste learning (see **Figure 2**) opens with posttranslation modifications within minutes after learning (e.g., ERK, eEF2; see **Figure 2** for more details), proceeds with other posttranslation modifications to synaptic proteins on a time scale of hours (e.g., tyrosine phosphorylation of the NR2B), and continues with protein expression on a time scale of hours to days following learning (e.g., the inductions of postsynaptic density 95 and C/EBPβ, which occur 3 and 18 h following learning, respectively). A major task will be to organize a temporal and spatial chart of these correlations in order to understand the stream of molecular and cellular events underlying taste memory formation.

In addition, the known identities of molecular events, and their time windows, are in accord with other brain structures that subserve other learning paradigms, for example, the expression of C/EBPβ in the relevant brain structure 18 h after learning. However, some of the events are different. For example, there are several indications that functional NMDA receptor in the GC is necessary for the consolidation phase, in contrast to other learning and LTP protocols, whereas the NMDA receptor is necessary mainly for the acquisition/induction phase. One possibility is that this is a unique feature of taste learning and that it is related to the ability of the taste memory trace to be associated after such a long delay. However, further analysis is needed to understand the role of NMDA receptor activity, localization, and posttranslation modification within the GC during taste memory formation.

6. In the last few years, the role of translation regulation in taste learning consolidation has been studied extensively. The results demonstrate intrinsic regulation of both the initiation and the elongation phases during taste memory formation. Moreover, it is clear that those genetic and pharmacological modifications that enhance the initiation phase also enhance performance. Much more research is needed to achieve understanding of translation regulation in memory consolidation to answer such questions as: What are the upstream neurotransmitters and signal transduction cascades? What are the targets and what are the mRNA populations and their localizations? However, the taste system can serve as a platform from which to study new targets for drugs, to be used as cognitive enhancers.

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4.14 Memory Reconsolidation

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4.14.1 Definitions and History of Memory Reconsolidation

Memory is an organism's ability to store, retain, and subsequently recall information.

The formation of memory takes place through complex and very dynamic processes. Following learning, the newly acquired information is in a fragile state and stabilizes over time to be finally stored as a long-lasting representation of the learned experience. This process of progressive stabilization of the memory is known as memory consolidation, and the internal representation of the memory is referred to as the memory trace or engram.

The concept of memory consolidation evolved from many studies over the last century in both humans and animal models. Toward the end of the nineteenth century, clinical observations showed that head injury or other traumatic insults to the brain lead to forgetting of more recent memories, while gradually sparing older ones. This gradient of retrograde amnesia (RA) was later reproduced using human verbal learning by the pioneering studies of Muller and Pilzecker around 1900. These authors reported that memory for a newly learned list of words is disrupted by the learning of a second list of words if it is presented shortly after the original one, a phenomenon known as retroactive interference. The authors concluded that neural processes activated by recently acquired information require time in order to become permanently 'fixated' or consolidated (Muller and Pilzecker, 1900).

The consolidation hypothesis has greatly influenced the neurobiological studies of memory throughout the last century. Starting from 1940, many laboratories investigated RA and experimentally reproduced the human data using animal models, particularly mice and rats. These animal studies confirmed that, if within a limited time window after acquiring the information the subject encounters some type of interfering stimuli, memory does not stabilize and the experience is forgotten. Stimuli able to disrupt memory consolidation can be different in nature and include, among others, brain trauma, new learning, seizure, electroconvulsive shock (ECS), brain cooling, and pharmacological treatments that inhibit basic molecular functions such as transcription and translation (Dudai, 2004). During the same years, in parallel with the multitude of studies on RA, an interesting complementary cellular view of how neuronal mechanisms store long-lasting changes was proposed. Inspired by Freud's earlier theories, in 1949 Hebb proposed that memories strengthen and stabilize through reverberating neural circuits and suggested a possible neuronal mechanism for the temporal gradient of RA: the disruption of reverberating circuits prevents the synaptic changes necessary for memory consolidation, and this effect gradually decreases as the changes progress over time. This concept of reverberating circuits, together with the conclusions from the findings on RA, led to the hypothesis that memory consolidation builds on biological modifications occurring over time, which become integrated and preserved as a lasting engram or impression (trace) in the brain.

One important issue that emerged from these original consolidation studies and still remains to be understood is the duration of the consolidation process. This has not yet been clarified, possibly because interferences of different nature likely affect different phases of the consolidation process itself. Various types of interfering stimuli have revealed at least

two levels, or phases, of consolidation. An early, rapid phase occurs over minutes or hours and is sensitive to molecular intervention, such as inhibition of RNA or protein synthesis or disruption of the expression of specific genes. In addition, at a later time, a second, slower phase of consolidation that can last for weeks, months, or even years in humans is believed to reorganize the circuits that bear the memory trace. This late consolidation phase can be disrupted by events that produce lesions or inactivation of neuronal circuits (e.g., hippocampal inactivation) (Squire et al., 2001). Despite that, this important issue remains unsettled: Until very recently, memory consolidation has been considered to be a one-time process that from its initial fragile state progressively strengthens memory over time (McGaugh, 2000). This chapter, unless otherwise specified, refers to the use of the term 'consolidation' that indicates the early phase of memory fragility, which depends upon new synthesis of RNA or proteins or the expression of specific molecules.

Discoveries reported in the 1960s and 1970s showed that, in contrast to the idea of a unitary process of memory consolidation, a memory that has become sufficiently stable and insensitive to various interfering stimuli, including the administration of ECS or RNA and protein synthesis inhibitors, could return to a fragile state for a limited time if the memory was retrieved or in some way 'reactivated.' During this fragile state, memory could be disrupted by the same interfering stimuli that were known to affect the initial consolidation. Consequently, a different hypothesis of memory stabilization was proposed: it is not the age of the memory that makes a memory more consolidated and resilient to disruption but, rather, its 'active' state (Lewis, 1979). Thus, a memory in its 'active' state (e.g., after training or retrieval) is fragile, whereas an 'inactive' memory is stable. It was also found that a memory could be reactivated by several means, the most common of which is the recall or retrieval of the learned experience (e.g., a conditioned stimulus (CS) exposure for Pavlovian conditioning memories). Memory reactivations, however, could also occur effectively by reexperiencing the motivational aspect of the learned event (e.g., drive states such as hunger) or by reliving the entire learning experience (e.g., another training trial). Thus, it was concluded that, after becoming again active and therefore fragile, memory needs to undergo again a stabilization process that, as revealed by RA, shares similarities to posttraining consolidation. For this reason, this process was later named reconsolidation.

After this series of experiments, in the 1970s, controversies set in, in part because of some failure to reproduce the findings of RA after memory reactivation and in part because, in several cases, spontaneous recovery from amnesia was reported. Therefore, alternative views that could explain postreactivation RA were put forward, and the lack of memory retention was considered a retrieval deficit rather than a failure of consolidation or reconsolidation (Riccio et al., 2006), raising a question that, as discussed below, still remains to be answered.

Subsequently, the issue of postreactivation memoryfragility quieted down for several decades, with the exception of a few sporadic studies, but then strongly reemerged in 2000, when an important study extended the notion that an established fear-conditioningmemory is disrupted if, after its retrieval, protein synthesis is inhibited in the amygdala (Nader et al., 2000a). The authors, in agreement with previous hypotheses, proposed that an established memory, once reactivated, becomes transiently labile and undergoes another consolidation phase in order to be maintained. This process was therefore named *reconsolidation* (Nader et al., 2000a,b; Sara, 2000a). In the last 6 years, a large number of manuscripts have been published on this subject, reflecting the exponential increase of the acquired knowledge about memory reconsolidation. All these studies have proven that numerous types of memories elicited with different protocols in different species ranging from snails to humans undergo reconsolidation (**Table 1**). In the following subchapters, the major recent findings about the mechanisms, features, circuits, and functions of memory reconsolidation are summarized.

4.14.2 Mechanisms and Circuits of Memory Reconsolidation

An important question that has been the focus of attention during the last 6 years and partially still remains to be fully understood is whether and to what extent the posttraining consolidation phase and reconsolidation are mechanistically similar at the molecular and cellular levels.

A premise that needs to be made here is that the identification of which molecular mechanisms underlie both memory consolidation and reconsolidation is one of the biggest technical challenges in neurobiology. Understanding the complexity of the

Table 1 Species, learning and memory tasks, brain regions, and molecules involved in memory reconsolidation

Species	*Tasks*	*Brain regions*	*Molecules*
Caenorhabditis elegans	Contextual fear conditioning	Hippocampus	C/EBPβ
Helix lucorum	Inhibitory avoidance	Amygdala	Zif268
Lymnaea stagnalis	Classical conditioning	Insular cortex	CREB
Chasmagnathus granulata	Implicit conditioning (mobile conjugate	Nucleus accumbens	NFκB
Honeybee	reinforcement)	Ventromedial prefrontal	PKA
Medaka fish	Radial arm-maze	cortex	MEK
Chick	Nonassociative learning (tap response)		SGK3
Mouse	Aerial respiratory operant conditioning		ERK
Rat	Appetitive odor discrimination		ERK2
Human	Object recognition		Choline
	Cue-induced drug seeking		AMPAR
	Conditioned withdrawal		CB-1R
	Conditioned place preference		NMDAR
	Motor skill finger tapping		mGluR5
			β-AR
			Glucocorticoid R
			$GABA_AR$

molecular changes accompanying behavioral responses and their functional role requires both the identification and functional testing of multiple molecules and mechanisms ongoing in different brain regions over time, an arduous task with the technology currently available. Currently, the most comprehensive approaches available can only identify correlative changes to behavioral responses occurring within specific brain regions or cell populations and are based on differential screenings using DNA microarrays or proteomics. These techniques offer the advantage of simultaneously evaluating the expression levels of thousands of genes or proteins, respectively, in a given experimental condition (e.g., time, space, treatment). The comparison among different experimental conditions (e.g., trained vs. controls) leads to the identification of specific genes or proteins. Although these techniques are incredibly powerful, presently they still possess several limitations: they are very laborious and expensive and are still not sufficiently sensitive for detecting small changes in heterogeneous tissues such as brain regions. Moreover, they can target only one time point at a time, and therefore each experiment only reveals a single snapshot of the ongoing molecular changes. Despite these limitations, several studies in the last few years have investigated comprehensive changes during posttraining consolidation using DNA microarrays or proteomics. However, perhaps due to its more recent reemergence, very little progress has been made investigating the reconsolidation process. The approaches thus far employed to uncover the nature of the molecular mechanisms underlying memory reconsolidation have been based on testing the potential roles of individual molecules which had been previously found to play a critical function during the consolidation phase. This approach, which has limited the potential for identifying new molecules and mechanisms, has been mainly used for another purpose: addressing the question of whether the reconsolidation process is a recapitulation of consolidation (Dudai and Eisenberg, 2004; Alberini, 2005; Nader et al., 2005). This question has been the focus of many debates. For example, it has been argued that it is expected that reconsolidation is not an exact copy of the posttraining consolidation, as new learning is distinct from a reactivation process, which often occurs through only a partial reexperience (i.e., reactivation by recall, in which, for example, for Pavlovian conditioning only a CS presentation occurs). However, for the precise reason that reactivation is generally different than training, other authors have felt that it is important to determine how similar or different their underlying mechanisms of stabilization actually are. With this in mind, several studies have examined whether the disruption of protein or RNA synthesis or of the expression of specific molecules that were known to block consolidation affected the reconsolidation of the same memories. Disruption of specific molecules was achieved by stereotactic injection of molecular inhibitors such as specific antagonists or antisense

sequences, or by region-restricted gene expression interference in transgenic animals. The conclusions from these studies revealed that a number of mechanisms are commonly used by both consolidation and reconsolidation, while other mechanisms are regulated and required for the consolidation of a particular memory but not during its reconsolidation. In summary, reconsolidation appears to be only a partial recapitulation of the initial posttraining consolidation process (von Hertzen and Giese, 2005). One study also reported that molecular mechanisms required in the hippocampus during either consolidation or reconsolidation are distinct or show distinct temporal dynamics. The genes examined thus far belong to different functional classes including kinases such as mitogen-activated protein kinase (MAPK), serum and glucocorticoid-induced kinase 3 (SGK3), and cAMP-dependent protein kinase A (PKA); transcription factors such as cAMP-response element binding protein (CREB), CCAAT enhancer binding protein β (C/EBPβ), zif 268, and nuclear factor kappa B (NFκB); neurotrophic factors such as brain-derived growth factor (BDNF); and membrane receptors, such as glutamate, noradrenergic, cannabinoid, and glucocorticoid receptors (**Table 1**).

In addition to specific mechanisms, it was also investigated whether similar or distinct brain areas (circuits) are recruited during consolidation or reconsolidation. These questions have been addressed by disrupting neuronal activity (inactivation) or specific molecules within restricted brain areas or even distinct cell populations within brain regions. The results indicate that not all the same brain regions engaged during memory consolidation are also involved during the reconsolidation of the same memory. Hence, in a variety of memories, similar to what has been observed for the molecular mechanisms, only a partial overlapping of circuitry between the two processes was revealed.

In summary, the two processes of consolidation and reconsolidation appear to only partially share molecular mechanisms and brain areas. Hence, the term 'reconsolidation' refers to the concept of memory restabilization and does not indicate the reiteration of underlying mechanisms.

Our understanding of how memory reconsolidation, but also consolidation, occurs mechanistically at the molecular and cellular levels is still very primitive. While, as mentioned above, several molecules required for memory reconsolidation have been pinpointed, the sequence of the molecular events that are activated during the onset and development of the process is unknown. Some recent studies have found intriguing correlations between the expression level of glutamate receptors and memory reconsolidation, suggesting that, like memory consolidation, a critical role might be carried out by the regulation of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors (Rose and Rankin, 2006). Further studies will be important for unraveling the sequence of molecular events necessary to restabilize a memory after it has been reactivated.

4.14.3 Temporal Constraints

Two types of temporal constraints have been found to characterize memory reconsolidation: (1) the persistence and duration of the amnesia and (2) the age of the memory.

4.14.3.1 Persistence and Duration of Amnesia Induced by Postreactivation Interference

Some researchers have found that, whereas the amnesia caused by protein synthesis inhibition after training is long lasting, the amnesia resulting from inhibition of protein synthesis after memory reactivation spontaneously recovers over time (Anokhin et al., 2002; Lattal and Abel, 2004; Salinksa et al., 2004) or recovers after exposure to a reminder event (Fischer et al., 2004). Thus, several authors believe that the amnesia caused by postreactivation interference results from a retrieval deficit. On the other hand, these results have not always been reproduced, and in several cases memory disruption after reactivation lasted for a long time (weeks or months in animals), and memory retention never returned even following a strong reminder. Several reasons may explain these opposing results. First, the question of the persistence of the amnesia induced by protein synthesis inhibitors after reconsolidation may be related to the degree and duration of protein synthesis inhibition. Unfortunately, very few studies have determined the rate of protein synthesis inhibition with the conditions and protocols used in reconsolidation experiments. The same issue had been under debate for the consolidation process a few decades ago, and a possible explanation offered for the temporary effect was that protein synthesis was only partially inhibited or inhibited for an insufficient length of time. Indeed, it has been reported

that stronger memories require longer (stronger) inhibition of protein synthesis to be permanently disrupted (Flood et al., 1973, 1975; Barraco and Stettner, 1976; Davis et al., 1978). In addition, in agreement with these results, it has been recently demonstrated that a partial inhibition of protein synthesis during the consolidation or reconsolidation phase precisely results in temporary impairment that recovers at later times (Milekic et al., 2006; Miller and Sweatt, 2006). A second possibility that can explain why amnesia results more frequently after postretrieval rather than posttraining interference is that disrupting a memory that had been already stabilized is more difficult than disrupting a newly acquired memory. Logically, a memory trace that has been previously established would be more resistant to disruption than a newly developing trace. Therefore, the same amnesic treatments may result in a total amnesia for new memories, but only a partial amnesia for a reactivated memory. In the latter case, a reminder may increase the strength of the memory trace and result in a memory recovery, which would be misinterpreted in favor of a potential retrieval failure rather than a loss of stored information. Third, by definition, it is always impossible to exclude that an amnesia results from a retrieval deficit, because even though no recovery might be observed under any circumstances, currently there is no direct method to prove that the memory trace has been completely erased.

4.14.3.2 Age of the Memory

The hypothesis of memory reconsolidation predicts that when a memory is active (following training or reactivation) it is in a labile state, and when inactive it is stable. This implies that *every time* a memory is reactivated it becomes transiently labile. A number of recent studies have tested whether this hypothesis is correct or whether the memory changes over time. The results have clearly shown that, in most cases, the passage of time influences the stability of a memory and that the older a memory becomes the less susceptible it is to disruption following its reactivation. These results have been found using several fear conditioning types of tasks in many different species including rat, mouse, chick, and Medaka fish. In general, an increasing resilience to disruption following memory reactivation has been reported over several weeks. For example, in rat inhibitory avoidance 2- and 7-day-old memories became fragile and are disrupted by protein synthesis inhibitors administered after recall, while older, 14- and 28-day-old memories are resistant to the same treatment (Milekic and Alberini, 2002) (**Figure 1**). Similarly, in Medaka fish, the administration of a Na^+ channel blocker upon reactivation disrupts a 4-day-old memory but has no effect on a 15-day-old memory and an intermediate effect on a 9-day-old memory (Eisenberg and Dudai, 2004). Likewise, numerous other studies in different species have confirmed the existence of a temporal gradient of progressive resilience to memory disruption after reactivation (Litvin and Anokhin, 2000; Suzuki et al., 2004; Boccia et al., 2006; Frankland et al., 2006). An important extension of these findings revealed that, in addition to the age of the memory, the strength of the memory and the reactivation intensity interact to influence the degree of vulnerability of a reactivated memory (Suzuki et al., 2004). In fact, whereas protein synthesis inhibitors can disrupt a 24-h- to 3-week-old contextual fear memory after reactivation in mice, an 8-week-old memory remains insensitive to disruption by a similar reactivation session. However, if the reactivation session is prolonged, even the older memory can be disrupted. Finally, weak (one shock) versus strong (three shocks) training protocols correlate with more or less susceptibility to disruption after reactivation, respectively. Interestingly, using associative learning in *Lymnaea*, it was found that reconsolidation at both 6 and 24 h, which requires protein synthesis, undergoes progressive molecular changes, because only reconsolidation at 6 h after training, but not at 24 h, requires protein kinase A activity. This phase-dependent differential molecular requirement for reconsolidation supports the idea that even

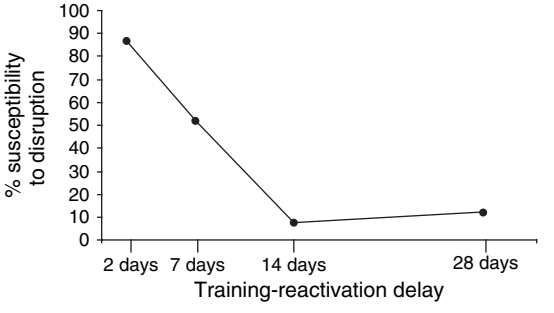


Figure 1 Temporally graded decrease of susceptibility to disruption of a reactivated memory. Susceptibility to disruption by anisomycin of reactivated inhibitory avoidance memories of different ages. Taken from Milekic MH and Alberini CM (2002) Temporally graded requirement for protein synthesis following memory reactivation. *Neuron* 36: 521–525, with permission from Elsevier.

seemingly consolidated memories undergo further selective molecular maturation processes (Kemenes et al., 2006).

In conclusion, an important temporal constraint characterizes memory reconsolidation: as the time interval since training lengthens, there is increasing resistance to postreactivation disruption. This indicates that the reconsolidation process is a manifestation of a lingering consolidation (Dudai and Eisenberg, 2004; Alberini, 2005).

Some authors disagree with this interpretation. Some studies show that 2-day- and 2-week-old memories of cued fear conditioning as well as a 45-day-old contextual fear conditioning memory in rats are disrupted by postretrieval bilateral injections of anisomycin into the amygdala or hippocampus, respectively (Nader et al., 2000a; Debiec et al., 2002). In contrast, other groups have reported opposite results using contextual fear conditioning in mice (Suzuki et al., 2004; Frankland et al., 2006). Furthermore, memories that are based on multiple training trials appear to show a long-lasting temporal window of postreactivation fragility: a 21-day-old appetitive instrumental response (sucrose self-administration) is disrupted by systemic injection of the beta-adrenergic antagonist propranolol, and a 27-day-old cocaine-induced conditioned place preference is disrupted by postreactivation amygdala injections of zif 268 antisense (Diergaarde et al., 2006; Lee et al., 2006). These opposing data can be explained by the findings discussed above showing that the nature of the learning, the intensity of training, and reactivation protocols produce gradients of protein synthesis requirements after reactivation that show different temporal evolutions. Hence, under certain experimental conditions (e.g., the reactivation intensity and time points chosen), the memory may still lie within the labile period. Following the same logic, it will be consistent with a lingering consolidation and the results of RA affecting remote memories that the stabilization process may take a long time, and therefore, some memories may remain sensitive to postretrieval interference for an extended period of time.

Importantly, the existence of a gradient does not exclude the possibility that reactivation of fully consolidated memories is accompanied by a phase of *de novo* protein synthesis. However, new molecular changes induced by the reactivation of a fully consolidated memory may occur without manifested fragility of the consolidated information. This view is in agreement with a hypothesis that had been proposed to explain memory stabilization over time, which suggested that memory stability correlates with the distributed nature of the memory trace (Nadel and Moscovitch, 1997) and is also in line with the hypothesis mentioned above of reverberating circuits proposed by Hebb (1949).

The mechanisms underlying the progressively decreasing gradient of memory fragility are not yet clear. However, future investigations should be able to address this question.

4.14.4 Functions of Memory Reconsolidation

The reason why memories become labile after reactivation is still unknown. Two main, non–mutually exclusive hypotheses have been proposed. First, as suggested above by the hypothesis of a lingering consolidation, a function of reconsolidation might be to strengthen memory retention (Sara, 2000b). A second possible role of reconsolidation may be to allow the integration of new information into the background of the past. In other words, reconsolidation might be required "to associate new learning with already established and reactivated memories" (Lewis, 1979; Sara, 2000a; Dudai, 2004). Although no direct proof has yet been provided, the results of several studies support the idea that memory reactivation leads to increased memory strength and prevents forgetting. For example, electrical stimulation of the mesencephalic reticular formation (MRF) improves the retention of memories, including fear conditioning and linear maze, when the stimulus is applied after memory reactivation (DeVietti et al., 1973; Sara, 2000b), indicating that memory retention can be enhanced during a postreactivation phase. In addition, recent studies in *Lymnea stagnalis* showed that the reexperience of a conditioned stimulus in the same context where the subject previously underwent a training that elicits an intermediate-term memory converts a residual memory trace into long-term memory (Parvez et al., 2006). Finally, several modulatory compounds, including angiotensin II and agonists of protein kinase A, have been found to increase memory retention if present during the reconsolidation phase (Frenkel et al., 2005; Tronson et al., 2006), suggesting once again that after reactivation, memory can be strengthened.

The second proposed function, that the reconsolidation process mediates the formation of associations between new and reactivated information, is now

beginning to be understood. Toward this end, one study utilized a second-order conditioning paradigm (Tronel et al., 2005) in which two associations are established in series. First, a conditioned stimulus (CS1) is paired with an unconditioned stimulus (US), such as when a given context (context A) is paired with a footshock. Then the established properties of CS1 are paired with a new stimulus, CS2, producing a CS1-CS2 association. For example, the subject that has learned the context A-US pairing is then exposed to a different context (context B) in which one cue of context A is presented. This experience reevokes the result of the CS1-US association, which now becomes linked with context B. Therefore, the subject learns to fear context B even though this context has never been associated with a shock.

Using inhibitory avoidance, it was found that systemic inhibition of protein synthesis after the exposure to context B (which also contains a cue from context A) disrupts both the new association (CS2-CS1) and the recalled memory (CS1-US). Region-restricted inhibition of the transcription factor C/EBPβ allows the functional dissociation of the consolidation and reconsolidation of inhibitory avoidance. Inhibition of C/EBPβ in the hippocampus disrupts inhibitory avoidance consolidation but not reconsolidation, and conversely, inhibition of C/EBPβ in the amygdala disrupts reconsolidation but not consolidation. If memory reconsolidation mediates the incorporation of new information into a reactivated memory, then it is predicted that after the exposure to context B the inhibition of C/EBPβ in the amygdala should disrupt both memories. Instead, it was found that such a C/EBPβ inhibition only and selectively disrupted the old memory for context A but spared the new memory for context B. Thus, the reconsolidation of the original memory takes place independently from the formation of the new association. Conversely, the memory for context B was selectively disrupted by hippocampal C/EBPβ inhibition, suggesting that the formation of the new association is mediated by molecular mechanisms similar to those underlying the initial consolidation of a new memory. In conclusion, although the old memory needs to be retrieved in order to associate new information, its reconsolidation process is not engaged in establishing the link between the new information and the reactivated memory.

Another study investigated memory 'updating' by approaching this question from a different angle. Repeated learning presentations of the same event using attenuation of neophobia (AN) in rats were used to test (1) how the acquisition of a new learning trial becomes linked to the consolidated memory established by the previous trials and (2) the fragility of both the updated and previously established memories (Rodriguez-Ortiz et al., 2005).

In AN, the animal gradually increases the intake of a tastant after repeated presentations of the tastant itself. Each additional learning could be considered as a memory updating event. Using multiple training trials, each separated by 24 h, it was found that, while after the first and second trials an infusion of anisomycin into the insular cortex completely disrupted memory retention, after the third trial a partial disruption was observed. Moreover, after six trials, a similar treatment had no significant effect. Thus, it was concluded that, while additional learning is acquired, part of the older, consolidated memory in the insular cortex becomes independent of protein synthesis. Furthermore, rats that after seven trials of AN were trained to conditioned taste aversion (CTA) for the same taste in the presence of anisomycin developed amnesia for CTA the following day. In light of these results, it was concluded that "protein synthesis is required to update previously consolidated memory trace regardless of the valence of the tastant." This study, in agreement with the first one, indicates that the formation of a new association linked to a previously established memory requires protein synthesis. Because it was not determined whether the protein synthesis required for the reconsolidation or persistence of the old memory is recruited to mediate the incorporation of new information, it is possible that AN updating, like inhibitory avoidance (IA) second-order conditioning, utilizes consolidation-like mechanisms and not the reconsolidation process of the original memory. In agreement with this hypothesis, the AN study shows that the old and new memories are dissociable processes because, while the old memory can become resistant (consolidated), the updating is sensitive to disruption. In conclusion, it is important to experimentally dissect what is being disrupted – the process that mediates an association between a new experience and a retrieved information, or the restabilization process of a reactivated, previously established memory.

4.14.5 Clinical Applications

The reemergence of research focusing on memory reconsolidation has stimulated interest in the medical community, particularly among mental health professionals treating disorders that are based on

pathogenic memories. Two examples of these directives are posttraumatic stress disorder (PTSD) and drug addiction, both of which can be viewed as the result of dysfunctional memory processes.

PTSD can develop after an exposure to an emotionally or physically traumatic event, and the illness is characterized by repeated, intrusive memories of the experienced trauma. Patients suffering from PTSD have difficulty sleeping and feel detached or estranged, and these symptoms can be so severe and persistent as to significantly impair the person's daily life. A behavioral model of PTSD onset has been proposed: the traumatic event (US) triggers a strong hormonal stress response, which mediates the formation of a robust and enduring memory for the trauma. Subsequent recall of the event in response to cues and reminders (CS) releases more stress hormones (conditioned response) and even further consolidates the memory leading to PTSD symptoms, such as flashbacks, nightmares, and anxiety (Pitman and Delahanty, 2005). The persistence of PTSD can be explained in terms of a trauma-induced strengthening of the memory trace. More specifically, it is hypothesized that noradrenergic hyperactivity and stress hormones facilitate the encoding and consolidation of the memory (Pitman, 1989; O'Donnell et al., 2004; Yehuda, 2006).

Although it is not possible to precisely reproduce PTSD in an animal model, fear conditioning in rodents can be used to mimic and elucidate some aspects of PTSD, including the processing of fearful stimuli and the encoding of emotional memory. As described above, fear conditioning is readily induced in laboratory animals by pairing a CS, such as contextual cues, with a fear-inducing US, such as a foot shock. Presentation of the neutral CS at some later time evokes a stereotypic fear response in the animal, including the stress response and activation of the autonomic nervous system. This is similar to the scenario in which contextual cues associated with a traumatic event can precipitate onset of PTSD symptoms in patients. Thus, it is possible to closely approximate some of the PTSD symptoms in an animal model that can be used for preclinical research. In principle, all pharmacological compounds thus far shown to have the ability to disrupt fear memory reconsolidation could potentially be useful for designing new drugs that can be used in clinical trials. Some of these compounds, including antagonists of beta-adrenergic or glucocorticoid receptors, which target the stress pathways, are already employed in clinical pharmacology for treating other diseases. Hence, they represent the most readily available potential therapies.

Targeting memory reconsolidation and the noradrenergic system in an attempt to treat PTSD has already been explored at the preclinical level and is currently being used in clinical trials. One method involves administration of the beta-adrenergic blocker propranolol, commonly used to treat hypertension, following recall of the traumatic memory. Several years ago, researchers first tested the effect of propranolol on memory reconsolidation using inhibitory avoidance in rats (Przybyslawski et al., 1999). Systemic administration of propranolol given after the reactivation of the inhibitory avoidance memory disrupted the memory at subsequent tests. More recently, using another paradigm known as auditory fear conditioning, other researchers have shown that propranolol injected either systemically or into the lateral nucleus of the amygdala (LA) after reactivation of a 2-month-old memory weakened the fear response when tested 48 h later (Debiec and LeDoux, 2004, 2006).

Another pharmacologically targeted pathway for the potential treatment of PTSD is the glucocorticoid pathway. The endogenous stress hormone corticosterone bidirectionally modulates memory retention (McGaugh and Roozendaal, 2002; Roozendaal, 2002). Low doses increase memory retention, while high doses disrupt it. Recent studies have reported that glucocorticoids, when administered after the reactivation of a contextual fear memory, have an amnesic effect on the original memory and provide evidence that a possible mechanism for this effect is an enhancement of extinction of the expression of the original memory (Cai et al., 2006). Another study, however, revealed that intra-amygdala blockade of glucocorticoid receptors with the antagonist mifepristone after reactivation of inhibitory avoidance memory significantly disrupts the original memory, probably interfering with its reconsolidation process (Tronel and Alberini, 2007). In light of these results, the role of the glucocorticoid pathway following memory reactivation has clearly been established and promises to be an additional site of pharmacologic intervention for PTSD.

Substance abuse generally leads to a chronic condition believed to result from an addict's inability to permanently abstain from drug use. Drug addicts repeatedly relapse to drug seeking even after years of abstinence, and this pathologic behavior is frequently induced by the recall of memories and environmental

stimuli intimately connected to the rewarding effects of the drug. Therefore, disruption of memory reconsolidation provides an unprecedented potential strategy to disrupt memories that facilitate drug addiction. Promising results have recently been reported in animals dependent on morphine or cocaine by injecting, after memory reactivation, inhibitors of protein synthesis or ERK (extracellular signal-regulated kinase), or by disrupting the expression of the immediate-early gene *zif268* both peripherally and within specific brain regions, such as the amygdala, hippocampus, or nucleus accumbens (Lee et al., 2005; Miller and Marshall, 2005; Milekic et al., 2006; Valjent et al., 2006). In some of these studies, inhibitors were administered to animals that had previously acquired a place preference in response to the drug of abuse, a learning known as conditioned place preference. Animals learned to associate the euphoria of the drug with a specific location, choosing to spend more time there. Administration of several of these inhibitors after reactivation of the drug-related memory interfered with its reconsolidation and abolished the place preference. Other studies investigated a different type of task in which animals form a CS–drug association during drug self-administration training, a model of drug seeking (Lee et al., 2005, 2006). These studies showed that infusion of zif268 antisense oligodeoxynucleotides into the basolateral amygdala, prior to the reactivation of a CS–cocaine association, abolishes its impact on the learning of a new cocaine-seeking response or maintenance of cocaine seeking as well as relapse to a previously established drug-seeking behavior. Furthermore, the same group later demonstrated that conditioned withdrawal could be disrupted following the reactivation of a CS–withdrawal association (Hellemans et al., 2006).

How might a clinical strategy for disruption of reconsolidation of a pathogenic memory be designed? An example of methodology involves pairing pharmacotherapy with memory retrieval within a carefully controlled clinical setting. For example, a patient's memory could be evoked through reexposure to key sensory components of the memory, or reminder cues, immediately followed by treatment with an antagonist or inhibitor that interferes with the reconsolidation of that particular memory. Another strategy might involve patient self-administration during a spontaneous memory flashback, presumably when the memory becomes pliable. Better understanding the mechanism of memory reconsolidation and the consequences of its disruption on human behavior will ultimately help clinicians to target mental illnesses based on pathogenic memories.

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4.15 Molecular Aspects of Memory Dysfunction in Alzheimer's Disease

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4.15.1 Introduction

Alzheimer's disease (AD) is the foremost human disease of memory. Independent streams of research on the molecular mechanisms of AD and the molecular basis of memory, which historically were quite distinct, are now converging in exciting ways. The study of AD may provide novel insights into basic mechanisms of memory, and even more importantly for the over 24 million patients with AD worldwide (Ferri et al., 2005), discoveries about the molecular basis of memory are advancing our understanding of the disease and yielding new treatment targets.

AD typically begins with mild forgetfulness in the elderly. Early on, before memory problems interfere with day-to-day functioning, a diagnosis of mild cognitive impairment (MCI) is often made. As years go by, deficits become more severe and patients are forced to curtail their usual activities. Deficits in other cognitive domains become increasingly apparent, including executive dysfunction, anomia and other language problems, visuospatial dysfunction, and ultimately, global cognitive impairment. Median survival is about 12 years after first symptom onset (Roberson et al., 2005).

Although the diagnosis of probable AD is made during life, definite diagnosis is made only at autopsy. The hallmark pathological features of AD are extracellular amyloid plaques and intracellular neurofibrillary tangles (NFTs) (**Figure 1**), combined with neuronal and synaptic loss, vascular amyloidosis, astrocytosis, and microgliosis. Most of these changes occur in a stereotypical regional distribution, with medial temporal structures involved in memory affected earliest and most severely.

Biochemical and genetic studies of AD have identified several molecules that may play a causal role in its pathogenesis (**Figure 2**). For the most part, these molecules have been studied primarily in the context of AD. In the first half of this chapter, we consider the impact of these AD-related molecules on memory. In addition, many molecules that have been studied primarily in the context of synaptic plasticity and memory, including a variety of receptors, channels, second messengers, kinases, and transcription factors, are also involved in AD. In the second half of the chapter, we consider AD-related memory impairment in relation to these molecules.

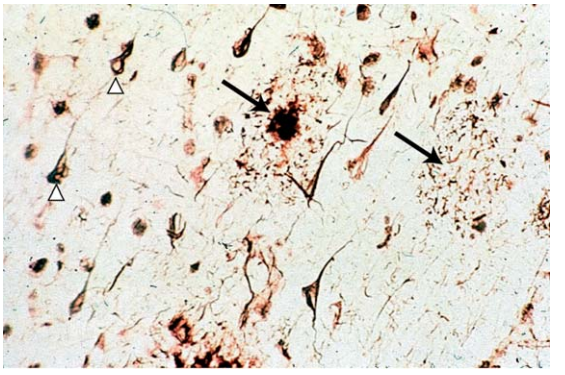


Figure 1 Plaques and tangles. This silver-stained section of cerebral cortex from a patient with Alzheimer's disease shows extracellular amyloid plaques (black arrows) and intracellular neurofibrillary tangles (white arrowheads). (Courtesy of Dr. Nitya R Ghatak, Virginia Commonwealth University Health System.)

4.15.2 Memory Impairment by AD-Related Molecules

In this section, we examine several molecules that likely contribute to the pathogenesis of AD, including the amyloid precursor protein (APP), amyloid-β peptide (Aβ), the β- and γ-secretases that release Aβ from APP by proteolytic cleavage, the microtubule-associated protein tau, the lipid carrier apolipoprotein E (apoE), and α-synuclein, which also plays an important role in Parkinson's disease. For each molecule, we briefly review its normal function and the evidence for its involvement in AD and then examine studies of its relationship to memory deficits in both humans and animal models.

4.15.2.1 APP and Aβ

APP is a ubiquitously expressed, multifunctional type I membrane protein with potential roles in signaling (Turner et al., 2003), axonal transport (Kamal et al., 2000; Satpute-Krishnan et al., 2006),

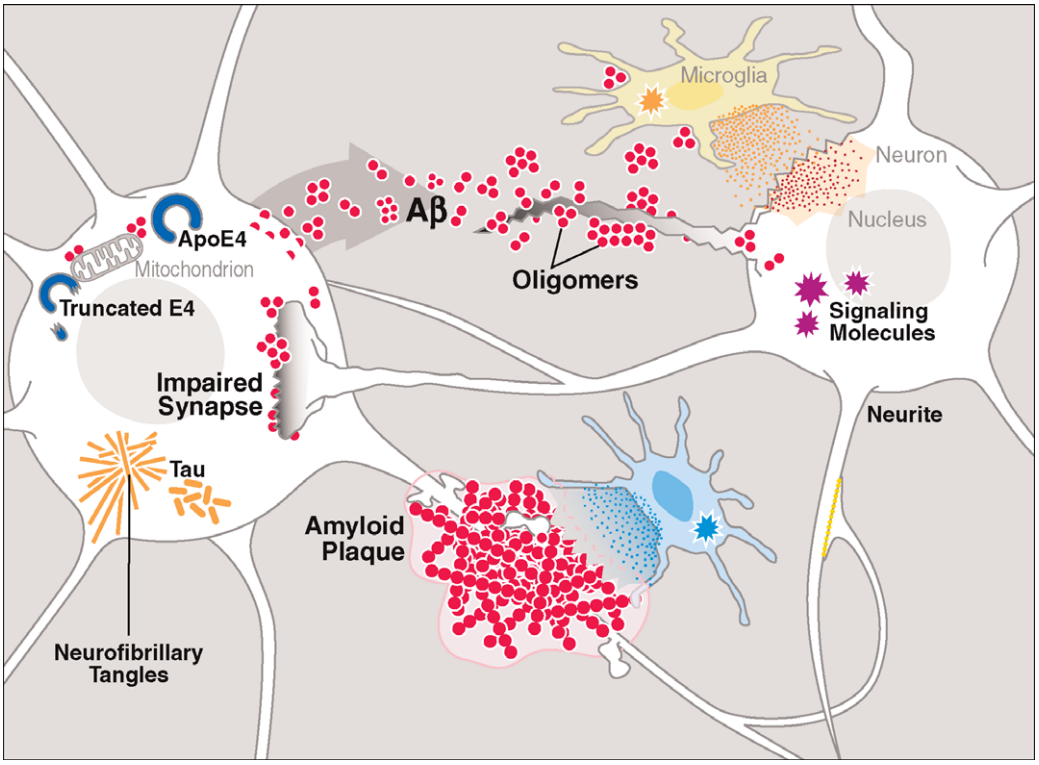


Figure 2 Key molecules involved in Alzheimer's disease pathogenesis. Aβ peptides produced by neurons and other brain cells aggregate into a variety of assemblies, including amyloid plaques containing Aβ fibrils and soluble nonfibrillar oligomers. Some of these Aβ assemblies impair synapses and neuronal dendrites, either directly or through the engagement of pathogenic glial loops. However, other glial activities protect neurons against dysfunction and degeneration. ApoE4 and tau promote Aβ-induced neuronal injury and also have independent adverse effects. (From Roberson ED and Mucke L [2006] 100 years and counting: Prospects for defeating Alzheimer's disease. *Science* 314: 781–784.)

transcriptional regulation (Cao and Südhof, 2001), neurite outgrowth (Leyssen et al., 2005), synaptogenesis (Mucke et al., 1994; Morimoto et al., 1998), and regulation of glutamate uptake (Masliah et al., 1998). APP-null mice have deficits in learning and memory, abnormal long-term potentiation (LTP), and loss of synapses (Dawson et al., 1999; Seabrook et al., 1999).

Proteolytic cleavage of APP produces several biologically active fragments. Among them are Aβ peptides, produced by the sequential action of two proteases, β- and γ-secretase, on APP (**Figure 3**). Aβ peptides of 40 (Aβ40) or 42 (Aβ42) amino acids in length are the primary species produced. Aβ42 is believed to be more pathogenic, since it is more prone to aggregation and more toxic when applied to cells than Aβ40 (Jarrett et al., 1993; Seilheimer et al., 1997). Aβ has been shown to decrease synaptic transmission (Hsia et al., 1999; Kamenetz et al., 2003; Priller et al., 2006) or LTP (Lambert et al., 1998; Walsh et al., 2002), which may represent physiological functions, pathogenic mechanisms, or both. Neuronal activity stimulates the production and release of Aβ, which in turn depresses excitatory synaptic transmission, at least at certain synapses (Kamenetz et al., 2003). Under normal circumstances, this feedback loop could help regulate synaptic activity. In AD, aberrant neuronal activity might turn this feedback loop into a vicious cycle that increases Aβ production and impairs neurotransmission (Palop et al., 2006, 2007).

A great deal of additional biochemical and genetic evidence suggests an involvement of Aβ in AD, which forms the basis of the 'amyloid hypothesis' of AD pathogenesis (Hardy and Higgins, 1992; Tanzi and Bertram, 2005). Aβ is the major constituent of amyloid plaques, one of the pathological hallmarks of AD (Glenner and Wong, 1984). Mutations in APP cause autosomal dominant AD (Goate et al., 1991); AD-causing APP mutations are concentrated around the Aβ domain, and most increase the Aβ42/Aβ40 ratio or total Aβ production (Theuns et al., 2006). Presenilin mutations, the other major known cause of autosomal dominant AD, also increase the relative

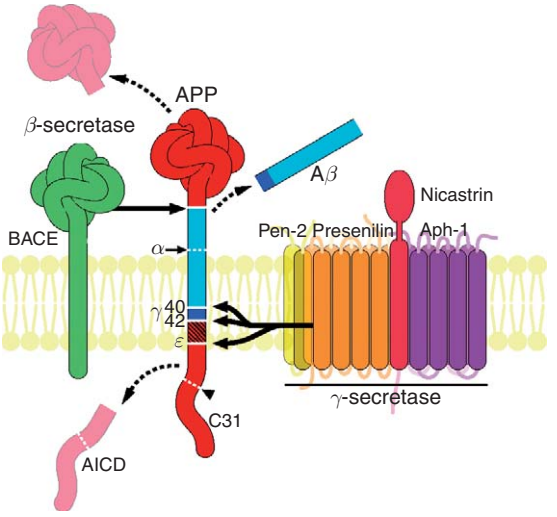


Figure 3 APP, Aβ, and the secretases. Aβ production depends on sequential proteolytic cleavage of APP by β-secretase, also known as β-site APP-cleaving enzyme 1 (BACE1), and the γ-secretase complex, composed of presenilin and other proteins. γ-Secretase can cleave APP at different locations to generate Aβ40, Aβ42, or the APP intracellular domain (AICD). Caspase cleavage at the carboxy-terminus yields the C31 fragment. (Modified from Roberson ED and Mucke L [2006] 100 years and counting: Prospects for defeating Alzheimer's disease. Science 314: 781–784.)

abundance of Aβ42 (see the section titled 'γ-Secretase') (Levy-Lahad et al., 1995; Rogaev et al., 1995; Sherrington et al., 1995). In addition, polymorphisms in the gene for insulin-degrading enzyme, which degrades Aβ, may contribute to AD risk (Ertekin-Taner et al., 2004).

The first animals with prominent AD-like pathology, and still the most widely studied, were transgenic mice with neuronal expression of human APP (hAPP) carrying familial AD mutations (Games et al., 1995; McGowan et al., 2006). These mice have high levels of Aβ and several, but not all, of the neuropathological hallmarks of AD. For example, age-dependent plaque deposition is a universal feature, whereas tangles do not develop. Because hAPP mice develop age-dependent learning and memory abnormalities (Kobayashi and Chen, 2005; McGowan et al., 2006), these models provide an opportunity to test hypotheses about how Aβ contributes to cognitive impairment in AD.

At a molecular level, there are a variety of hypotheses for how Aβ might interfere with normal neuronal function and survival. For example, Aβ can bind directly to lipid bilayers, altering membrane fluidity (Müller et al., 2001). It also forms pores or otherwise permeabilizes membranes, increasing their conductance to ion flow (Pollard et al., 1995; Kayed et al., 2004). In addition, Aβ binds to and influences the function of a variety of cellular proteins (Verdier et al., 2004), including the α7 nicotinic acetylcholine receptor (Oddo and LaFerla, 2006), integrins (Sabo et al., 1995), the receptor for advanced glycation end-products (RAGE; Yan et al., 1996), mitochondrial enzymes (Lustbader et al., 2004), and the APP holoprotein (Shaked et al., 2006). Several of these interactions are discussed in more detail in the section titled 'Memory-related molecules in AD.'

4.15.2.1.1 Aβ and plaques

The deposition of Aβ into amyloid plaques has been the focus of much AD research, given the prominence of plaques in AD patients and related animal models and their relative ease of detection. Plaque deposition in AD brains has historically been studied with neuropathological methods, including histochemical staining with amyloid-binding dyes and immunostaining with Aβ antibodies. It can now be imaged *in vivo* in living human subjects by positron emission tomography with a plaque-binding agent termed Pittsburgh compound B (PIB) (Klunk et al., 2004) (**Figure 4**).

The leading hypothesis for how plaques might disrupt neuronal function is by inducing changes in surrounding neurites. A subset of amyloid plaques in both AD patients and mouse models, termed neuritic plaques, are surrounded by swollen and tortuous neuronal processes (Knowles et al., 1999; Tsai et al., 2004). Plaque-associated neuritic dystrophy may alter the electrophysiological properties of the circuits involved (Knowles et al., 1999; Stern et al., 2004).

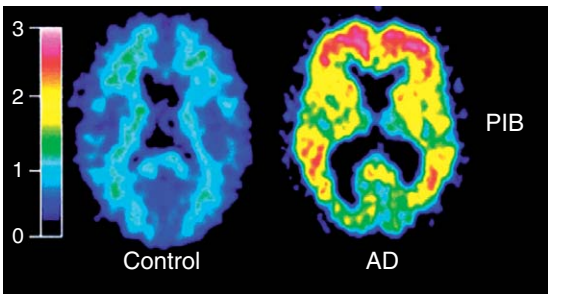


Figure 4 *In vivo* imaging of amyloid plaques with Pittsburgh compound B (PIB). PIB–positron emission tomography scan of a 79-year-old patient with AD shows a robust signal attributable to amyloid plaque binding, compared with a 67-year-old control subject. (From Klunk WE, Engler H, Nordberg A, et al. [2004] Imaging brain amyloid in Alzheimer's disease with Pittsburgh Compound-B. *Ann. Neurol.* 55: 306–319.)

However, plaque deposition does not correlate well with memory deficits in AD. Amyloid deposition tends to be an early event in AD pathogenesis that plateaus and does not worsen as cognition deteriorates throughout the disease course (Arriagada et al., 1992; Ingelsson et al., 2004; Giannakopoulos et al., 2007). Also, plaques do not have the preferential distribution in medial temporal structures involved in memory that is seen with NFTs (see the section titled 'NFTs, neuronal death, and memory loss'). Rather, plaques are seen first in the neocortex, particularly in prefrontal and posterior parietal regions (Price et al., 1991; Buckner et al., 2005; Kemppainen et al., 2006). Data from animal models also support a mechanistic dissociation between plaques and cognitive deficits (Holcomb et al., 1998; Westerman et al., 2002; Palop et al., 2003; Kobayashi and Chen, 2005; Lesné et al., 2006; Cheng et al., 2007). While neuritic dystrophy correlates with cognitive deficits better than plaque load (McKee et al., 1991), many dystrophic neurites in AD brains are not associated with plaques (Wang and Munoz, 1995). Recent findings in hAPP transgenic mice suggest that vascular amyloidosis and oxidative stress may contribute to the pathogenesis of plaque-independent neuritic dystrophy (Esposito et al., 2006).

4.15.2.1.2 Soluble Aβ oligomers

Because deposition of fibrillar Aβ in plaques does not correlate well with cognitive deficits, interest has grown in nonfibrillar forms of Aβ. Increasing evidence points to a pathogenic role for oligomeric Aβ assemblies that are soluble in aqueous solutions. Multiple types of Aβ oligomers have been described, including low-molecular-weight species (dimers and trimers) and larger globular species (including dodecamers and Aβ-derived diffusible ligands) (Podlisny et al., 1995; Lambert et al., 1998; Lesné et al., 2006). Putative Aβ oligomers have been detected in the cerebrospinal fluid (CSF) and brain tissue of AD patients (Podlisny et al., 1995; Kuo et al., 1996; Pitschke et al., 1998; Kayed et al., 2003; Lacor et al., 2004) and hAPP transgenic mice (Podlisny et al., 1995; Lambert et al., 1998; Lesné et al., 2006; Cheng et al., 2007). Extracellularly, oligomers seem to bind preferentially to dendritic and synaptic regions (Lacor et al., 2004; Barghorn et al., 2005). Aβ42 is more prone to oligomer formation than Aβ40 (Dahlgren et al., 2002; Bitan et al., 2003), which may relate to their relative pathogenicity (Aβ42 > Aβ40).

In mixed neuronal/glial cultures and brain slices, oligomeric Aβ is a potent neurotoxin, inducing neuronal death within 24 h (Roher et al., 1996; Lambert et al., 1998; Chen et al., 2005a). Direct experimental comparisons suggest that oligomeric Aβ is more toxic than fibrillar or monomeric Aβ (Klein et al., 2001; Dahlgren et al., 2002).

At physiological concentrations, Aβ oligomers interfere with synaptic plasticity, and memory. Both low-molecular-weight and larger globular oligomers interfered with LTP when acutely applied to hippocampal slices, whereas monomers did not (Lambert et al., 1998; Wang et al., 2004; Barghorn et al., 2005; Walsh et al., 2005). Injection of oligomeric Aβ into the cerebral ventricles blocked LTP *in vivo* and impaired learning and memory (Walsh et al., 2002; Cleary et al., 2005; Lesné et al., 2006). The effects of Aβ on LTP are acute and independent of neuronal death (Chen et al., 2000). Although oligomer-induced blockade of LTP in hippocampal slices could not be reversed by washing out Aβ (Townsend et al., 2006), *in vivo* impairment of LTP by oligomers could be blocked when Aβ antibodies were injected after the Aβ (Klyubin et al., 2005).

4.15.2.1.3 Neuronal dysfunction versus neuronal death

The ability of Aβ to induce both neuronal dysfunction and neuronal death raises the question of which effect is primarily responsible for the memory deficits in AD. Evidence from animal models points toward an important role for neuronal dysfunction. First, although hAPP mice have memory deficits, most lines do not have prominent neuron loss in the areas most affected in AD, including entorhinal cortex and hippocampal area CA1 (Irizarry et al., 1997a,b; Takeuchi et al., 2000). Also, memory impairment in hAPP mice can be rapidly reversed by treatments that lower Aβ levels but are not likely to change neuron numbers (Dodart et al., 2002; Kotilinek et al., 2002). Rather than frank neuronal loss, hAPP mice have synaptodendritic changes, including reduction of presynaptic terminals, loss of postsynaptic spines, and simplification of dendritic arborization, that may contribute to cognitive impairment (Mucke et al., 2000; Buttini et al., 2002; Lanz et al., 2003; Chin et al., 2004; Moolman et al., 2004; Wu et al., 2004; Spires et al., 2005). Whether these alterations would ultimately lead to neuronal loss if mice had a longer lifespan is unknown. It is also unknown how long it takes neurons to die in the brains of humans with AD.

Differentiating the relative contributions of neuronal dysfunction and neuronal loss to cognitive deficits in AD patients has been difficult, because

neuron loss is commonly found early in the disease (Price et al., 2001). However, several observations suggest that dysfunction may at least add to the impairments that are likely to result from progressive neuronal loss. Synaptodendritic alterations similar to those observed in hAPP mice are also observed in AD (Terry et al., 1991; Masliah et al., 2001a; Shim and Lubec, 2002; Moolman et al., 2004). Synaptic loss is an early event that correlates with cognitive impairment in AD (Terry et al., 1991; Masliah et al., 2001a). In addition, synaptic failure may precede and contribute to neuronal death (Selkoe, 2002). Finally, the frequent fluctuations in cognition seen in AD patients cannot be explained by sudden changes in neuronal numbers and are likely a manifestation of neuronal dysfunction (Palop et al., 2006).

4.15.2.1.4 Other APP fragments

Aβ is not the only biologically active APP fragment. γ-Secretase cleavage liberates a carboxy-terminal fragment known as the APP intracellular domain (AICD). The AICD activates the adapter protein Fe65, which translocates to the nucleus and regulates gene expression (Cao and Südhof, 2001, 2004). The AICD/Fe65 pathway stimulates expression of many genes, including neprilysin, a peptidase that degrades Aβ (Pardossi-Piquard et al., 2005), the P53 tumor suppressor, which controls programmed cell death (Alves da Costa et al., 2006), and components of the cytoskeleton (Müller et al., 2006). The AICD also modulates calcium stores in the endoplasmic reticulum (Leissring et al., 2002). While AICD-dependent changes in gene expression may be a factor in patients with APP mutations (Wiley et al., 2005), their importance in sporadic AD remains unclear. AICD levels and some cytoskeletal target genes are unchanged, while others are upregulated, and neprilysin expression is decreased (Wang et al., 2005; Müller et al., 2006).

The ectodomain of APP can be shed by α- or β-secretase, generating secreted APP fragments known as sAPPα and sAPPβ, respectively. Ectodomain shedding is a prerequisite for γ-secretase cleavage and, thus, Aβ and AICD production. sAPP may have independent effects, particularly neuroprotective and neurotrophic activities (Mattson, 1997; Kerr and Small, 2005; Zheng and Koo, 2006).

Finally, APP is cleaved by caspase(s) at a site 31 amino acids from the carboxy terminus; the resulting C-terminal fragment is termed C31 (Gervais et al., 1999; Lu et al., 2000). Transgenic mice expressing hAPP with a mutation that prevents this cleavage have less pronounced cognitive impairment than mice expressing caspase-sensitive hAPP (Galvan et al., 2006). The favorable effect of this mutation may be a result of reduced generation of C31, which is neurotoxic (Lu et al., 2000; Galvan et al., 2002). It may also be caused by changes in protein–protein interactions involving the APP carboxy terminus, including APP multimerization (Lu et al., 2003) and interactions with motor proteins (Satpute-Krishnan et al., 2006).

4.15.2.2 BACE

The first step in production of Aβ from APP is shedding the large APP ectodomain by β-secretase (**Figure 3**). The primary β-secretase enzyme is an aspartyl protease termed β-site APP cleaving enzyme (BACE1, also known as Asp2, memapsin 2) (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999). Genetic deletion of BACE1 dramatically reduces Aβ levels (Roberds et al., 2001) and prevents Aβ-dependent cognitive deficits in hAPP mice (Ohno et al., 2006). Thus, BACE1 inhibition is an attractive potential AD therapy.

Two aspects of BACE physiology are of particular interest in relation to synaptic transmission and neuronal function. First, BACE activity, at least its cleavage of APP, is dynamically regulated and increases with neuronal activity (Kamenetz et al., 2003). This effect contributes to a putative feedback loop whereby increased neuronal activity stimulates Aβ production, which in turn can suppress synaptic transmission (Kamenetz et al., 2003). Alterations in BACE regulation may be important in AD pathogenesis, as BACE activity is increased in AD (Holsinger et al., 2002) and after other types of neuronal injury (Blasko et al., 2004). Second, BACE1 has other substrates beyond APP, many of which affect neuronal function (**Table 1**). Among these are neuregulin 1, a

Table 1 Selected BACE1 substrates

Aβ
APP
APP-like proteins
Low density lipoprotein receptor-related protein
Neuregulin-1
P-selectin glycoprotein ligand-1
ST6Gal I sialyltransferase
Voltage-gated sodium channel β subunit

Adapted from Willem M, Garratt AN, Novak B, et al. (2006) Control of peripheral nerve myelination by the β-secretase BACE1. *Science* 314: 664–666.

ligand in the ErbB signaling pathway (Willem et al., 2006). In the absence of BACE cleavage, lack of neuregulin/ErbB signaling results in peripheral nerve hypomyelination (Willem et al., 2006). Cleavage of substrates besides APP may also contribute to AD. Mice overexpressing human BACE1 have neurodegeneration and memory deficits (Rockenstein et al., 2005). However, these effects are not a result of increases in Aβ levels, which are actually lower in BACE-overexpressing mice than in mice with normal BACE levels (Lee et al., 2005; Rockenstein et al., 2005), presumably because BACE overexpression promotes APP processing in an earlier component of the secretory pathway (Lee et al., 2005).

4.15.2.3 Presenilins

Shortly after the discovery that mutations in the APP gene cause autosomal dominant AD, two other genes were found in other families with early-onset AD: presenilin 1 (PS1) on chromosome 14 and presenilin 2 (PS2) on chromosome 1 (Levy-Lahad et al., 1995; Rogaev et al., 1995; Sherrington et al., 1995). PS1 mutations have since proven to be the most common cause of dominantly inherited AD, responsible for more than 50% of cases. Over 150 different mutations spanning the protein have been identified. PS2 mutations are much less common and tend to produce a somewhat less severe phenotype (Bertram and Tanzi, 2004). PS1 and PS2 are 67% identical and are ubiquitously expressed in the brain and other tissues.

The normal functions of the presenilins were unknown at the time of their discovery and are still being elucidated today. Most attention has focused on presenilin's role in Aβ production as a key component of γ-secretase. However, diverse γ-secretase-independent roles of presenilins continue to emerge, and controversy remains about the degree to which these roles contribute to AD and whether the AD-linked mutations cause primarily a gain or loss of function.

4.15.2.3.1 γ-Secretase

The presenilins are membrane-embedded aspartyl proteases that form the catalytically active center of the γ-secretase complex, along with nicastrin, Aph-1, and Pen-2 (De Strooper et al., 1998; Edbauer et al., 2003). γ-Secretase cleaves type I membrane proteins within their transmembrane domains (reviewed in Wolfe, 2006). Its substrate selectivity is rather broad, with the main requirement being a short extracellular domain (Struhl and Adachi, 2000). Thus, γ-secretase has many substrates (**Table 2**).

Table 2 Selected γ-secretase substrates

γ-protocadherin
APLP1
APLP2
APP
CD43
CD44
DCC
Delta
E-Cadherin
ErbB-4
Jagged
LRP
Voltage-gated sodium channel β2 subunit
N-Cadherin
Nectin-1α
Notch
NRADD
P75
Syndecan-1
Tyrosinase
Tyrosinase-related proteins 1 and 2

From Vetrivel KS, Zhang YW, Xu H, et al. (2006) Pathological and physiological functions of presenilins. *Mol. Neurodegener.* 1:4.

One of these is, of course, APP. Shedding of the large extracellular domain of APP by α- or β-secretase generates carboxy-terminal fragments (CTFs) that make suitable substrates for γ-secretase (**Figure 3**). The γ-secretase complex can cleave β-CTF at different sites, generating either Aβ40 or Aβ42. PS mutations favor production of Aβ42 over Aβ40 (Borchelt et al., 1996; Scheuner et al., 1996). Given the considerable evidence that Aβ42 is more pathogenic (see the section titled 'APP and Aβ'), this effect is believed to be an important mechanism by which PS mutations lead to AD. γ-Secretase is also responsible for generation of the AICD (see the section titled 'Other APP fragments').

4.15.2.3.2 γ-Secretase-independent roles of presenilins

In addition to their role in Aβ production, presenilins have several other functions, many of which are independent of γ-secretase, as they are not blocked by γ-secretase inhibitors or by point mutations that abolish secretase activity.

Several of these functions relate to calcium regulation. Presenilin mutations increase calcium release from the endoplasmic reticulum induced by inositol-1,4,5-trisphosphate (IP_3) (reviewed in LaFerla, 2002). One mechanism for this effect seems to be overfilling endoplasmic reticulum (ER) Ca^{2+} stores (Leissring et al., 2000). Presenilins form calcium leak channels

in the ER membrane; this function is lost in AD-associated mutants, leading to overfilling of the ER with Ca^{2+} (Tu et al., 2006). Others have pointed to an upregulation of IP_3 receptors in presenilin-deficient cells as another possible cause for increased calcium release (Kasri et al., 2006). Whatever the underlying mechanism, the resulting increases in intracellular calcium release induced by presenilin mutations are likely to contribute to neuronal dysfunction.

Presenilin also regulates intracellular signaling pathways that control tau phosphorylation. Presenilin stabilizes cadherin–cadherin complexes that interact with and activate phosphatidylinositol-3 kinase, stimulating Akt activity, which, in turn, suppresses glycogen synthase kinase (GSK) activity and tau phosphorylation (Baki et al., 2004). This γ-secretase-independent effect of presenilin in preventing tau phosphorylation is lost in AD-associated mutants and, thus, may enhance tau-mediated neurotoxicity (Baki et al., 2004).

Conditional PS1/PS2 double-knockout mice have learning and memory impairments, LTP deficits, aberrant tau phosphorylation, and neurodegeneration, although their Aβ levels are not increased (Saura et al., 2004), suggesting that mutations impairing APP-independent PS functions could also contribute to AD-related deficits.

4.15.2.4 Tau

Tau is a small microtubule-associated protein (MAP) and a member of the MAP2 superfamily (Weingarten et al., 1975; Cleveland et al., 1977; Dehmelt and Halpain, 2005). It has a variety of functions, including stabilizing microtubules, enabling neurite outgrowth, regulating axonal transport and controlling neuronal susceptibility to overexcitation (Shahani and Brandt, 2002; Avila et al., 2004; Roberson et al., 2007). Tau knockout mice are surprisingly normal, with no abnormalities in general health, fertility, longevity, gross brain cytoarchitecture, or learning and memory (Harada et al., 1994; Ikegami et al., 2000; Dawson et al., 2001; Tucker et al., 2001; Roberson et al., 2007). This may be, at least in part, the result of compensation by other MAPs, since double knockouts lacking both tau and MAP1B have 80% mortality in the first few weeks postnatally (Takei et al., 2000). Microdeletions on chromosome 17q21 including the tau gene are associated with mental retardation in humans (Lupski, 2006), but these deletions also involve several other genes. The fact that tau knockout mice have a very mild phenotype suggests that the loss of other genes might underlie the deficits associated with these deletions.

Tau was first implicated in AD by the discovery that NFTs are composed of heavily phosphorylated tau forming paired helical filaments (Grundke-Iqbal et al., 1986; Kosik et al., 1986; Wood et al., 1986; Lee et al., 1991). Interestingly, mutations in tau cause frontotemporal dementia, but not AD (Rademakers et al., 2004). However, the tau gene contains several polymorphisms that are in linkage disequilibrium, creating several unique haplotypes, one of which is associated with AD (Myers et al., 2005). The high-risk haplotype, known as H1c, is associated with roughly 10% more tau expression than other haplotypes (Kwok et al., 2004). This is consistent with the observation that reducing tau expression is protective in mouse models of AD (Roberson et al., 2007). The H1c haplotype also affects splicing of tau. Transcripts of the single tau gene are alternatively spliced to generate six different isoforms in adults (**Figure 5**). The most important distinction is between those isoforms with three copies of the microtubule-binding domain (termed 3R tau) and those with four (4R). The H1c haplotype is associated with slightly higher production of 4R than 3R tau (Myers et al., 2007). The mechanism by which these differences in tau expression raise AD risk is unclear.

4.15.2.4.1 NFTs, neuronal death, and memory loss

The study of tau's contribution to memory dysfunction in AD has concentrated largely on NFTs. Interest in NFTs dates all the way back to Alzheimer's original report (1907), which focused more on tangles than on plaques. Two observations form the basis of the hypothesis that tangles produce memory deficits in AD. First, the regional distribution of NFTs, which evolves in a stereotypical manner over the course of the illness, begins in medial temporal structures involved in memory (Braak and Braak, 1991). Tangles first appear in the transentorhinal region (stage I), then the entorhinal cortex (stage II), hippocampus (stage III), temporal neocortex (stage IV), and eventually other neocortical areas (stages V–VI) (**Figure 6**). Second, NFT counts correlate with clinical dementia severity, unlike amyloid plaque deposition, which does not (Giannakopoulos et al., 2007; but see Näslund et al., 2000).

Neuron loss may be a key mechanism underlying the connection between tau aggregation into NFTs and memory deficits. NFT burden correlates with the severity of neuronal loss (Giannakopoulos et al., 2007). NFT counts also correlate with levels of CSF tau, which increase in AD, possibly as a result of tau release

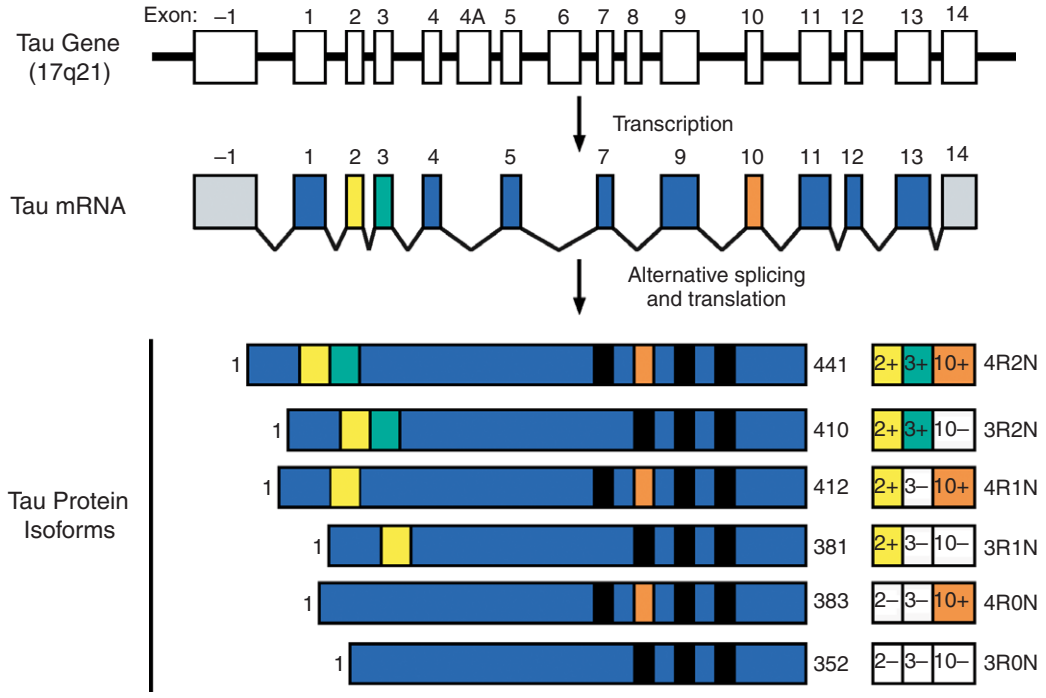


Figure 5 Alternative splicing of tau isoforms. The tau RNA can be alternatively spliced to include zero, one, or two amino-terminal inserts encoded by exons 2 and 3. Isoforms contain either three or four microtubule-binding domains, depending on whether exon 10 is included. (Modified from Buée L, Bussière T, Buée-Scherrer V, et al. [2000] Tau protein isoforms, phosphorylation and role in neurodegenerative disorders. *Brain Res. Rev.* 33: 95–130.)

from dying neurons (Arai et al., 1995; Tapiola et al., 1997; Giannakopoulos et al., 2007). Increased CSF tau is associated with poorer cognitive performance (Wallin et al., 2006) and a higher risk of progressing from MCI to AD (Blennow and Hampel, 2003).

Animal and cellular models also support a link between tau aggregation and neuron death (McGowan et al., 2006). Many transgenic mouse lines expressing human tau with mutations that favor tau aggregation display memory deficits and neuron loss (Lewis et al., 2000; Tatebayashi et al., 2002; Pennanen et al., 2004; McGowan et al., 2006). Overexpression of tau in large neurons of the lamprey leads to fibrillar tau aggregates and neuronal degeneration, an effect blocked by compounds that inhibit tau aggregation (Hall et al., 2001, 2002). Expression of aggregation-prone tau mutants in cultured neuroblastoma cells also causes toxicity that can be reversed by point mutations or small molecules that block aggregation (Khlistunova et al., 2006).

4.15.2.4.2 Tangle-independent roles for tau

There are limitations to the data suggesting a connection between NFTs and cognitive decline. Because much of the human data are correlational, they cannot establish causal relationships. Notably, animal model data indicate that aggregation is not the only means by which tau can impair neuronal and cognitive functions. Tau overexpression induced neurodegeneration in *Drosophila* in the absence of NFTs (Wittmann et al., 2001). Even in tau transgenic mice that have NFTs, neurodegeneration affects cells that do not have tau aggregates (Andorfer et al., 2005; Spires et al., 2006). Nonfibrillar tau might induce neuronal death by stimulating cell cycle reentry in normally postmitotic neurons (Andorfer et al., 2005; Khurana et al., 2006), although other mechanisms are possible also. The rTg4510 model has an inducible mutant tau transgene that, when turned on, causes NFT formation, severe neuronal loss, and spatial memory impairment (Ramsden et al., 2005; SantaCruz et al., 2005). However, suppressing transgene expression reverses the memory deficits, even though NFT formation continues, dissociating these processes (SantaCruz et al., 2005).

The mechanisms underlying these effects of tau on neuronal function have not yet been determined, although some leads exist. Abnormal tau can interfere with axonal transport (Ebneth et al., 1998; Ishihara et al., 1999), which seems consistent with its role as a

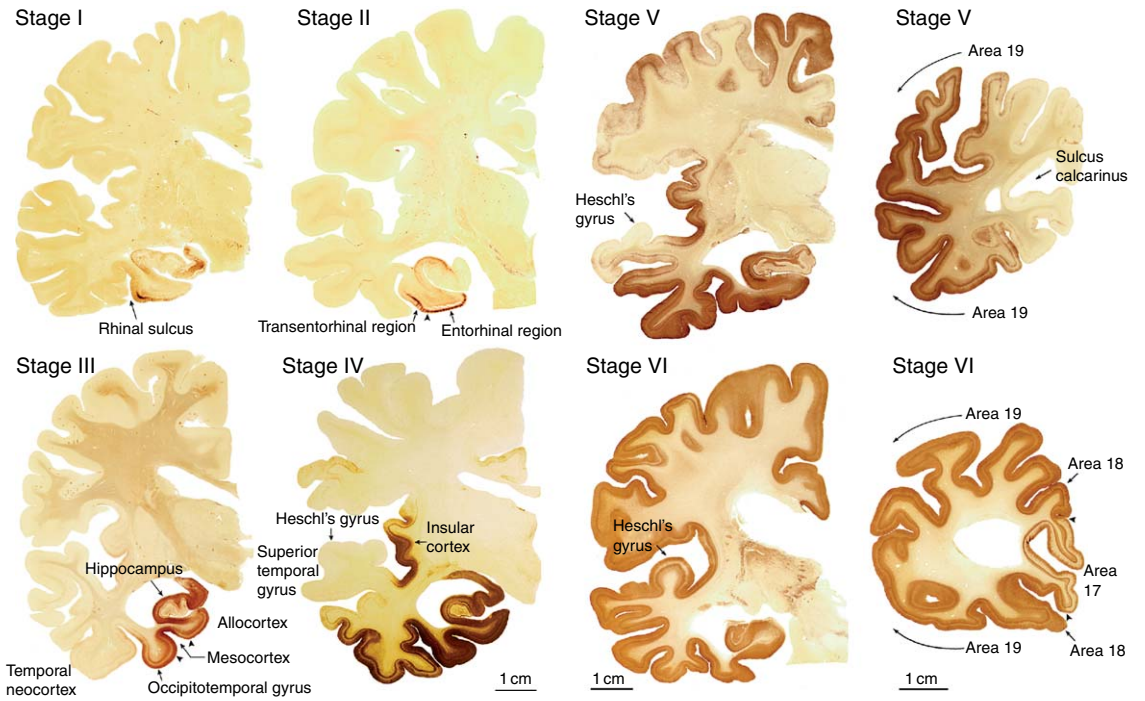


Figure 6 Stages of neurofibrillary pathology in Alzheimer's disease. Whole-brain sections were immunostained with antibody to phosphorylated tau. In stage I, involvement is limited to the transentorhinal region. Neurofibrillary pathology spreads to the entorhinal cortex in stage II. Stage III involves the hippocampus. Stage IV involves spread to the insula and inferior temporal neocortex. Finally, in stages V–VI even more neocortical areas are affected. (From Braak H, Rüb U, Schultz C, et al. [2006]. Vulnerability of cortical neurons to Alzheimer's and Parkinson's diseases. *J Alzheimers Dis* 9:35–44, with permission from IOS Press.)

microtubule-binding protein. Tau's influence on microtubule stability could also affect plasticity-related structural rearrangements, and some of these effects may relate to normal tau functions. Reversible synaptic regression during hibernation in certain rodents is associated with changes in tau, especially changes in its phosphorylation (Arendt et al., 2003). At young ages, before formation of aggregates or NFTs, tau transgenic mice have better-than-normal LTP in the dentate gyrus and longer-lasting memory than nontransgenic littermates (Boekhoorn et al., 2006), suggesting a role of soluble tau in cognitive function.

4.15.2.4.3 Tau phosphorylation and other posttranslational modifications

Aberrant tau phosphorylation is a hallmark of AD and seems to be carried out by many of the kinases involved in learning and memory (see the section titled 'Kinases'). Fully 19% of the amino acids in tau are potential phosphorylation sites (Ser, Thr, and Tyr), and many are in fact phosphorylated in AD brains (Stoothoff and Johnson, 2005). The vast majority of the phosphorylation sites are highly conserved across species and surround tau's microtubule-binding domains (**Figure 7**). Much effort has been devoted to

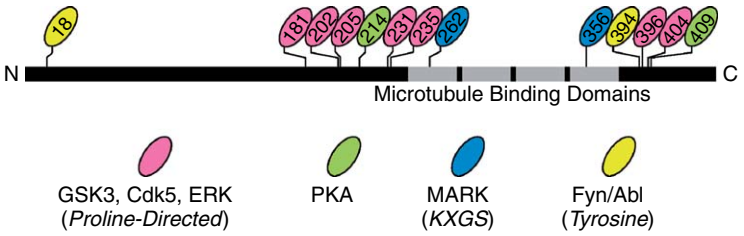


Figure 7 Tau phosphorylation sites. The four microtubule-binding domains are indicated by shading in a schematic line drawing of tau protein. Most tau phosphorylation sites surround the microtubule-binding regions.

sorting out which sites and kinases are most important in the pathogenesis of AD.

- Proline-directed kinase sites. Many tau phosphorylation sites are substrates for proline-directed kinases, which target Ser/Thr residues directly adjacent to a proline. These sites are substrates for glycogen synthase kinase 3 (GSK3), cyclin-dependent kinase 5 (Cdk5), and extracellular-signal regulated kinase (ERK), among others. Phosphorylation at these sites seems to be involved in the tau aggregation/cell death pathways mentioned above (Lucas et al., 2001; Augustinack et al., 2002; Cruz et al., 2003; Noble et al., 2003). For example, GSK3 stimulates tau aggregation (Sato et al., 2002), and GSK3 inhibition reduces tau aggregation and neurodegeneration (Noble et al., 2005).
- PKA sites. Tau is phosphorylated by cyclic AMP-dependent protein kinase (PKA), preferentially at Ser214 (Scott et al., 1993). Phosphorylation of Ser214 inhibits tau's microtubule binding and stabilizing activity (Illenberger et al., 1998). The PKA sites are near the proline-directed sites, and tau phosphorylation by PKA facilitates phosphorylation by proline-directed kinases (Singh et al., 1996; Liu et al., 2004) but may inhibit aggregation (Schneider et al., 1999).
- KXGS sites. The PKA and proline-directed sites are concentrated at both ends of the four microtubule-binding domains of tau. Within these domains are sites with a Lys-Xxx-Gly-Ser (KXGS) consensus sequence, most notably Ser262 and Ser356. These sites are phosphorylated by microtubule-affinity regulating kinase (MARK), which dramatically reduces tau's ability to stabilize microtubules (Drewes et al., 1997) and facilitates neurite outgrowth (Biernat et al., 2002). This phosphorylation may also be a prerequisite for phosphorylation at proline-directed kinase sites (Nishimura et al., 2004; but see Biernat and Mandelkow, 1999).
- Tyrosine phosphorylation. In AD brains, tau is also phosphorylated on tyrosine residues, primarily Tyr18 by Fyn and Tyr394 by Abl (Williamson et al., 2002; Lee et al., 2004; Derkinderen et al., 2005). There is considerable evidence for a role of Fyn in AD (Lambert et al., 1998; Chin et al., 2004, 2005; Lee et al., 2004), as reviewed further in the section titled 'Fyn.' Interestingly, interactions between tau and Fyn may contribute to the subcellular localization of Fyn and, thus, affect its substrate availability (Lee et al., 1998). In addition, disease-associated tau phosphorylation and mutations strongly increase Fyn binding (Bhaskar et al., 2005).
- Tau proteolysis. The carboxy terminus of tau can be cleaved by activated caspases, producing a truncated tau that aggregates more easily than full-length tau (Cotman et al., 2005). This may be an important step leading to development of tangles in AD, as tau cleavage at this site is a relatively early event in NFT formation (Rissman et al., 2004; Guillozet-Bongaarts et al., 2005). Tau is also cleaved by calpain, which produces a 17-kD tau fragment that is toxic to cultured neurons (Park and Ferreira, 2005).

4.15.2.4.4 *Tau and Aβ*

Given the prominence of Aβ and tau in AD pathology, their relationship is of considerable interest. Although expression of human Aβ does not induce NFTs in hAPP transgenic mice, it increases tangle formation in hAPP mice coexpressing mutant human tau; in contrast, expression of mutant human tau does not seem to worsen Aβ-dependent pathologies in hAPP mice (Lewis et al., 2001; Götz et al., 2001). In such multiple transgenic models, Aβ immunotherapy decreases tau pathology, further suggesting that Aβ acts upstream of tau (Oddo et al., 2004). Plausible mechanisms include Aβ-induced kinase activation and resulting tau phosphorylation (see the section titled 'Kinases'). In addition, caspase activation by Aβ may stimulate tau proteolysis that favors aggregation (Cotman et al., 2005).

Calpain-mediated tau proteolysis seems to play an important role in Aβ toxicity *in vitro*. Tau-deficient primary neurons are resistant to the rapid neurodegeneration induced by Aβ application, apparently because they lack calpain-induced tau fragments that are toxic to neurons in culture (Rapoport et al., 2002; Park and Ferreira, 2005). Reducing tau even by just 50% ameliorated Aβ-induced memory deficits in a mouse model of AD (Roberson et al., 2007). Interestingly, this effect did not seem to involve removal of a tau species with Aβ-induced posttranslational modifications. Rather, tau reduction prevented Aβ-induced epileptiform activity and compensatory inhibitory remodeling of hippocampal circuits (Palop et al., 2007; Roberson et al., 2007).

4.15.2.5 ApoE

ApoE is a multifunctional lipoprotein originally discovered for its role in intercellular transport and distribution of cholesterol throughout the body (Mahley, 1988). The brain is second only to the liver

as a producer of apoE. In addition to mediating lipid transport within the central nervous system, apoE is involved in the response to neural injury and regulation of neurite outgrowth (Mahley and Rall, 2000).

Three main apoE isoforms are produced from different alleles ($\varepsilon 2$, $\varepsilon 3$, and $\varepsilon 4$) of a single *APOE* gene on chromosome 19; apoE2 and apoE4 differ from each other and from the more frequent apoE3 by single amino acid substitutions, which have major effects on apoE structure and function (**Figure 8**) (Hatters et al., 2006; Mahley et al., 2006).

ApoE was implicated in AD by the near-simultaneous discoveries that it binds Aβ and colocalizes to amyloid plaques (Namba et al., 1991; Strittmatter et al., 1993) and that *APOE* genotype has dramatic effects on AD risk and onset (Corder et al., 1993). Individuals with one, and particularly those with two, $\varepsilon 4$ alleles are much more likely to develop AD and have an earlier age of onset compared with $\varepsilon 3$ carriers, while $\varepsilon 2$ carriers are most resistant to the disease (**Figure 9**) (Corder et al., 1993). *APOE* $\varepsilon 4$ appears to be the most important genetic risk factor for sporadic AD (Farrer et al., 1997; Raber et al., 2004; Bertram et al., 2007); 40–60% of all sporadic AD patients have at least one $\varepsilon 4$ allele (Saunders et al., 1993).

These genetic studies established that apoE4 decreases the age at which AD becomes manifest. Interestingly, even in the absence of frank AD, individuals with apoE4 have abnormalities in cognitive performance and functional neuroimaging. In apoE4 carriers, the normal age-related decline in performance on episodic memory tasks occurs at an earlier age and progresses at a faster rate than in noncarriers, while other cognitive domains do not appear to be affected (Caselli et al., 1999, 2004). ApoE4 carriers are also more likely to develop cognitive deficits following open heart surgery or head injury (Tardiff et al., 1997; Teasdale et al., 1997). Even before cognitive impairment is detectable, apoE4 carriers have hypometabolism in the same regions affected in AD, including posterior parietal, posterior cingulate, and frontal cortex (Reiman et al.,

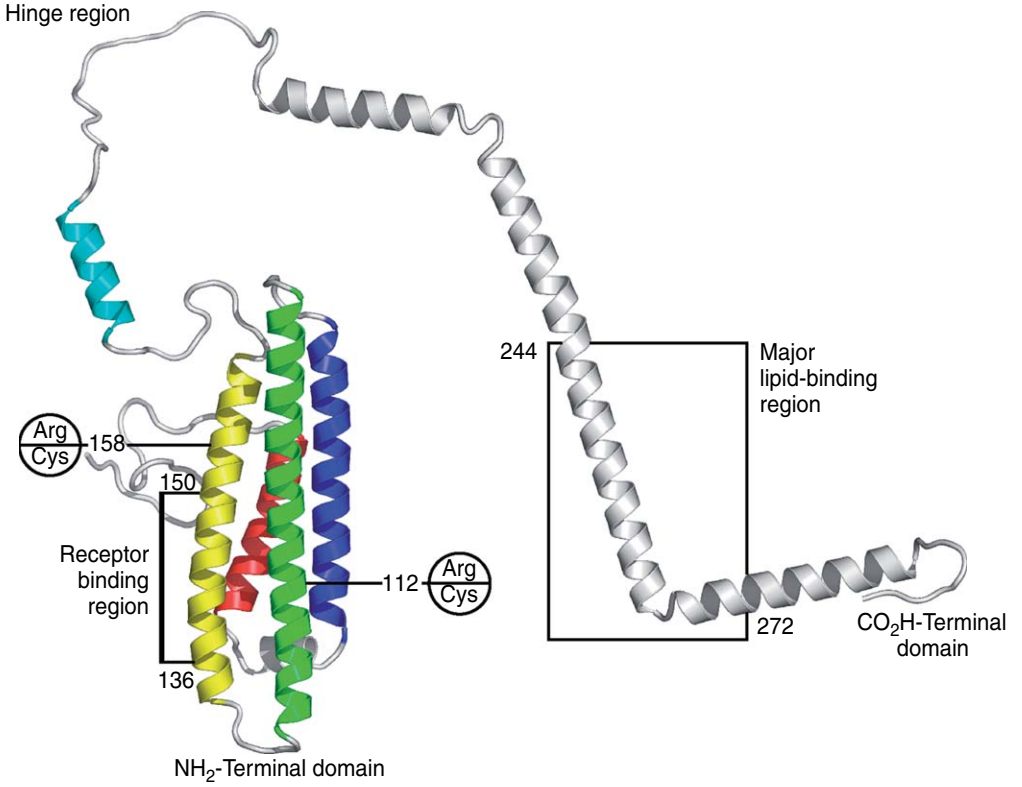


Figure 8 ApoE structure and isoforms. The apoE molecule consists of a globular amino-terminal domain that mediates receptor binding, while the lipid-binding region is in the carboxy-terminal domain. ApoE2 and apoE4 isoforms differ from the most common apoE3 by single amino acid substitutions. At amino acid positions 112 and 158, apoE3 contains Cys and Arg, whereas apoE2 contains Cys and Cys and apoE4 contains Arg and Arg. (From Hatters DM, Peters-Libeu CA, and Weisgraber KH [2006] Apolipoprotein E structure: Insights into function. *Trends Biochem. Sci.* 31: 445–454.)

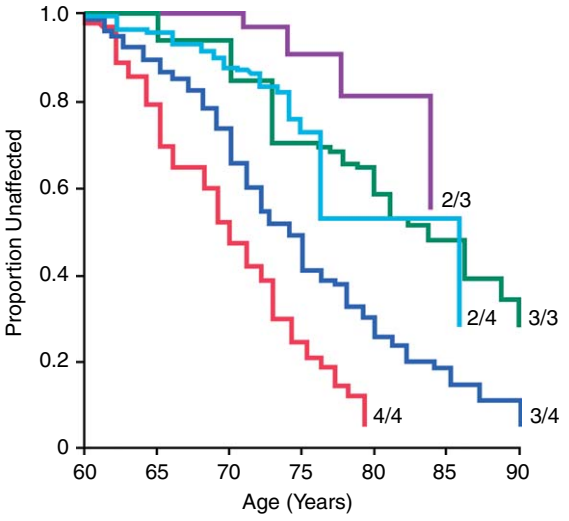


Figure 9 Effect of apoE genotype on AD risk. ApoE4 increases AD risk in a gene dose–dependent manner, while apoE2 lowers risk. (From Strittmatter WJ and Roses AD [1996] Apolipoprotein E and Alzheimer's disease. *Annu. Rev. Neurosci.* 19: 53–77.)

2004, 2005). These abnormalities are seen in apoE4 carriers as young as 20–39 years (Reiman et al., 2004).

4.15.2.5.1 Interactions between Aβ and apoE

ApoE4-related cognitive impairment is also seen in animal models, including mice expressing human apoE4 with or without hAPP/Aβ in neurons. ApoE3, but not apoE4, protects against hAPP/Aβ-induced cognitive deficits (Raber et al., 2000) and synaptic loss (Buttini et al., 2002). Remarkably, this effect is seen well before such mice form amyloid plaques. Consistent with these results, oligomeric Aβ impairs LTP more in slices from apoE4 knockin mice than apoE3 knockin mice (Trommer et al., 2005).

In addition, apoE has a prominent effect on Aβ aggregation and deposition. ApoE binds directly to Aβ and is a component of amyloid plaques (Namba et al., 1991; Strittmatter et al., 1993). ApoE4 is associated with increased deposition of amyloid plaques both in AD (Schmechel et al., 1993) and after head trauma (Nicoll et al., 1995). ApoE-deficient hAPP mice have almost no amyloid plaques (Bales et al., 1997). Compared with apoE3, apoE4 greatly increases amyloid plaque deposition in aged hAPP mice (Holtzman et al., 2000; Buttini et al., 2002). Given their effects on Aβ aggregation into fibrils and plaques, apoE isoforms may also have differential effects on Aβ aggregation into oligomers, although this has not yet been shown.

In addition to direct effects on Aβ aggregation, apoE4 increases Aβ production by stimulating endocytosis of APP-containing vesicles, which enter the endosomal pathway where much of Aβ is produced (Ye et al., 2005). ApoE also increases intracellular Aβ by enhancing its uptake from the extracellular space via the LDL receptor-related protein (LRP), although this effect is not isoform-dependent (Zerbinatti et al., 2006). Finally, once Aβ has been taken up into lysosomal vacuoles, apoE4 potentiates Aβ-induced lysosomal leakage and apoptosis (Ji et al., 2006).

4.15.2.5.2 Aβ-independent mechanisms for apoE4-induced neuronal impairments

Compared with apoE-deficient mice, female mice with neuronal expression of apoE4 develop age-dependent deficits in learning and memory even in the absence of Aβ (Raber et al., 1998, 2000), presumably because of apoE4-induced reductions in andogen receptor levels in the brain (see following). ApoE4 knockin mice also have LTP impairments not seen in apoE3 mice (Trommer et al., 2004). Neuronal expression of human apoE3, but not apoE4, protected mice lacking endogenous apoE against synaptodendritic damage elicited by excitotoxic drugs (Buttini et al., 1999). Notably, these excitoprotective effects of apoE3 were eliminated when apoE3 and apoE4 were coexpressed in the same mice, suggesting a dominant adverse effect of apoE4 (Buttini et al., 2000).

ApoE is also found in NFTs and has effects on tau (Namba et al., 1991). ApoE3 binds to tau, whereas apoE4 does not (Strittmatter et al., 1994). ε4 carriers have more NFTs than age-matched ε3 homozygotes (Ohm et al., 1999). ApoE4 also stimulates microtubule depolymerization (Nathan et al., 1995), possibly through effects on tau. Neuronal, but not astroglial, expression of apoE4 in transgenic mice increases tau phosphorylation (Tesseur et al., 2000). Neuronal expression of endogenous apoE occurs primarily after neuronal injury (Xu et al., 2006b). In neurons, apoE4 undergoes cleavage by a chymotrypsin-like protease activity, and E4 is more susceptible to cleavage than E3 (Huang et al., 2001). The resulting C-terminally truncated apoE stimulates tau phosphorylation and NFT formation (Huang et al., 2001; Brecht et al., 2004). Truncated apoE can escape the secretory pathway and enter the cytosol, where it binds to mitochondria and impairs their function (Mahley et al., 2006).

ApoE also has cerebrovascular effects that may contribute to memory dysfunction. ApoE4 carriers have higher plasma cholesterol levels (Hallman et al., 1991) and are at higher risk of carotid atherosclerosis (Terry

et al., 1996), coronary artery disease (Chen et al., 2003), and ischemic stroke (McCarron et al., 1999). The overlap between these cerebrovascular risks and AD is becoming increasingly clear (Martins et al., 2006).

There are interesting gender differences in the effects of apoE that point toward important interactions with sex hormones. Among ε4 carriers, women are more likely to develop AD than men (Payami et al., 1996), and female apoE4 mice are more susceptible to memory deficits than their male counterparts (Raber et al., 1998, 2000; Grootendorst et al., 2005). This difference may be the result of effects of apoE4 on androgen receptors. ApoE4 decreases androgen receptor levels in males and females, and females may be more susceptible to this effect because of their lower circulating androgen levels (Raber et al., 2002; Raber, 2004).

Last, apoE4 is intrinsically less stable than apoE3 (Morrow et al., 2002). As a result, brain apoE levels are lower in individuals with the ε4 allele, an isoform difference that has also been identified in apoE4 and apoE3 knockin mice (Gregg et al., 1986; Ramaswamy et al., 2005). Thus, in addition to the adverse gain-of-function effects described above, apoE4 may contribute to neurological impairments by a loss-of-function mechanism. Consistent with this notion, ApoE-deficient mice display age-dependent synaptic loss, deficient regenerative axonal sprouting after perforant pathway transection, and greater susceptibility to diverse neural injuries (Masliah et al., 1995a, b; Buttini et al., 1999, 2000; Krzywkowski et al., 1999).

4.15.2.6 α-Synuclein

α-Synuclein is a small, cytosolic protein that is enriched in presynaptic terminals; it regulates presynaptic function and neurotransmitter release (Chandra et al., 2004; Fortin et al., 2005). It is also the main component of Lewy bodies, neuronal inclusions associated with most forms of Parkinson's disease (PD) and other Lewy body diseases (Spillantini et al., 1997). Mutations in α-synuclein are linked to rare forms of autosomal dominant PD (Polymeropoulos et al., 1997). α-Synuclein also seems to play a role in the intriguing clinical and neuropathological overlap between PD and AD. Many AD patients have Lewy bodies, sometimes known as the Lewy body variant of AD, and many PD patients develop AD-like dementia (Perl et al., 1998), emphasizing the fact that AD is a polyproteinopathy combining the accumulation of abnormal assemblies or fragments of Aβ, tau, apoE, and α-synuclein.

The initial link between α-synuclein and dementia was established through the discovery of a so-called 'nonamyloid component' (NAC) of plaques in AD brains, which turned out to be a fragment of α-synuclein (Uéda et al., 1993; Iwai et al., 1995). α-Synuclein promotes Aβ aggregation *in vitro* (Yoshimoto et al., 1995), although it doesn't seem to increase amyloid plaque deposition *in vivo* (Masliah et al., 2001b). α-Synuclein does, however, worsen Aβ-induced neuronal deficits independently of plaques. Doubly transgenic mice expressing hAPP/Aβ and wild-type human α-synuclein in neurons displayed more synapse loss, greater reductions in choline acetyltransferase-positive neurons, and more severe cognitive impairments than the singly transgenic parental strains, even though α-synuclein had no effect on plaque formation *in vivo* (Masliah et al., 2001b). This study also revealed that Aβ strongly promotes α-synuclein aggregation, both *in vitro* and *in vivo*. α-Synuclein concentrations are elevated in synaptic boutons in AD brains, suggesting that α-synuclein also plays a role in synaptic pathology in the human condition (Masliah et al., 1996). Whether the underlying mechanisms relate to α-synuclein's normal functions or to its abnormal aggregation remains to be determined.

4.15.3 Memory-Related Molecules in AD

Although some of the major players in the pathobiochemistry of AD have been identified, it is just now beginning to be understood how these molecules affect neuronal function and impair learning and memory. Mouse models of AD recapitulate many aspects of the human disease, both in terms of pathology and in relation to behavioral/memory impairments. Indeed, transgenic mouse models have provided a unique opportunity for synergy between two historically separate fields of biomedical research – AD research and the basic scientific analysis of learning and memory. As reviewed below, research in the last 15 years has revealed that AD-relevant molecules such as Aβ, tau, and apoE affect several aspects of neuronal function and cellular mechanisms of plasticity that have been implicated in the formation of long-lasting memories.

4.15.3.1 Neurotransmitter Release

Reliable neurotransmission requires a steady supply of synaptic vesicles filled with neurotransmitter to be ready for release at the presynaptic terminal. Since trafficking of new synaptic vesicles from the cell body

can take several hours, nerve terminals are equipped with a special machinery that allows for local recycling and refilling of synaptic vesicles, a process that replenishes vesicle pools within seconds to minutes (Südhof, 2004; Fernandez-Alfonso and Ryan, 2006; Kavalali, 2006; Ryan, 2006). The number of recycling vesicles and the efficiency with which they fuse with (exocytosis) and are retrieved from (endocytosis) the presynaptic terminal plasma membrane set the boundaries on the duration and frequency of neurotransmission, particularly during repetitive stimulation. Thus, factors that affect vesicle cycling, particularly steps that are rate limiting, can have profound consequences on synaptic efficacy.

The fusion of synaptic vesicles with the presynaptic membrane and their recovery through endocytosis require a number of neuron-specific proteins. The levels of many proteins that coordinate the docking and fusion of synaptic vesicles are decreased in AD as well as in experimental models (Honer, 2003; Scheff and Price, 2003). Synaptophysin, one of the most abundant membrane proteins on synaptic vesicles, is integral to the vesicle fusion process for neurotransmitter release. Decreases in synaptophysin have been used extensively as a measure of synaptic impairments in both AD and transgenic mouse models of the disease (Masliah et al., 1993; Mucke et al., 2000; Honer, 2003; Scheff and Price, 2003). Decreased levels of proteins involved in vesicle release, such as synaptophysin, may represent decreased expression at intact synapses, synaptic degeneration, or both.

In addition, several proteins involved in the local endocytosis and recycling of vesicles, including synaptotagmin, AP2, AP180, and dynamin I, are altered in AD and in some mouse models of the disease, as illustrated in **Figure 10** (Honer, 2003; Yao, 2004; Nixon, 2005). Animal models in which any of these proteins are mutated or ablated exhibit abnormal synaptic vesicle size and number, synaptic transmission deficits, and even mortality in extreme cases (reviewed in Yao, 2004; Kavalali, 2006). For some factors, both protein and mRNA levels are decreased in AD, suggesting that the expression of the corresponding genes may be dysregulated. Other factors may be depleted by increased cleavage and degradation (Yao, 2004; Kelly et al., 2005; Kelly and Ferreira, 2006; but see Yao et al., 2005). Impaired vesicle recycling may also contribute to some of the

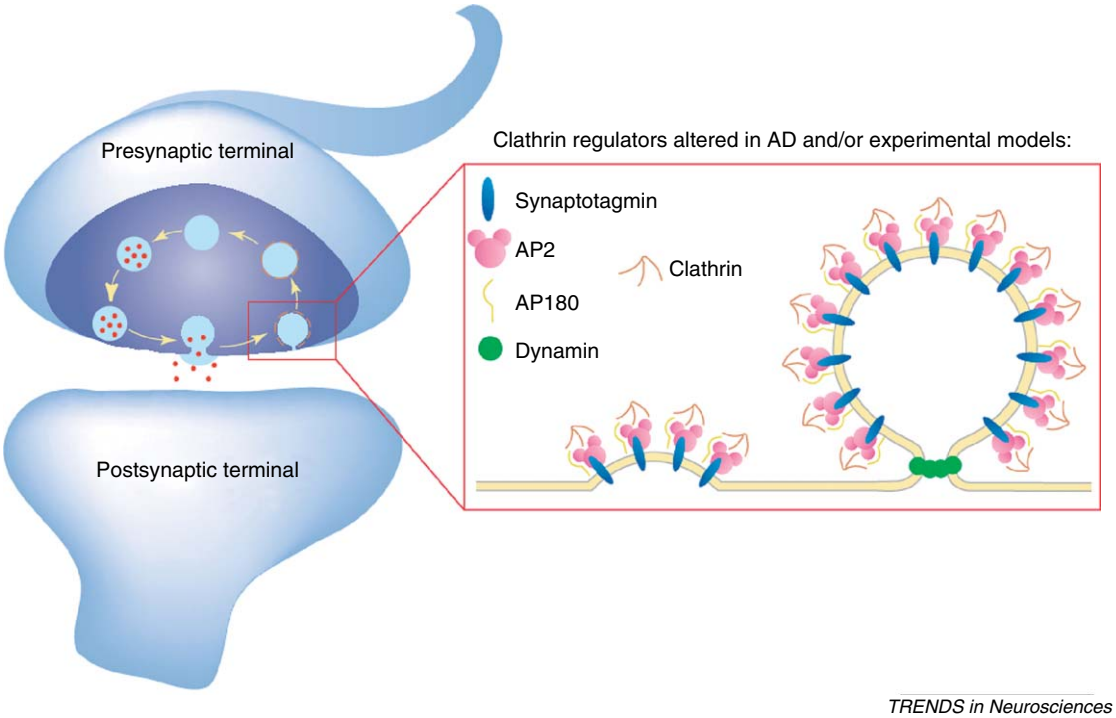


Figure 10 Alzheimer's disease (AD) affects clathrin-mediated synaptic vesicle recycling in synapses. Synaptic vesicles are recycled through clathrin-mediated endocytosis, which requires clathrin and regulatory proteins. The levels of several key regulators (see red box) of this process are decreased in AD as well as in experimental models of the disease. (From Yao P [2004] Synaptic frailty and clathrin-mediated synaptic vesicle trafficking in Alzheimer's disease. *Trends Neurosci.* 27: 24–29.)

ultrastructural changes found in AD brains and related mouse models. Decreases in synaptic density, attributed to synapse loss, are accompanied by an increase in size of the remaining synapses (reviewed in Scheff and Price, 2003). Although it has been hypothesized that such increases represent compensatory changes to maintain overall synaptic contact area, it has also been proposed that impaired endocytosis for local generation of vesicles leads to an overall accumulation of membrane and enlargement of nerve terminals (Yao, 2004).

In summary, alterations in levels of key components in synaptic vesicle trafficking may contribute to deficits in neurotransmitter release in AD.

4.15.3.2 Receptors and Channels

A large number of cell surface receptors and channels are located on the postsynaptic membrane, ready to receive and transduce input from presynaptic contacts. The strength of any given synapse can remain stable over time, increase, or decrease based on the number, localization, and activity of the neurotransmitter receptors and ion channels at the postsynaptic membrane. In this section, we review several receptors and channels that play important roles in synaptic plasticity and describe how alterations in their levels, localization, or function may contribute to AD-related cognitive dysfunction.

4.15.3.2.1 NMDA receptors

N-methyl-D-aspartate (NMDA) receptors play a critical role in the induction of LTP by acting as coincidence detectors of presynaptic glutamate release and postsynaptic depolarization (reviewed in Wang et al., 2006). Subsequent influx of calcium through the NMDA receptor triggers a series of intracellular signaling events that culminate in the induction of gene expression required for long-term changes in synaptic efficacy.

Currently approved therapies for the treatment of AD include an NMDA receptor antagonist, which is thought to protect neurons against increased calcium permeability of NMDA receptors and to increase the synaptic signal-to-noise ratio (Tariot and Federoff, 2003; Jacobsen et al., 2005). Studies in transgenic mouse models of AD and cell culture experiments are beginning to unravel the mechanisms by which AD-relevant molecules such as Aβ might alter NMDA receptor functions and impact synaptic plasticity.

As reviewed in the section titled 'Soluble Aβ oligomers,' many studies have reported that Aβ potently inhibits the induction of LTP both *in vitro* and *in vivo* (reviewed in Walsh and Selkoe, 2004). Although it is uncertain whether Aβ directly binds NMDA receptors, several lines of evidence demonstrate that at least part of Aβ's effect on LTP may be a result of alterations in the level, availability, or activity of NMDA receptors at the postsynaptic membrane.

In vitro, low concentrations of synthetic Aβ peptides acutely augment NMDA receptor–mediated calcium influx and synaptic transmission (Wu et al., 1995; Wu and Dun, 1995; Kelly and Ferreira, 2006). Furthermore, Aβ-dependent degradation of dynamin and the scaffolding protein PSD-95 can be blocked by NMDA receptor antagonists (Almeida et al., 2005; Roselli et al., 2005; Kelly and Ferreira, 2006). However, Aβ produces a delayed NMDA receptor–dependent reduction in synaptic transmission (Cullen et al., 1996). Because prolonged NMDA receptor stimulation leads to down-regulation of receptor activity through the recruitment of negative feedback loops (Oster and Schramm, 1993; Resink et al., 1995; Salter and Kalia, 2004; Braithwaite et al., 2006), it is possible that acute, Aβ facilitates glutamatergic transmission and sensitizes neurons to excitotoxic events (Mattson et al., 1993), whereas chronically, it leads to a down-regulation of NMDA receptor activity.

In vivo, hAPP transgenic mice that produce high Aβ levels have decreased levels of phosphorylation of Tyr-1472 of NR2B subunits of the NMDA receptor, particularly in the dentate gyrus (Palop et al., 2005). Phosphorylation at this residue affects the gating of the channel and is positively correlated with NMDA receptor currents (Lu et al., 1999; Alvestad et al., 2003; reviewed in Salter and Kalia, 2004), suggesting that the decreased phosphorylation found in hAPP mice may contribute to an attenuation of NMDA receptor-dependent signaling. Importantly, decreased levels of Tyr-1472 phosphorylation were associated with decreased activity of Fyn, a src-kinase family member that phosphorylates NR2B subunits at this residue, and increased expression of striatal-enriched phosphatase (STEP), a tyrosine phosphatase that negatively regulates Fyn activity (Chin et al., 2005). STEP can dampen NMDA receptor-dependent activity when engaged by high levels of stimulation (Pelkey et al., 2002; Braithwaite et al., 2006). The regulation of tyrosine kinases, such as Fyn, by Aβ is discussed in more detail in the section titled 'Kinases.'

Phosphorylation at Tyr-1472 also regulates the interaction of NMDA receptors with the scaffolding protein PSD-95 and with AP-2, an adaptor molecule that triggers clathrin-mediated endocytosis (Lavezzari

et al., 2003). *In vitro* experiments demonstrate that Aβ-dependent dephosphorylation of Tyr-1472 induces endocytosis of NMDA receptors, resulting in decreased surface expression of this key regulator of LTP induction (Snyder et al., 2005). The results of this study suggested a model in which extracellular Aβ binds α7 subunit-containing nicotinic acetylcholine receptors and thereby activates the phosphatases PP2B (calcineurin) and STEP, resulting in dephosphorylation of NR2B (**Figure 11**). Thus, Aβ may impair synaptic plasticity by inducing the dephosphorylation of Tyr-1472 and attenuating NMDA receptor activity through a variety of mechanisms.

The attenuation of glutamatergic transmission by Aβ may have different consequences depending on the brain region affected. If brain regions that control neuronal excitability on a global scale are particularly susceptible to Aβ-induced impairments of glutamatergic transmission, aberrant increases in overall brain activity may result. Such a notion is supported by findings that hAPP mice with high levels of Aβ exhibit nonconvulsive seizure activity in EEG recordings, which was associated with the induction of compensatory inhibitory mechanisms and impairments in synaptic plasticity (Palop et al., 2007).

4.15.3.2.2 AMPA receptors

α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors are glutamate-gated channels that mediate most of the fast excitatory synaptic transmission in the brain and provide the primary means of postsynaptic depolarization in glutamatergic neurotransmission. AMPA receptor localization is dynamically regulated by neuronal activity, and rapid insertion and removal of these receptors into/from the postsynaptic membrane are key mechanisms by which long-term changes in

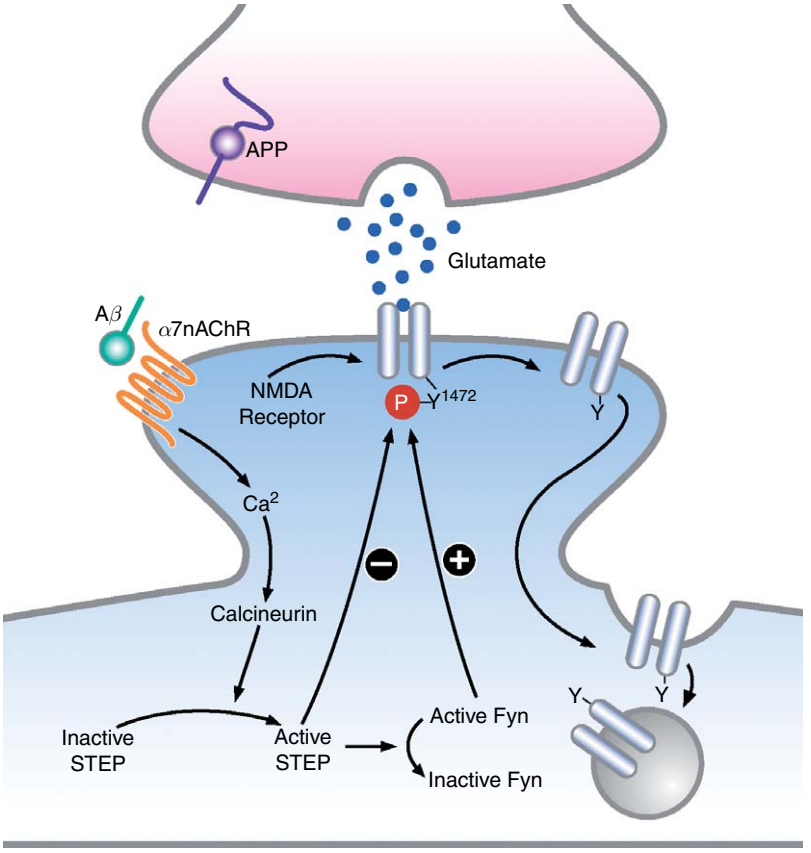


Figure 11 Aβ attenuates *N*-methyl-D-aspartate (NMDA) receptor signaling. Under normal circumstances, phosphorylation of the NMDA receptor at Tyr1472 is controlled by a balance between phosphorylation by Fyn and dephosphorylation by STEP. STEP also negatively regulates Fyn. In models of AD, Aβ activates α7 nAChRs and increases STEP activity, resulting in a net decrease in Fyn activity and Tyr1472 phosphorylation. In the absence of Fyn-mediated tyrosine phosphorylation, NMDA receptors are endocytosed.

synaptic strength (LTP and LTD) are expressed (Bredt and Nicoll, 2003; Esteban, 2003).

Such dynamic regulation of AMPA receptor density at the synapse requires that a pool of receptors be available for use at any given time. Indeed, recycling endosomes in dendritic compartments maintain a pool of AMPA receptors that can be rapidly mobilized and shuttled to the synaptic membrane in response to NMDA receptor activation to effect increases in synaptic strength (**Figure 12**) (Park et al., 2004; reviewed in Kennedy and Ehlers, 2006), whereas LTD-inducing stimuli result in endocytosis and removal of AMPA receptors from the synapse (Bredt and Nicoll, 2003).

In addition to their role in rapidly modifying synaptic strength, AMPA receptors play a critical role in another, slower form of plasticity called synaptic scaling (reviewed in Turrigiano and Nelson, 2004). In this type of homeostatic plasticity, the overall synaptic strength of a neuron is modulated to regulate its excitability depending on its history of activity. Periods of reduced activity result in increased levels of AMPA receptors at the synapse, whereas periods of increased activity lead to removal of AMPA receptors from the synapse. Moreover, regulation of AMPA receptor density at the postsynaptic membrane partly underlies distance-dependent scaling, in which synapses that lie farther from the soma are endowed with increased synaptic strength so they can transmit information with similar fidelity as synapses located closer to the soma (Andrasfalvy and Magee, 2001; Smith et al., 2003).

Several factors contribute to the ability of AMPA receptors to fulfill these critical roles in dictating synaptic strength and homeostatic control of neuronal activity, including expression levels, dendritic transport, and local synaptic trafficking within the endosomal pathway. Thus, impairments in any one of these processes may lead to deficits in synaptic plasticity and in the regulation of activity levels.

It is particularly interesting in this regard that reductions in several AMPA receptor subunits have been documented in the entorhinal cortex and hippocampus in AD and related transgenic mouse models (Yasuda et al., 1995; Chan et al., 1999; Wakabayashi et al., 1999; Carter et al., 2004; Chang et al., 2006; Palop et al., 2007). In AD, AMPA receptors also appear to be cleaved by caspases (Chan et al., 1999). In primary neurons or slices treated with Aβ or isolated from hAPP transgenic mice, surface expression of AMPA

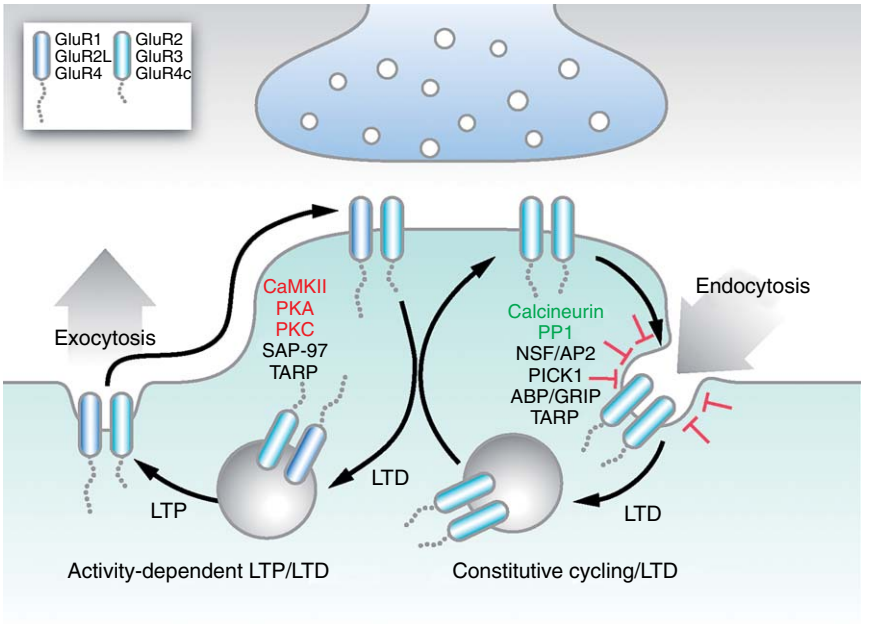


Figure 12 α-Amino-3-hydroxy-5-methyl-4-isoxazole-proprionic acid (AMPA) receptor trafficking. The trafficking of AMPA receptors between the synaptic membrane and recycling endosomes regulates synaptic strength. The insertion of AMPA receptors into the synapse (in long-term potentiation [LTP]) and the endocytosis of synaptic AMPA receptors (in long-term depression [LTD]) are governed by the activities of several kinases, phosphatases, and binding proteins. In Alzheimer's disease or related models, the activities of several kinases are decreased (red font), whereas the activities of several phosphatases are increased (green font), which may contribute to overall decreases in synaptic strength.

receptors is decreased (Almeida et al., 2005; Roselli et al., 2005; Hsieh et al., 2006). Such alterations may contribute to the decreased AMPA-mediated currents and increased NMDA/AMPA current ratios found in several *in vitro* and transgenic mouse models of AD (Hsia et al., 1999; Chang et al., 2006; Shemer et al., 2006).

Since AMPA receptors play such key roles in synaptic and homeostatic plasticity, alterations in their expression or trafficking may contribute to deficits in synaptic plasticity and learning and memory in AD and related models. As reviewed in the next section, it has been hypothesized that increased cholinergic activity in early stages of AD may be recruited to support synaptic scaling, perhaps in response to the loss of normal mechanisms underlying this homeostatic plasticity (Small, 2004).

4.15.3.2.3 Nicotinic acetylcholine receptors

Neuronal nicotinic acetylcholine receptors (nAChRs) are key modulators of neurotransmission. Presynaptically localized receptors enhance neurotransmitter release, postsynaptic receptors transduce fast excitatory transmission and calcium-regulated signaling, and perisynaptic or nonsynaptic receptors modulate neuronal excitability (Dani and Bertrand, 2006).

Although cholinergic activity is increased in early stages of AD, the loss of cholinergic neurons, particularly in the basal forebrain, is a characteristic neuropathological feature of AD that is thought to contribute to cognitive decline (Auld et al., 2002). The levels of acetylcholine receptors in AD brains, particularly the primarily presynaptically localized α7 subunit–containing nAChRs, are also decreased in postmortem tissues and by *in situ* imaging of receptor binding in live patients (Burghaus et al., 2000; Guan et al., 2000; reviewed in Auld et al., 2002; and Oddo and LaFerla, 2006). It is unclear whether this decrease simply reflects the loss of neurons expressing the receptors. Some studies report that α7nAChR mRNA levels are increased in the hippocampus of AD patients (Hellstrom-Lindahl et al., 1999; but see Mousavi et al., 2003), suggesting that decreases in protein levels and receptor binding may indeed reflect synaptic loss or neuronal degeneration. The use of acetylcholinesterase inhibitors to boost cholinergic signaling has long been a standard treatment for mild to moderate AD (reviewed in Jacobsen et al., 2005; Roberson and Mucke, 2006), but these regimens do not appear to provide long-lasting benefits.

A number of studies have shed light on how Aβ peptides may alter cholinergic signaling in AD. *In vitro* evidence demonstrated that Aβ binds α7nAChRs with high affinity, although experimental conditions and cell types can determine whether Aβ inhibits or stimulates the receptor (Wang et al., 2000; Liu et al., 2001a; Dineley et al., 2001, 2002; Pettit et al., 2001). Aβ-induced stimulation of α7nAChRs can appear to block receptor function but may simply occlude the effect of other ligands such as nicotine by potently stimulating the receptor and inducing calcium influx (Dougherty et al., 2003). Unlike the majority of membrane-bound receptors, nAChR expression is increased upon receptor stimulation (Fenster et al., 1999), which may account for several reports that Aβ increases α7nAChR levels *in vitro* and in hAPP transgenic mice (Dineley et al., 2001; Chin et al., 2005; Snyder et al., 2005).

Moreover, α7nAChRs can be targeted to somatodendritic compartments and have been found in perisynaptic regions on postsynaptic membranes, where they are in a strategic position to modulate intracellular signaling processes through their high calcium permeability (Fabian-Fine et al., 2001; Xu et al., 2006a). In primary cortical neurons, Aβ activates the calcium-dependent phosphatase PP2B (calcineurin) by engaging postsynaptic α7nAChRs (Snyder et al., 2005). One consequence of this activation is activation of the phosphatase STEP and subsequent dephosphorylation of the Tyr-1472 of the NR2B subunit of the NMDA receptor, either through direct dephosphorylation by STEP or through STEP-mediated desphosphorylation and inactivation of tyrosine kinases such as Fyn that are known to phosphorylate the NMDA receptor at this residue (see **Figure 11**).

These *in vitro* findings are in line with observations in hAPP mice, in which increased levels of α7nAChRs are concomitant with increased STEP levels, Fyn suppression, and decreased phosphorylation of Tyr-1472 of NR2B subunits (Chin et al., 2005; Palop et al., 2005). It remains to be determined whether the engagement of α7nAChRs by Aβ in AD and in experimental models of the disease represent primary pathogenic mechanisms or compensatory mechanisms to boost synaptic scaling (Small, 2004; Geerts and Grossberg, 2006) or combat excitotoxicity (Palop et al., 2007).

4.15.3.2.4 Potassium channels

The activities of potassium (K^+) channels, which efflux K^+ and hyperpolarize cells, play an important

role in neuronal survival, because they govern membrane excitability and because the intracellular potassium level is a determinant of apoptosis (Yu, 2003). New discoveries have highlighted how diverse K^+ channels in neuronal dendrites fine-tune excitability and affect neuronal information processing by regulating the induction of NMDA receptor–dependent synaptic plasticity in the hippocampus (reviewed in Yuan and Chen, 2006).

Phosphorylation-dependent modulation of K^+ channel activity, particularly of the Kv4.x family, is a major means by which synaptic plasticity is regulated and dendritic information processing is achieved (reviewed in Birnbaum et al., 2004; Yuan and Chen, 2006). Based on the kinetics of time-dependent inactivation, K^+ currents can be separated into different components. One such component, the fast-inactivating A-type K^+ current, plays a particularly important role in regulating membrane excitability, because it responds quickly to subthreshold depolarization and its activation delays the generation of action potentials (**Figure 13**). Inhibition of this current by pharmacological agents that block ion flux or stimulate phosphorylation of the channel markedly increases intracellular calcium (Hoffman et al., 1997; reviewed in Yuan and Chen, 2006).

Interestingly, Aβ inhibits A-type K^+ currents in primary hippocampal and neocortical neurons, increasing dendritic calcium influx and neuronal excitability (Good et al., 1996; Ye et al., 2003; Chen, 2005). This process may contribute to the observation that nonfibrillar Aβ assemblies increase neuronal excitability in various *in vitro* models (Hartley et al., 1999; Jhamandas et al., 2001; Turner et al., 2003; Ye et al., 2004; but see Yun et al., 2006). Aβ may inhibit K^+ currents through the aberrant engagement of tyrosine kinases, as tyrosine kinase inhibitors abrogated the ability of Aβ to increase excitability of cholinergic neurons (Jhamandas et al., 2001). Aβ treatment also induces K^+ channel abnormalities in cultured fibroblasts that are similar to K^+ channel abnormalities detected in fibroblasts isolated from AD patients (Etcheberrigaray et al., 1993, 1994). While regulated increases in intracellular calcium are important for synaptic plasticity, sustained increases in intracellular calcium through Aβ's effect on K^+ channels could impair synaptic plasticity and sensitize neurons to excitotoxic injuries (Xie, 2004).

Although A-type K^+ channel currents are generally inhibited by Aβ, the expression levels of K^+ channel subunits that contribute to the A-type current are increased in early stages of AD and in primary cerebellar neurons after treatment with Aβ (Angulo et al., 2004; Plant et al., 2006). Such increases in subunit expression may represent compensatory mechanisms aimed at restoring membrane excitability.

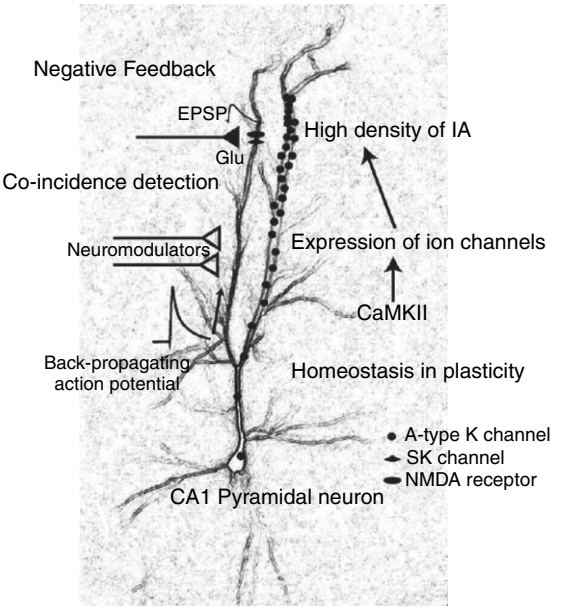


Figure 13 Dendritic K^+ channels influence neuronal information processing. SK and Kv4.2-encoded A-type K^+ channels are expressed at high levels in dendrites. Propagation of back-propagating action potentials and release of glutamate and neuromodulators within an appropriate time window ensures *N*-methyl-D-aspartate (NMDA) receptor activation through three coinciding events (as depicted in left dendrite above). Regulation of K^+ channel expression is also a homeostatic mechanism regulated by CaMKII. Activation of CaMKII promotes expression of both α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptors and A-type K^+ channels, resulting in antagonizing effects on neuronal responsiveness. Alzheimer's disease–related decreases in the activity of A-type K^+ channels increase neuronal excitability and intracellular calcium and may contribute to excitotoxicity. (From Yuan L and Chen X [2006] Diversity of potassium channels in neuronal dendrites. *Prog. Neurobiol.* 78: 374–389.)

4.15.3.3 Calcium Signaling

Downstream of cell surface receptors and ion channels at the postsynaptic membrane, a myriad of intracellular signaling molecules await instruction. Depending on the dynamics of receptor/channel activity, local Ca^{2+} plumes of various magnitudes are generated to direct the kinase cascades and other signaling pathways that transduce signals to the nucleus or other subcellular compartments. In

this section, we discuss several aspects of calcium homeostasis that impact the dynamics of postsynaptic signaling and describe kinase pathways that play key roles in the induction and maintenance of long-term changes in synaptic efficacy. Special emphasis is placed on those aspects that are altered in AD and related models and on how their dysregulation contributes to plasticity deficits. Finally, we review the mechanisms by which extracellular, neuromodulatory factors such as brain-derived neurotrophic factor (BDNF) and Reelin influence synaptic plasticity and how AD-related alterations in these factors exacerbate impairments in synaptic function.

Calcium (Ca^{2+}) plays fundamental roles in synaptic plasticity and neuronal survival. Calcium signals, either from the extracellular milieu or from intracellular stores, must be precisely regulated both temporally and spatially to achieve tight control over intracellular signaling pathways that transduce signals from extracellular sources (Yuste et al., 2000; Berridge et al., 2003). Disruptions in neuronal Ca^{2+} homeostasis and calcium-regulated signaling likely play important roles in normal aging and AD, impairing synaptic plasticity and cognitive function, and contributing to neuronal loss in vulnerable regions (Mattson and Chan, 2003; Xie, 2004; Smith et al., 2005a; Kelly et al., 2006). Acute treatment of primary neurons and cell lines of neuronal origin with Aβ induces rapid, transient increases in intracellular calcium levels, whereas chronic exposure to Aβ leads to slower, more progressive increases in resting calcium levels (Xie, 2004; Kelly and Ferreira, 2006). Aβ peptides, particularly oligomeric assemblies, have been suggested to insert into neuronal cell membranes and form calcium-fluxing pores (reviewed in Pollard et al., 1995; Glabe and Kayed, 2006). Alternatively, such assemblies may increase intracellular calcium levels by increasing influx through calcium channels, releasing calcium from intracellular stores, or modulating intracellular calcium dynamics through alterations in endogenous calcium binding proteins (**Figure 14**).

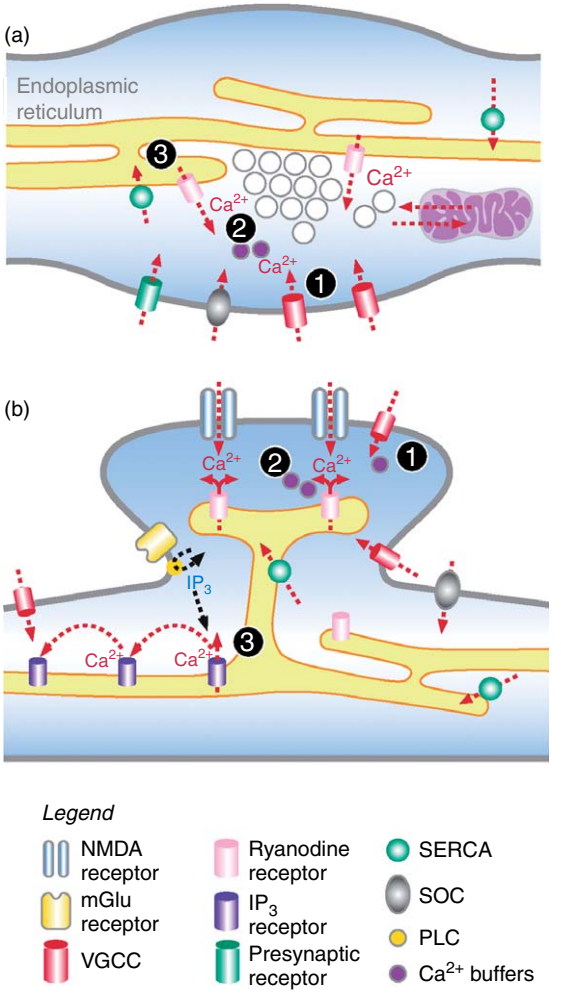


Figure 14 Ca^{2+} dysregulation in AD. Intracellular Ca^{2+} levels in the presynaptic terminal (top) and postsynaptic spine (bottom) are regulated by the influx of Ca^{2+} through Ca^{2+} channels (1), the buffering of free Ca^{2+} by Ca^{2+}-binding proteins (2), and the release of Ca^{2+} from intracellular Ca^{2+} stores (3). AD-related alterations in the levels and/or activities of Ca^{2+} channels, receptors, and Ca^{2+}-binding proteins perturb calcium homeostasis and may contribute to deficits in synaptic plasticity as well as increase susceptibility to excitotoxicity. (Modified from Bardo S, Cavazzini MG, and Emptage N [2006] The role of the endoplasmic reticulum Ca^{2+} store in the plasticity of central neurons. *Trends Pharmacol. Sci.* 27: 78–84).

4.15.3.3.1 Calcium channels

Ca^{2+} channels play diverse roles in synaptic transmission, on both the presynaptic and the postsynaptic side (Augustine et al., 2003). Presynaptic N- and P/Q-type voltage-sensitive Ca^{2+} channels are primarily involved in triggering synaptic vesicle exocytosis, whereas postsynaptic L-type voltage-sensitive Ca^{2+} channels contribute to the integration of synaptic activity and the transduction of signals that trigger a transcriptional response. Synaptic activity induces Ca^{2+} entry through NMDA receptors and L-type channels. The magnitude and temporal dynamics of the combined calcium influx determine which signaling pathways become engaged (Bradley and Finkbeiner, 2002; Deisseroth et al., 2003; Thiagarajan et al., 2006).

Aβ potentiates currents through L-type voltage-sensitive Ca^{2+} channels in primary neurons (Brorson

et al., 1995; Ueda et al., 1997; Ekinci et al., 1999; Fu et al., 2006). Although the underlying mechanisms have not been fully characterized, one possibility is Aβ-stimulated phosphorylation of L-type Ca^{2+} channels, which would increase conductance through the channel (Ekinci et al., 1999). In addition, because L-type Ca^{2+} channels are voltage sensitive, Aβ could indirectly modulate Ca^{2+} currents by blocking voltage-gated potassium channels (see earlier section), altering neuronal excitability and prolonging membrane depolarization (Good et al., 1996; Birnbaum et al., 2004). Finally, Aβ exposure leads to increased expression of particular L-type Ca^{2+} channel subunits on surface membranes in neuronal cell lines (Scragg et al., 2005; Chiou, 2006), which may contribute to long-term changes in Ca^{2+} homeostasis. Because Ca^{2+} influx through L-type channels could contribute to deranged Ca^{2+} homeostasis and signaling, L-type Ca^{2+} channel blockers are now in clinical trials for the treatment of AD (reviewed in Jacobsen et al., 2005; Roberson and Mucke, 2006).

4.15.3.3.2 Calcium-binding proteins

The level of intracellular free Ca^{2+} is governed by a balance between the entry of Ca^{2+} into the cytoplasm, either from extracellular sources or from intracellular stores, and the removal of Ca^{2+} by buffers, pumps, and exchangers (reviewed in Berridge et al., 2003). Many cell types contain particular calcium-binding proteins that act as buffers by rapidly binding and sequestering free Ca^{2+}. The primary cytosolic calcium-buffering proteins include calbindin-D_{28K}, calretinin, and parvalbumin, which are differentially expressed in various populations of neurons and play important regulatory roles in the maintenance of Ca^{2+} homeostasis (Hof et al., 1999). Parvalbumin is expressed in interneurons that modulate local circuitry in the neocortex and hippocampus, whereas calbindin and calretinin are expressed by both interneurons and pyramidal cells in the neocortex and hippocampus. Since disruption of neuronal Ca^{2+} homeostasis appears to contribute to AD pathogenesis, the levels and distribution of these types of calcium buffers in various areas of the AD brain and AD models have been the focus of a rapidly increasing number of studies.

Neuronal populations that express the calcium buffers calretinin and parvalbumin appear to be relatively preserved in AD (Hof et al., 1993; Fonseca and Soriano, 1995; Sampson et al., 1997). Losses of parvalbumin-expressing neurons have been documented in the entorhinal cortex and hippocampus of AD brains, but this loss appears to occur at late stages of the disease and may be secondary to degeneration of principal neurons in the same region (Solodkin et al., 1996; Brady and Mufson, 1997; Mikkonen et al., 1999). Such findings supported the hypothesis that neurons containing high levels of calcium-buffering proteins, and presumably a high calcium-buffering capacity, are relatively resistant to AD-related neurotoxicity (Hof et al., 1993). Late-stage loss of calcium-binding proteins was related to loss of neurons producing these proteins (Solodkin et al., 1996).

The calcium-binding protein calbindin is expressed in local circuit interneurons and pyramidal cells of the neocortex. In addition, it is very highly expressed in granule cells of the dentate gyrus and in Purkinje cells of the cerebellum (Celio, 1990). Calbindin regulates intracellular Ca^{2+} levels and is important for synaptic plasticity and learning and memory (Molinari et al., 1996). Calbindin levels in dentate granule cells are depleted in hAPP mice with high hippocampal levels of Aβ, and the magnitude of this depletion correlates tightly with cognitive deficits (Palop et al., 2003). Transgenic mice expressing the carboxy terminus of hAPP also exhibit depletions of calbindin in the dentate gyrus (Lee et al., 2006). Similar calbindin depletions occur in the dentate gyrus of AD patients, in whom the greatest depletions were seen in individuals with the most severe dementia (Palop et al., 2003). Moreover, calbindin mRNA levels in the hippocampus are also reduced in AD and in hAPP mice, supporting the hypothesis that calbindin depletions result from decreased expression of the calbindin gene rather than from loss of dentate granule cells (Iacopino and Christakos, 1990; Sutherland et al., 1993; Palop et al., 2003).

Similar calbindin depletions in the dentate gyrus have been observed after chronic neuronal overexcitation, for example, in human temporal lobe epilepsy, $GABA_B$ receptor-deficient mice, and models of kindling or kainate-induced chronic excitotoxicity (Tonder et al., 1994; Magloczky et al., 1997; Nägerl et al., 2000; Ruttimann et al., 2004; Palop et al., 2007). Calbindin reductions in the dentate gyrus of hAPP mice and in AD brains may also result from an imbalance between excitatory and inhibitory inputs (Palop et al., 2003, 2006, 2007). The ability of granule cells to downmodulate calbindin over a wide dynamic range may explain, at least in part, why these neurons are relatively resistant to degeneration in AD (West et al., 1994; Irizarry et al., 1997b; Palop et al., 2003), since calbindin reduction can lead to an

inactivation of voltage-gated calcium channels, limiting calcium entry and protecting against excitotoxicity (Nägerl et al., 2000).

4.15.3.3.3 Intracellular stores

In addition to influx from the extracellular compartment, the release of Ca^{2+} from intracellular stores in the ER is a major source of free Ca^{2+} that is available for signaling and synaptic plasticity (Rose and Konnerth, 2001; Berridge et al., 2003; Bardo et al., 2006). To maintain such a supply of Ca^{2+}, the ER faces the daunting task of sustaining an immense concentration gradient of Ca^{2+} across its membrane: The concentration of free Ca^{2+} in the ER lumen is about one thousand times greater than resting levels in the cytosol. Sarco-ER Ca^{2+} ATPases (SERCAs) actively transport Ca^{2+} into the ER to clear Ca^{2+} from the cytosol and fill ER stores. The liberation of Ca^{2+} from these internal stores is regulated by two types of channels in the ER membrane: the ryanodine receptor (RyR) and the inositol triphosphate receptor (IP_3R).

RyRs are activated by cytosolic Ca^{2+}, and their sensitivity is modulated by several factors: caffeine binding, oxidation, and high luminal Ca^{2+} levels increase sensitivity, whereas phosphorylation (by PKA) and calmodulin binding decrease activity (reviewed in Berridge et al., 2003; Bardo et al., 2006). IP_3Rs must be activated by the second messenger IP_3, which is generated upon stimulation of Gq-coupled receptors on the plasma membrane, such as metabotropic glutamate receptor types 1 and 5 ($mGluR_{1,5}$), serotonin receptors ($5\text{-}HT_2$), and muscarinic receptors (M1–3). The binding of IP_3 to IP_3Rs then sensitizes the receptors to Ca^{2+}, which increases receptor activity at low concentrations but inhibits it at high concentrations, such as those reached after release of Ca^{2+} from the ER (Berridge et al., 2003). IP_3Rs are often tethered to IP_3-producing cell surface receptors by scaffolding proteins such as Homer, linking the source of IP_3-production to its site of action (Ehrengruber et al., 2004). IP_3R activity can also be influenced by phosphorylation, which modulates the sensitivity of IP_3Rs in different directions, depending on the kinase involved. For example, phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) decreases activity, whereas phosphorylation by Fyn kinase increases activity (Cui et al., 2004; Bare et al., 2005). Together, the activities of SERCAs, RyRs, and IP_3Rs monitor intracellular Ca^{2+} levels and regulate release of Ca^{2+} from the ER in a process called Ca^{2+}-induced Ca^{2+} release (CICR).

Several aspects of Ca^{2+} dysregulation in AD and AD mouse models have been linked to alterations in ER Ca^{2+} signaling. AD-related mutations in presenilin 1, presenilin 2, and APP increase cellular sensitivities to IP_3, caffeine activation of RyRs, and blockade of SERCA pumps, enhancing Ca^{2+} liberation from the ER (Smith et al., 2005a; Stutzmann, 2005). Overfilling of ER stores or excess phosphorylation of IP_3Rs or RyRs through aberrant activation of kinases by Aβ or other AD-related molecules may result in ER hypersensitivity and exaggerated Ca^{2+} release upon physiological stimulation of these receptors (discussed in the section titled 'Intracellular calcium stores'). As a result, even normal stimuli, such as synaptic activity and activation of mGluRs, could disrupt the intracellular Ca^{2+} homeostasis.

Mutations in presenilin 1 have been particularly linked to dysregulation of ER Ca^{2+} signaling by mechanisms that are unrelated to γ-secretase activity. As mentioned in Section 4.15.2.3.2, wild-type, but not AD-mutant, presenilin 1 and 2 can act as low-conductance Ca^{2+}-permeable ion channels, which may account for the majority of passive Ca^{2+} leaks from the ER (Tu et al., 2006). This Ca^{2+}-fluxing activity is important for maintaining normal steady-state intraluminal levels of Ca^{2+} and is exhibited by the unprocessed, holoprotein form of presenilin in the ER (Tu et al., 2006). In contrast, the secretase activity of presenilin emerges only in later compartments (trans-Golgi network, endosome) after assembly with other components of the γ-secretase complex (Tandon and Fraser, 2002). AD-related mutations in PS1 (PS1-M146V) or PS2 (PS2-N141I) abrogate the Ca^{2+} fluxing properties of the presenilins and abolish the passive efflux of Ca^{2+} out of the ER, overloading the ER with Ca^{2+}.

AD-related increases in levels of RyRs may also contribute to ER hypersensitivity and exaggerated Ca^{2+} release. RyR binding is increased in the entorhinal cortex and hippocampus in early stages of AD, suggesting increased levels of RyRs in these areas (Kelliher et al., 1999). Presenilin mutations are associated with increased levels of RyRs in transgenic mice and cell culture models (Chan et al., 2000; Smith et al., 2005b; Stutzmann et al., 2006). hAPP mice and primary cortical neurons treated with Aβ also show increases in RyRs (Supnet et al., 2006), suggesting that increased levels of Aβ are the unifying mechanism.

Together, these studies indicate that dysregulation of ER Ca^{2+} dynamics may contribute to impairments in synaptic plasticity and cognitive function associated with AD.

4.15.3.4 Kinases

Kinase activity is often coupled to the activity of receptors and channels at the plasma membrane and is crucial to the transduction of extracellular signals to cytosolic or nuclear targets. Signaling specificity is conferred by the type of receptor activated by synaptic activity, the dynamics and distribution of the ensuing Ca^{2+} influx, and the scaffolding of particular kinases to receptors/channels. Phosphorylation events triggered by active kinases can alter enzymatic activities or protein conformations of target molecules, setting in motion molecular cascades that culminate in cytoplasmic changes or nuclear events including gene transcription. Over the years, researchers have uncovered important roles for many kinases in synaptic plasticity and demonstrated how the orchestration of their activities leads to the induction, expression, and maintenance of long-term changes in synaptic efficacy. Notably, the levels, localization, or activities of many of these kinases are disrupted in AD, providing clues into the mechanisms by which AD impairs synaptic and cognitive function. In this section, we discuss several kinases whose roles in synaptic plasticity have been well characterized and how AD-related alterations in these kinases or associated signaling pathways may contribute to synaptic dysfunction.

4.15.3.4.1 MAPKs

The mitogen-activated protein kinase (MAPK) superfamily comprises three major subclasses of Ser/Thr kinases that are involved in the regulation of growth, differentiation, and cellular responses to stress and/or inflammatory cytokines. The extracellular signal-regulated kinases (ERKs) regulate growth, proliferation, and differentiation in many cell types and are essential for short-term increases in synaptic efficacy and for the expression and maintenance of LTP (Pearson et al., 2001; Thomas and Huganir, 2004; Davis and Laroche, 2006). The p38 branch of the MAPK family was originally characterized as key transducers of stress and inflammatory responses to cytokines but has recently been discovered to also mediate the induction and expression of LTD (Pearson et al., 2001; Thomas and Huganir, 2004). The activity or localization of ERK and p38 family members are altered in AD and related models, and their misregulation has been implicated in impairments of synaptic plasticity (Johnson and Bailey, 2003; Haddad, 2004). The third branch of the MAPK family is made up of the c-Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPKs), which transduce stress signals, including oxidation and DNA damage, as well as growth and differentiation signals (Raivich and Behrens, 2006). Aβ-induced generation of reactive oxygen species activates JNK/SAPK, and such activation has been documented in AD and in AD models (reviewed in Zhu et al., 2004; Smith et al., 2006). Aβ-related engagement of JNK/SAPKs has been associated with overt cell death rather than more subtle effects on synaptic/neuronal functions. We will thus focus our discussion of MAPKs in AD-related synaptic impairments on ERK and p38.

ERK1/2 is activated rapidly after the induction of LTP. Although ERK1/2 activity is not necessary for the induction of LTP, it is critical for its maintenance and for learning and memory (English and Sweatt, 1997; reviewed in Thomas and Huganir, 2004; Davis and Laroche, 2006). In combination with other signaling pathways, LTP-induced ERK1/2 activation increases the expression of proteins necessary for long-term changes in synaptic efficacy, including the activity-regulated cytoskeletal protein (Arc/Arg3.1) (Roberson et al., 1999; Waltereit et al., 2001; Ying et al., 2002). Additional targets of ERK1/2 that are important for the expression or maintenance of LTP include cytoskeletal proteins such as MAP-2 and Tau, which modulate the structural organization of neurites; Kv4.2 potassium channels, which control dendritic depolarization and neuronal excitability; AMPA receptors, which are inserted into the membrane and increase synaptic strength; and mTOR, a component of the ribosomal machinery that controls synthesis of new proteins (reviewed in Haddad, 2004; Birnbaum et al., 2004; Kelleher et al., 2004; Sweatt, 2004). Clearly, tight regulation of ERK1/2 activity, dynamics, and localization is necessary to orchestrate its many effects on synaptic efficacy (**Figure 15**).

Increased levels of active ERK1/2 in AD brains are typically associated with neurofibrillary tangles and amyloid plaques (Trojanowski et al., 1993; Pei et al., 2002; Haddad, 2004; Webster et al., 2006). ERK1/2 phosphorylation of tau, described above in the section titled 'Tau phosphorylation and other posttranslational modifications,' has been well documented as a means by which hyperphosphorylated tau is generated in AD (reviewed in Haddad, 2004). In addition, the dysregulation of ERK1/2 activity may contribute in other ways to synaptic dysfunction, as indicated by studies in animal and *in vitro* models of AD. ERK1/2 activity has been shown to mediate the effects of Aβ on synaptic plasticity, including Aβ's effect on L-type calcium channels

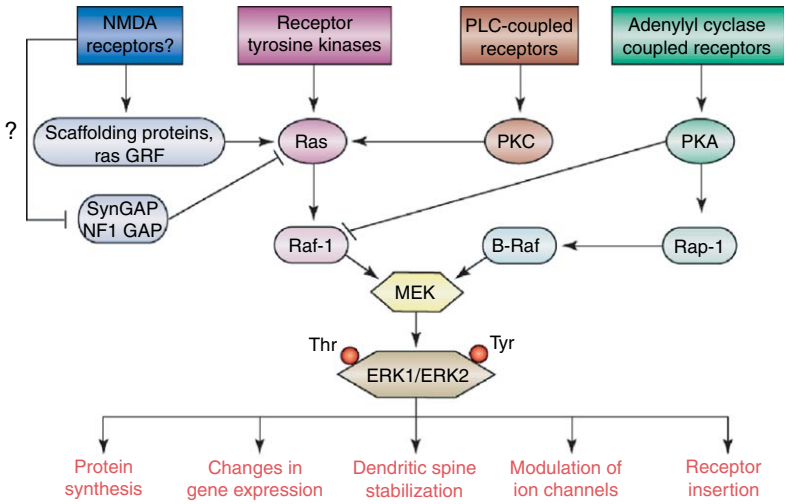


Figure 15 Regulation and targets of extracellular signal-regulated kinase (ERK)1/2 signaling in neurons. The ERK/mitogen-activated protein kinase cascade is activated by a number of receptors and pathways, and therefore plays a critical role in the integration of a wide variety of signals. The targets of ERK1/2 modulate processes that are crucial for synaptic plasticity (red font), all of which are impaired in Alzheimer's disease and experimental models. (From Sweatt JD [2004] Mitogen-activated protein kinases in synaptic plasticity and memory. *Curr. Opin. Neurobiol.* 14: 311–317.)

(reviewed in the section titled 'Calcium channels'). Interestingly, the kinetics of ERK1/2 activity depend on the duration of Aβ exposure and on Aβ's assembly state. Exposure of primary neurons or hippocampal slices to oligomeric Aβ acutely activated ERK1/2, but chronically decreased ERK1/2 activity, while exposure to fibrillar Aβ progressively increased ERK1/2 activity (Rapoport and Ferreira, 2000; Bell et al., 2004). Similarly, young hAPP transgenic mice exhibit increased ERK1/2 activity, whereas older hAPP mice exhibit decreased ERK1/2 activity, in particular in hippocampal subregions (Dineley et al., 2001; Chin et al., 2005; Palop et al., 2005). Certain brain regions may be able to downregulate ERK1/2 activity through compensatory mechanisms. For example, the phosphatase STEP dephosphorylates and inactivates ERK1/2 and is increased by Aβ *in vitro* and in the hippocampus of hAPP mice (Chin et al., 2005; Snyder et al., 2005; Braithwaite et al., 2006). Although the downregulation of aberrant ERK1/2 activity may be neuroprotective, it may also increase Aβ production (Kim et al., 2006) and decrease the expression of gene products required for the formation of long-term memories.

Although much emphasis has been placed on p38-mediated phosphorylation of tau, this process does not contribute greatly to the hyperphosphorylation of tau in AD (reviewed in Johnson and Stoothoff, 2004). p38 signaling also regulates synaptic plasticity by mediating long-term depression (LTD) of synaptic strength (reviewed in Thomas and Huganir, 2004). Inhibitors of p38 activity block LTD mediated by mGluRs or NMDARs (Bolshakov et al., 2000; Zhu et al., 2002), and inhibition of either p38 or mGluR activity prevents Aβ-induced LTP deficits in hippocampal slices (Wang et al., 2004). Furthermore, levels of phosphorylated, active p38 are increased in AD brains and related mouse models (Hensley et al., 1999; Zhu et al., 2000; Savage et al., 2002; reviewed in Johnson and Bailey, 2003; Hwang et al., 2005). Thus, Aβ-induced neuronal p38 activation may impair synaptic function in AD by promoting LTD.

4.15.3.4.2 CaMKII

Calcium/calmodulin-dependent protein kinase II is a major constituent of the postsynaptic density (PSD) that interacts with NMDA receptors and the cytoskeletal protein α-actinin (*See* Chapter 4.23). When activated by calcium influx during high-frequency stimulation, CaMKII translocates to the PSD and undergoes autophosphorylation at Thr286 of the αCaMKII subunit, resulting in prolonged calcium/calmodulin-independent CaMKII activity. This process is thought to underlie, at least in part, the conversion of a transient calcium signal to long-lasting enhancement of synaptic strength. Genetic or pharmacological manipulations that decrease levels of CamKII or prevent its autophosphorylation abolish LTP and

impair learning and memory (reviewed in Colbran and Brown, 2004).

In addition to its influence on gene transcription, CaMKII's cytoplasmic targets have also received great attention for their roles in the expression and maintenance of LTP. CaMKII promotes the insertion of alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate receptors (AMPARs) into the synapse, surface expression of Kv4.2 potassium channels, and activity of R-type calcium channels (reviewed in Colbran and Brown, 2004). Moreover, CaMKII-dependent modulation of cytoskeletal proteins regulates modifications of dendritic spine morphology associated with LTP (reviewed in Carlisle and Kennedy, 2005).

Although levels of CaMKII are relatively preserved in AD brains, levels of active, autophosphorylated CaMKII are significantly decreased (Mah et al., 1992; Simonian et al., 1994; Amada et al., 2005). These changes may be subregion specific, as decreases in autophorylated CaMKII were found in the hippocampus, but not in the amygdala (Amada et al., 2005). Consistent with these findings, Aβ acutely inhibits the ability of high-frequency stimuli to induce αCaMKII autophosphorylation and subsequent LTP in hippocampal slices (Zhao et al., 2004).

Autophosphorylation of αCaMKII, and thus CaMKII activity, are negatively regulated by the phosphatase PP1, which acts downstream of the calcium-dependent phosphatase calcineurin (Blitzer et al., 1998; Hedou and Mansuy, 2003). Particularly interesting in this regard are the findings by multiple groups that Aβ activates calcineurin *in vitro* and in transgenic mouse models of AD (Chen et al., 2002; reviewed in Xie, 2004; Cardoso and Oliveira, 2005; Snyder et al., 2005). Calcineurin levels and activity are also increased in AD (Hata et al., 2001; Liu et al., 2005; but see Lian et al., 2001). Together, these results suggest that enhanced negative regulation of CaMKII may diminish its activity in AD and impair synaptic plasticity.

4.15.3.4.3 PKC

Considerable evidence indicates that protein kinase C (PKC) is critical for long-term synaptic plasticity (Hvalby et al., 1994; Bortolotto and Collingridge, 2000). Indeed, ablation of PKC or inhibition of its activity impairs LTP as well as learning and memory (reviewed in Battaini and Pascale, 2005). Part of PKC's role in LTP may relate to its actions on AMPARs (Chung et al., 2000; Boehm et al., 2006). In addition, crosstalk between the PKC and PKA pathways can amplify ERK1/2 signaling (Roberson et al., 1999).

PKC is kept in a folded, inactive conformation by the binding of its pseudosubstrate domain to the substrate-binding site in the catalytic domain. Activation of the conventional, calcium-dependent isoforms of PKC, which are highly expressed in the brain, is regulated by binding to the second messengers calcium and diacylglycerol (DAG, reviewed in Battaini and Pascale, 2005). Upon binding and activation by second messengers, PKC translocates to the membrane via interactions with the scaffolding protein RACK1, which stands for receptor for activated C kinase (reviewed in Sklan et al., 2006). Interactions with RACK1 therefore aid in localizing PKC to its substrates.

Decreased activity of PKC has been implicated in the pathogenesis of AD. PKC levels are reduced in AD brains (Cole et al., 1988). In addition to deficits in PKC activation, which may result from decreased synaptic transmission and depletion of growth factors, PKC does not translocate effectively from the cytosolic to the membrane fraction in samples from AD brains, possibly because of decreased levels of RACK1 (Wang et al., 1994; Battaini et al., 1999).

Increased PKC immunoreactivity has been found in some AD cases and in hAPP transgenic mice at the beginning stages of amyloid deposition (Saitoh et al., 1993; Rossner et al., 2001), suggesting an early hyperactivation of PKC by Aβ, which may be followed by chronic suppression. Indeed, chronic activation is known to downregulate PKC activity (reviewed in Battaini and Pascale, 2005).

The consequences of reduced PKC activity are several-fold. In addition to decreasing the potential for synaptic plasticity, reduction of PKC activity may enhance the production of neurotoxic Aβ peptides. PKC increases the processing of APP by α-secretase in the nonamyloidogenic pathway, releasing the neurotrophic sAPPα fragment and precluding Aβ production (reviewed in Olariu et al., 2005). Treatment of APP/PS1 transgenic mice with small molecule activators of PKC significantly increased sAPPα, decreased Aβ levels, and reduced premature mortality (Etcheberrigaray et al., 2004). In addition, PKC regulates Aβ levels by increasing its clearance. Overexpression of the epsilon isoform of PKC activated endothelin-converting enzyme, an Aβ-degrading enzyme, and decreased Aβ levels, plaque deposition, neuritic dystrophy, and reactive astrocytosis in hAPP transgenic mice (Choi et al., 2006).

Together, these results suggest that enhancement of PKC activity may provide some benefit in AD.

4.15.3.4.4 PKA

Since the discovery in the 1980s that activation of cyclic AMP-dependent PKA increases synaptic efficacy in *Aplysia* neurons, numerous roles for PKA have been described in both short-term and long-term plasticity (reviewed in Nguyen and Woo, 2003; Waltereit and Weller, 2003). PKA is activated rapidly by calcium influx, which leads to increased synthesis of cyclic adenosine monophosphate (cAMP) by calcium/calmodulin-sensitive adenyl cyclases. Short-term actions of PKA include phosphorylation of potassium channels, to acutely increase excitability, and phosphorylation of synaptic vesicle proteins, to increase neurotransmitter release.

LTP-inducing stimuli lead to the degradation of the regulatory subunits of PKA, resulting in sustained activity of the catalytic subunits, which translocate to the nucleus and phosphorylate the transcription factor cAMP response element binding protein (CREB) to initiate gene transcription (reviewed in Kandel, 2001). Activity of PKA is necessary for long-lasting LTP in the hippocampus: it initiates gene transcription by direct phosphorylation of transcription factors and synergizes with other kinases, such as PKC, to activate ERK1/2 signaling (Roberson et al., 1999; Impey et al., 1998; reviewed in Waltereit and Weller, 2003). The combined actions of PKA and ERK1/2 are necessary for the transcription of immediate-early genes such as Arc/Arg3.1 that are critical to memory consolidation (Waltereit et al., 2001). PKA also increases current conductance and synaptic strength via phosphorylation of synaptic AMPA receptors (reviewed in Nguyen and Woo, 2003).

Alterations in PKA signaling have been implicated in several aspects of AD. PKA contributes to tau hyperphosphorylation by direct phosphorylation and by rendering tau susceptible to phosphorylation by GSK-3 (Liu et al., 2004; reviewed in Gong et al., 2005). Although PKA is responsible for a large proportion of tau hyperphosphorylation in AD, PKA activity is decreased in AD as well as in animal and cell culture models of the disease (Kim et al., 2001; Vitolo et al., 2002; Gong et al., 2006), possibly because Aβ inhibits the proteasomal degradation of PKA's regulatory subunits (**Figure 16**) (Vitolo et al., 2002). Increasing cAMP levels by inhibition of phosphodiesterases that break down cAMP ameliorates Aβ-induced deficits in synaptic plasticity and

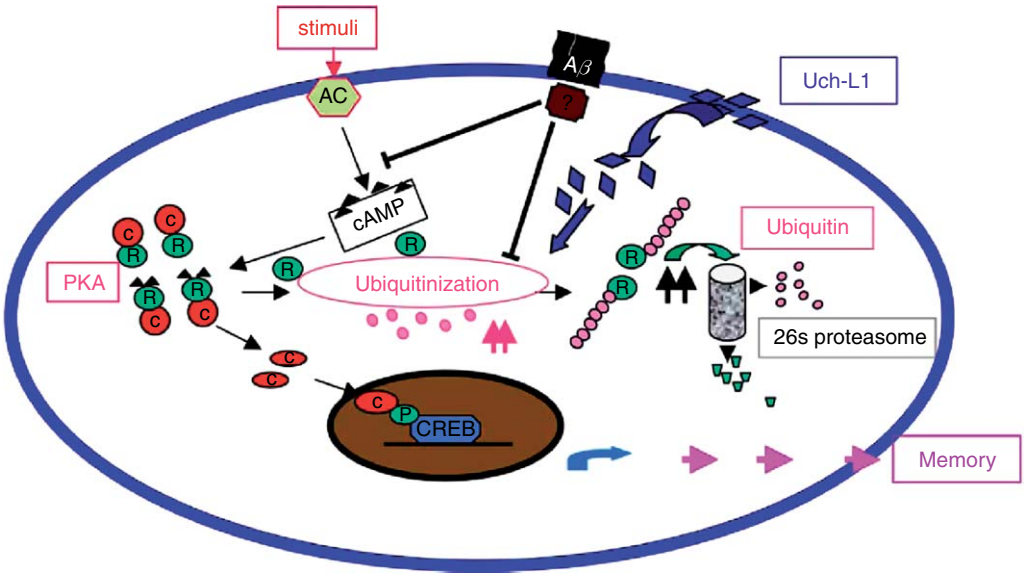


Figure 16 Aβ modulation of the ubiquitin-proteasome-protein kinase A (PKA)-cyclic adenosine monophosphate response element binding protein (CREB) pathway. Aβ inhibits adenylate cyclase activity and proteasomal degradation of the regulatory subunits of PKA, resulting in their accumulation and a shift in the PKA complex toward the inactive tetramer. Consequently, CREB phosphorylation and initiation of transcription is impaired. Transduction of Uch-L1 promotes proteasomal activity, normalizing levels of the PKA regulatory subunit and freeing the active catalytic subunit. (From Gong B, Cao Z, Zheng P, et al. [2006] Ubiquitin hydrolase Uch-L1 rescues β-amyloid-induced decreases in synaptic function and contextual memory. *Cell* 126: 775–788.)

learning and memory (Vitolo et al., 2002; Gong et al., 2004). Exogenous ubiquitin C-terminal hydrolase L1 (Uch-L1), which boosts proteasome activity, reversed Aβ-induced LTP deficits in hippocampal slices (Gong et al., 2006). Moreover, endogenous Uch-L1 activity was decreased in APP/PS1 transgenic mice, and treatment of these mice with exogenous Uch-L1 restored PKA activity and contextual memory (Gong et al., 2006).

4.15.3.4.5 Fyn

The tyrosine kinase Fyn can be activated through diverse receptors and participates in signaling pathways that control a broad spectrum of biological activities, including long-term changes in synaptic efficacy (Thomas and Brugge, 1997; Roskoski, 2004; Salter and Kalia, 2004). Ablation of Fyn abolishes LTP and impairs spatial learning and memory (Grant et al., 1992). Postnatal overexpression of Fyn can restore LTP, indicating that Fyn is a critical modulator of long-term synaptic efficacy (Kojima et al., 1997). Anchored to NMDA receptor complexes through interactions with PSD-95, Fyn phosphorylates the NR2B subunit at tyrosine residue 1472 (Tyr1472), which increases calcium conductance by altering channel gating properties and controls the internalization of the receptors by preventing AP-2 binding, a signal for endocytosis (reviewed in Salter and Kalia, 2004; Prybylowski et al., 2005). In addition, Fyn can modulate cytoskeletal dynamics by altering the phosphorylation and/or localization of cytoskeletal elements such as tau, adducin, and β-catenin (Williamson et al., 2002; Lilien and Balsamo, 2005; Gotoh et al., 2006). Moreover, phosphorylation by Fyn influences the integrity of synaptic AMPA receptors by rendering them less susceptible to proteolytic cleavage (Rong et al., 2001).

A number of findings suggest that misregulation of Fyn activity may play a role in AD. The distribution and levels of Fyn are altered in AD brains (Shirazi and Wood, 1993; Ho et al., 2005), and the toxic effects of Aβ oligomers on hippocampal slices can be blocked by the genetic ablation of Fyn (Lambert et al., 1998). Ablation of Fyn decreases – whereas overexpression of Fyn increases – Aβ-induced synaptotoxicity and premature mortality in hAPP transgenic mice (Chin et al., 2004). In addition, Fyn phosphorylates tau and binds it in a manner that is modulated both by AD-related hyperphosphorylation and by disease-related mutations in tau (Bhaskar et al., 2005; Lee, 2005). Together with *in vitro* studies demonstrating that acute application of Aβ leads to activation of Fyn signaling pathways and increased interactions with binding partners, these results suggested that Aβ may derange synaptic functions by aberrantly engaging Fyn-related pathways (Zhang et al., 1996; Williamson et al., 2002). Indeed, the overexpression of Fyn in hAPP mice with moderate levels of Aβ rendered the mice as severely impaired, with respect to biochemical and behavioral alterations, as hAPP mice with high levels of Aβ (Chin et al., 2005). These results suggest that Fyn activity sensitizes neurons to Aβ-induced neuronal impairments. Fyn also exacerbates Aβ-induced aberrant increases in neuronal activity (Palop et al., 2007).

Interestingly, aberrant engagement of Fyn activity also appears to trigger compensatory mechanisms that limit Fyn activity in the presence of elevated Aβ levels. hAPP transgenic mice exhibit significant increases in levels of the phosphatase STEP, which dephosphorylates and inactivates Fyn, and have corresponding decreases in levels of active Fyn (see **Figure 11**) (Chin et al., 2005). The increase in STEP and decrease in Fyn activity are most prominent in the dentate gyrus, a region particularly susceptible to Aβ-related synaptic dysfunction. The compensatory downregulation of Fyn activity in this region has consequences on NMDA receptor phosphorylation, calcium gating, and receptor internalization that likely further contribute to deficits in synaptic plasticity and learning and memory (Chin et al., 2005; Palop et al., 2005; Snyder et al., 2005).

4.15.3.4.6 Cdk5

Cyclin-dependent kinase 5 is an unusual Cdk that lacks a role in the cell cycle and is activated by two noncyclin activators, p35 and p39. With an extensive list of substrates, the primarily neuronal Cdk5 regulates cell death and survival, as well as a variety of specific cellular functions (reviewed in Cheung and Ip, 2004; Cruz and Tsai, 2004). Cdk5 activity is increased in AD brains and in neurons and cell lines treated with Aβ and mediates tau hyperphosphorylation (reviewed in Giese et al., 2005). It also modulates synaptic plasticity, with consequences on learning and memory (reviewed in Cruz and Tsai, 2004; Cheung et al., 2006; Angelo et al., 2006).

Presynaptic roles for Cdk5 that influence synaptic transmission include the regulation of synaptic vesicle exocytosis through phosphorylation of P/Q-type calcium channels and synapsin 1, which increases calcium influx and releases synapsin's tethering of synaptic vesicles in a reserve pool (reviewed in Angelo et al., 2006). Cdk5 also modulates endocytosis for the recycling of synaptic vesicles by phosphorylating dynamin

I and amphiphysin I, proteins necessary for clathrin-mediated endocytosis (reviewed in Angelo et al., 2006).

Postsynaptic roles for Cdk5 in synaptic plasticity include phosphorylation of PSD-95, which suppresses its multimerization and decreases PSD-95-dependent clustering of NMDA receptors and Kv1.4 potassium channels (Morabito et al., 2004). Cdk5 also phosphorylates NR2A subunits of NMDA receptors and increases calcium conductance (Li et al., 2001). Moreover, Cdk5 activity regulates dendritic spine remodeling through actions on proteins that modulate cytoskeletal dynamics, including Rho GTPases and PAK1 (Nikolic et al., 1998).

Direct evidence for a role of Cdk5 in learning and memory has come from studies of transgenic mice expressing p25, a truncated form of the Cdk5 activator p35 that results in constitutive activation of Cdk5. Transient expression of p25 led to improved synaptic plasticity and learning in hippocampus-dependent tasks (Fischer et al., 2005). These improvements were accompanied by increased spine density and synapse formation.

Thus, Cdk5 activity must be well regulated in order to maintain control over the numerous aspects of neuronal function that it modulates. p25 levels are higher in AD brains than in normal controls (Lee et al., 1999; Patrick et al., 1999; reviewed in Giese et al., 2005). Although transient expression of p25 increases synaptic plasticity, prolonged neuronal expression of p25 in transgenic mice leads to synaptic impairments, learning and memory deficits, neurofibrillary tangle formation, and neurodegeneration (Cruz and Tsai, 2004; Fischer et al., 2005). In addition, sustained Cdk5 activity increases Aβ production (Cruz et al., 2006). Thus, the generation of p25 may be initiated in early stages of AD as a compensatory mechanism to support waning plasticity and memory, but its continued presence and the resulting overactivation of Cdk5 may eventually contribute to synaptic impairments and other neuronal deficits.

4.15.3.5 Neurotrophic and Neuromodulatory Factors

During development of the nervous system, secreted neurotrophic and neuromodulatory factors regulate axonal outgrowth, dendritic maturation, synapse formation, and synaptic strength. These processes overlap widely with those necessary for synaptic plasticity and the maintenance of long-term changes in synaptic function. Therefore, it is not surprising that nature has recycled many of these same neurotrophic and neuromodulatory factors to effect synaptic plasticity in adult organisms. Two such neuromodulatory factors are brain-derived neurotrophic factor (BDNF) and Reelin. We discuss their roles in synaptic plasticity and how AD-related alterations in their levels may contribute to synaptic and cognitive dysfunction.

4.15.3.5.1 BDNF

BDNF belongs to the neurotrophin family of signaling proteins that also includes nerve growth factor (NGF), neurotrophin 3 (NT-3), and neurotrophins 4/5(NT-4/5), all of which participate in regulating the survival and differentiation of specific neuronal populations during development. However, BDNF is unique among its family members in its ability to modulate activity-dependent synaptic plasticity in the developing and the adult brain (reviewed in Lu, 2003).

The actions of BDNF have long been studied in the context of learning and memory in animal models, but the recent discovery that a Val→Met mutation in the prodomain of BDNF is linked to memory impairment and susceptibility to neuropsychiatric disorders in humans has fueled additional research (reviewed in Bath and Lee, 2006). This mutation affects the trafficking of BDNF to the secretory pathway, resulting in reduced secretion of BDNF in target regions. Moreover, BDNF mRNA and protein levels are decreased in brain regions that are vulnerable in AD, suggesting that alterations in BDNF may reflect or contribute to cognitive impairments in AD (reviewed in Murer et al., 2001; Allen and Dawbarn, 2006).

BDNF has multiple distinct functions in synaptic plasticity that can be divided into two broad categories: permissive and instructive (reviewed in Schinder and Poo, 2000; Bramham and Messaoudi, 2005). Permissive actions of BDNF prepare synapses to be LTP-competent but do not actually generate LTP. Such actions include the maintenance of the presynaptic release machinery (vesicle docking and vesicle pool dynamics), which allows neurons to follow high-frequency stimuli.

Instructive signals from BDNF are initiated in response to high-frequency stimuli that induce LTP and result in the activity-dependent expression and release of BDNF. Some of these signals modulate postsynaptic calcium influx through voltage-gated sodium channels, reducing the amount of stimulation necessary for LTP induction (gating) (reviewed in Blum and Konnerth, 2005). The majority of BDNF's

effects are mediated by TrKB receptors and subsequent signaling events that engage ERK1/2 and regulate the expression of genes, including the immediate-early gene Arc/Arg3.1 (reviewed in Blum and Konnerth, 2005; Bramham and Messaoudi, 2005). BDNF also regulates the translation of dendritically localized mRNAs associated with synapse-specific LTP (reviewed in Schuman et al., 2006). Together, BDNF's actions lead to long-lasting increases in synaptic strength.

Regulated BDNF expression also plays a central role in homeostatic synaptic scaling, through which the overall activity of a neuronal network is maintained over time (reviewed in Turrigiano and Nelson, 2004). As mentioned above, BDNF expression is activity dependent. Under situations of reduced activity, BDNF expression is reduced. GABA expression in inhibitory interneurons is then reduced, diminishing inhibition and promoting the firing rate of pyramidal neurons (Rutherford et al., 1997, 1998; reviewed in Turrigiano and Nelson, 2004).

The role of BDNF in scaling is particularly interesting in light of the decreased levels of BDNF observed in vulnerable brain regions in AD. Does the decrease in BDNF result from a primary insult and exacerbate synaptic deficits and plasticity in AD, or does it represent the attempts of an impaired network to increase neuronal activity and maintain synaptic connections? Answers to these questions are pending.

An additional complexity in considering the role of BDNF in AD is that the regulation and effects of BDNF have different outcomes depending on the state of the neuronal network on which it acts (Turrigiano and Nelson, 2004). The role of BDNF reductions in homeostatic scaling described above is evident in conditions in which normal activity has been abolished. However, in situations containing normal levels of background activity, BDNF's potentiating activity prevails. Transgenic mice overexpressing BDNF have increased seizure severity after kainic acid challenge and develop hyperexcitability in the entorhinal cortex and the CA regions of the hippocampus (Croll et al., 1999). Transgenic mice overexpressing BDNF's receptor TrKB also have a reduced threshold for kainate-induced seizures (Lahteinen et al., 2003). These studies suggest that a reduction of BDNF in AD may represent a compensatory mechanism against hyperexcitability. Consistent with this idea, removal of one BDNF allele in mice leads to increased synaptic inhibition (Olofsdotter et al., 2000).

4.15.3.5.2 Reelin

Reelin is a large glycoprotein of the extracellular matrix involved in neuronal migration and positioning during development (Tissir and Goffinet, 2003). In the mature brain, it modulates neuronal function and synaptic plasticity and regulates tau phosphorylation as well as axonal growth and dendritic spine morphology (Hiesberger et al., 1999; Liu et al., 2001b; Fatemi, 2005; Herz and Chen, 2006; Qiu et al., 2006b).

In most of the brain, Reelin is expressed by GABAergic interneurons that regulate the activity and function of neighboring glutamatergic neurons (Pesold et al., 1998; Ramos-Moreno et al., 2006). Interestingly, Reelin is also expressed highly by glutamatergic pyramidal neurons in layer II of the entorhinal cortex (Pesold et al., 1998; Perez-Garcia et al., 2001; Ramos-Moreno et al., 2006), a population of neurons that is affected early and severely by AD (Blennow et al., 2006). These neurons project primarily to the dentate gyrus and area CA1 of the hippocampus (Ramos-Moreno et al., 2006; van Groen et al., 2003), which are also vulnerable to AD (Blennow et al., 2006; Palop et al., 2003). Although Reelin does not appear to undergo calcium-dependent exocytosis (Lacor et al., 2000), its localization in secretory vesicles, axons, and dendritic spine-rich neuropils suggests that it may be released from both the cell soma and synaptic terminals (Pesold et al., 1998; Lacor et al., 2000; Pappas et al., 2001; Ramos-Moreno et al., 2006). Reelin immunoreactivity is also present in the axonal projections of glutamatergic pyramidal neurons in layer II of the entorhinal cortex (Ramos-Moreno et al., 2006), suggesting that Reelin produced by these cells is transported down axons and may impact neuronal function in target regions such as the dentate gyrus and CA1.

Two neuronal cell surface receptors that bind apoE and transport cholesterol into neurons, very low density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2), bind Reelin and cooperate to transduce its signals (reviewed in Herz and Chen, 2006). The close proximity between Reelin receptors and NMDA receptors allows crosstalk between the two signaling pathways: Through a series of phosphorylation events, Reelin increases NMDA receptor function and thereby enhances the induction of LTP (**Figure 17**) (Weeber et al., 2002; Beffert et al., 2005; Chen et al., 2005b). Reelin also enhances

synaptic function by stimulating the translation of dendritically expressed mRNAs, such as Arc/Arg3.1 mRNA (Dong et al., 2003). Reelin-deficient mice have a diminished capacity for hippocampus-dependent memory, indicating that Reelin is necessary for normal plasticity and memory formation (Qiu et al., 2006a).

The localization of Reelin expression and its roles in regulating synaptic plasticity as well as tau phosphorylation suggest that decreases in Reelin might exacerbate synaptic impairments in AD. In addition to memory deficits, Reelin-deficient mice exhibit robust increases in levels of hyperphosphorylated tau (Hiesberger et al., 1999; reviewed in Herz and Chen, 2006), suggesting a role for Reelin in the generation of tau pathology in AD. Furthermore, apoE competes with Reelin for binding to VLDLR/ApoER2 receptors and decreases Reelin signaling (D'Arcangelo et al., 1999; Herz and Bock, 2002). It has been suggested that apoE4 competes more efficiently than apoE3 (reviewed in Herz and Bock, 2002), providing yet another mechanism by which apoE4 may increase the susceptibility to AD (**Figure 17**; see also the section titled 'Aβ-Independent mechanisms for apoE-induced neuronal impairments').

A few recent studies have begun to examine whether AD is associated with Reelin alterations hAPP transgenic mice with high levels of Aβ were found to have significantly fewer Reelin-expressing

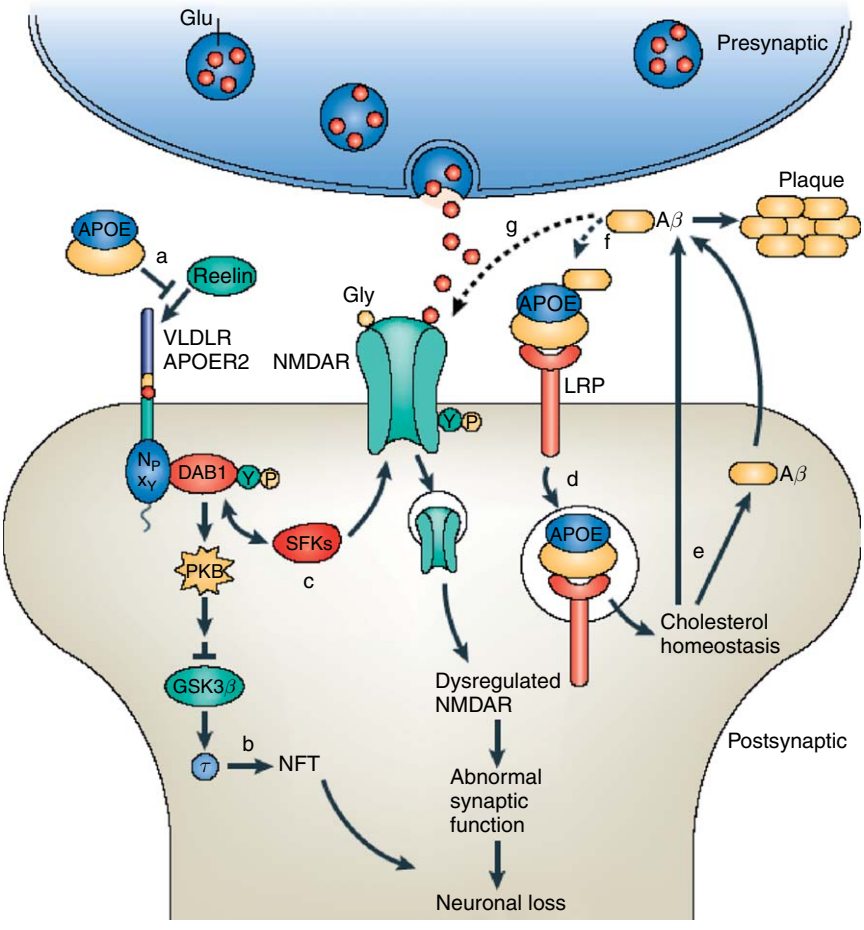


Figure 17 Reelin and ApoE signaling – implications for AD. Binding of Reelin to VLDLR and ApoER2 receptors initiates a cascade of events that leads to modulation of NMDA receptor function and enhanced long-term potentiation. ApoE can impede Reelin signaling by competing for receptor binding. Impaired Reelin signaling results in impaired synaptic plasticity as well as in elevated tau phosphorylation, which could contribute to neurofibrillary tangles associated with AD. In addition, binding of ApoE to the LRP lipoprotein receptor results in internalization of the ligand-bound receptor. Cholesterol homeostasis modulates the production and trafficking of Aβ. Secreted Aβ can bind apoE and be cleared through receptor-mediated endocytosis, promote the internalization of NMDA receptors, and deposit into plaques (From Herz J and Chen Y [2006] Reelin, lipoprotein receptors and synaptic plasticity. *Nat. Rev. Neurosci.* 7: 850–859.)

pyramidal cells in the entorhinal cortex and corresponding reductions in Reelin levels in the hippocampus relative to nontransgenic mice (Chin et al., 2007). In contrast, the number of Reelin-expressing GABAergic interneurons was not altered in either the entorhinal cortex or the hippocampus. Underscoring the relevance of these findings, qualitatively similar reductions of Reelin-expressing pyramidal neurons were found in the entorhinal cortex of AD brains (Chin et al., 2007). Increased fragments of Reelin were found in the CSF of AD patients, suggesting altered processing of Reelin (Saez-Valero et al., 2003). Increased levels of Reelin were found in the frontal cortex of AD brains (Botella-López et al., 2006). Conceivably, increases in Reelin in frontal brain regions reflect the kind of hyperactivation of frontal areas that is presumed to compensate for the failure of more vulnerable brain regions in AD (Buckner, 2004; Pariente et al., 2005; Palop et al., 2006).

4.15.3.6 Gene Expression

Short-term synaptic plasticity is effected by noncovalent modifications of existing proteins, such as ion channels, receptors, or components of the vesicle-release machinery that lead to acute modulation of synaptic strength. Long-lasting synaptic plasticity, however, requires protein synthesis and structural changes for the long-term maintenance of changes in synaptic strength (reviewed in Kandel, 2001; Carlisle and Kennedy, 2005). It is clear from the discussion in the section titled 'Kinases' that the derangement of kinase pathways in AD may disrupt the expression of pertinent genes. We consider here two proteins that play particularly important roles in gene transcription necessary for long-term plasticity (CREB) and in consolidation of long-term memories (Arc/Arg3.1), and how alterations in their expression or localization may contribute to AD-related synaptic and cognitive impairments.

4.15.3.6.1 CREB

The transcription factor CREB is essential for many forms of learning and memory (reviewed in Lonze and Ginty, 2002; and Tully et al., 2003) (*See* Chapter 4.27). CREB is a nuclear protein that modulates the transcription of genes containing cAMP-responsive elements (CREs). Phosphorylation of CREB at Ser133 leads to the recruitment of other components of the transcription machinery to CREs. Both PKA and Ca^{2+}/calmodulin kinase type IV (CaMKIV) can phosphorylate CREB at Ser133 and are responsible for rapid initial increases in CREB phosphorylation in response to neuronal activity, whereas ERK1/2-dependent phosphorylation of Ser 133 occurs with slower kinetics, involves an intermediate such as RSK or MSK family kinases, and leads to prolonged phosphorylation of CREB.

CREB target genes encompass diverse proteins, ranging from proteins involved in neurotransmission, the transcriptional machinery, or signal transduction to growth factors such as BDNF, structural proteins, channels, and transporters (reviewed in Lonze and Ginty, 2002). Consequently, disruptions in CREB activation impair numerous neurological functions.

Decreased activity of PKA and ERK1/2 in AD and related experimental models is accompanied by decreased levels of phosphorylated CREB (Dineley et al., 2001; Vitolo et al., 2002; Gong et al., 2004, 2006). Efforts are being made to determine whether promoting CREB activity is an effective means to enhance memory in normal aging and in AD (Tully et al., 2003). Indeed, treatment of transgenic mouse models of AD with agents that boost cAMP levels (and thus PKA activity) do restore PKA/CREB signaling and are associated with an amelioration of deficits in synaptic plasticity and hippocampus-dependent memory (Vitolo et al., 2002; Gong et al., 2004, 2006).

4.15.3.6.2 Arc/Arg3.1

The activity-regulated cytoskeletal protein/activity-regulated gene 3.1 is an immediate early gene (IEG) that is critical for LTP maintenance and for the consolidation of memories (Tzingounis and Nicoll, 2006). IEGs are rapidly and transiently activated at the transcriptional level after neuronal stimulation by neurotransmitters or growth factors. Some IEGs, for example, c-fos, encode transcription factors that modulate the expression of genes for proteins that affect synaptic strength, while others, such as Arc/Arg3.1, are effector IEGs whose products directly effect or maintain long-term changes in synaptic strength.

Arc/Arg3.1 was identified in 1995 by two independent groups searching for an IEG that might serve as an effector of long-term changes in synaptic strength (Link et al., 1995; Lyford et al., 1995). Both groups posited that the expression of such an effector should be (1) rapidly stimulated by neuronal activity, (2) blocked by NMDA receptor antagonists, and (3) localized to dendritic compartments. Using these criteria, both groups identified the same IEG, now known by the combined name of Arc/Arg3.1.

Arc/Arg3.1 is rapidly activated by patterned synaptic activity, including seizure activity, LTP, exploration of a novel environment, and memory-inducing behavioral paradigms (reviewed in Guzowski, 2002; Tzingounis and Nicoll, 2006). Newly synthesized Arc/Arg3.1 mRNA is rapidly transported to dendrites and accumulates in the particular synapses that were previously activated (Steward and Worley, 2001a,b). Because of these properties, the expression of Arc/Arg3.1 has been used to image behaviorally relevant activity in neuronal networks (Guzowski et al., 1999; Temple et al., 2003; Burke et al., 2005; Tagawa et al., 2005; Zou and Buck, 2006). For example, the sequential exposure of rats to two different environments results in the activation of distinct patterns of Arc/Arg3.1 expression in the hippocampus, particularly in the dentate gyrus, suggesting that Arc/Arg3.1 represents the activity of neuronal ensembles involved in the encoding of contextual information (Guzowski et al., 1999; reviewed in Guzowski et al., 2005).

Arc/Arg3.1 expression has also been used to examine the susceptibility of particular neuronal populations to Aβ-induced impairments in transgenic mouse models of AD (**Figure 18**). The induction of Arc/Arg3.1 after various stimuli is diminished in hAPP mice and hAPP/PS1 mice (Dickey et al., 2004; Chin et al., 2005; Palop et al., 2005). After exploration of a novel environment, Arc/Arg3.1 expression is reliably induced in the dentate gyrus of nontransgenic rodents (reviewed in Guzowski et al., 2005), but not in hAPP transgenic mice (Chin et al., 2005; Palop et al., 2005). Basal levels of Arc/Arg3.1 were also markedly reduced in the dentate gyrus. These alterations in Arc/Arg3.1 expression were accompanied by

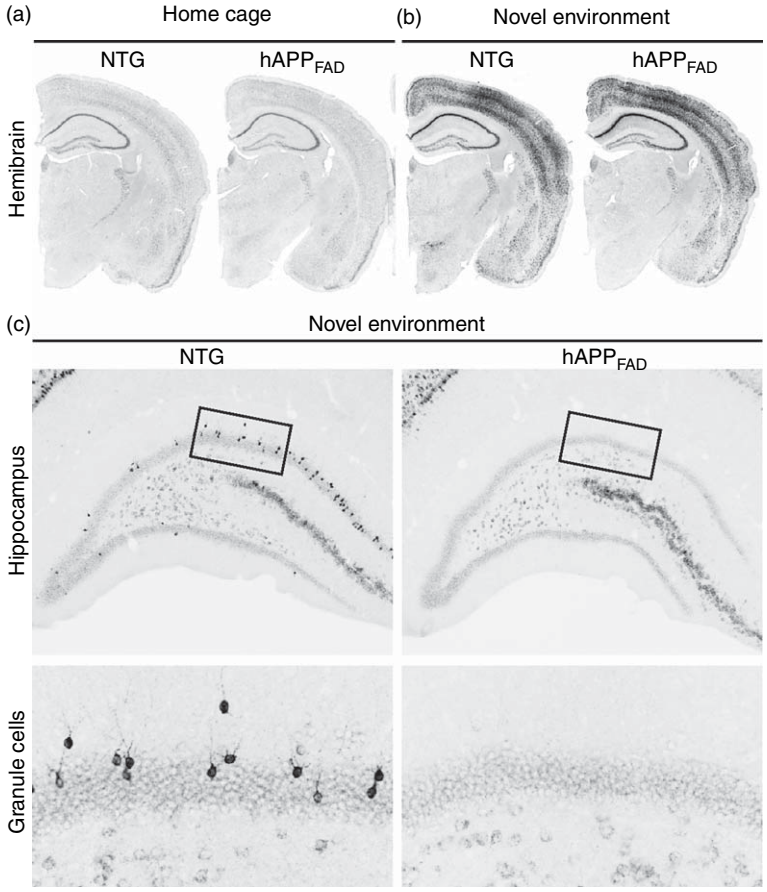


Figure 18 Imaging neuronal network activity using Arc/Arg3.1 expression. (a, b) After exploration of a novel environment, Arc/Arg3.1 expression is reliably induced in nontransgenic (NTG) mice in several brain regions including neocortex and hippocampus. hAPP transgenic mice show normal induction in the neocortex and CA1, but a striking lack of induction in the dentate granule cells of the hippocampus (a–c). (From Palop JJ, Chin J, Bien-Ly N, et al. [2005] Vulnerability of dentate granule cells to disruption of Arc/Arg3.1 expression in human amyloid precursor protein transgenic mice. *J. Neurosci.* 25: 9686–9693. Copyright 2005 by the Society for Neuroscience.)

decreased activities of NMDA receptors and ERK1/2, which regulate Arc/Arg3.1 expression. Other calcium-regulated proteins, for example, Fos and calbindin, are also depleted in the dentate gyrus of hAPP mice, and these depletions correlate well with deficits in learning and memory (Palop et al., 2003). Together, these studies indicate that the dentate gyrus is particularly vulnerable to Aβ-induced deficits in synaptic function, encoding of spatial information, and learning and memory.

Recent work by several groups has begun to shed light on Arc/Arg3.1 activities that may be particularly relevant to the synaptic and cognitive deficits observed in AD. Arc/Arg3.1 regulates AMPA receptor trafficking through interactions with endophilin and dynamin, proteins critically involved in the endocytotic recycling of synaptic vesicles (Chowdhury et al., 2006). Overexpression of Arc/Arg3.1 increases recycling rates and decreases surface expression of AMPA receptors, with corresponding decreases in AMPA receptor-mediated currents (Chowdhury et al., 2006; Rial Verde et al., 2006). In contrast, ablation of Arc/Arg3.1 increases surface expression of AMPA receptors and impairs long-term memory (Plath et al., 2006; Shepherd et al., 2006). Notably, the regulation of Arc/Arg3.1 and AMPA receptors is bidirectional, as AMPA receptor activity downregulates Arc/Arg3.1 expression (Rao et al., 2006).

The dynamic interactions between Arc/Arg3.1 and AMPA receptors may play a critical role in homeostatic scaling of synaptic strength (reviewed in Turrigiano and Nelson, 2004; Davis, 2006). Synaptic scaling is an important mode of plasticity by which neuronal networks maintain an optimal equilibrium of activity over time, and impairments of this plasticity may exacerbate synaptic and cognitive deficits in AD (Small, 2004; Palop et al., 2006).

4.15.4 Conclusions

We have surveyed here a multitude of molecules whose functions are perturbed in AD and animal models of the disease. The complexity of the pathways and interactions involved can be overwhelming, and it may be tempting to ask whether any molecule in the brain is left unaffected by the disease. Such a question has several potential answers.

Perhaps not coincidentally, this situation evokes similarities to LTP and the molecular basis of synaptic plasticity, where an equally long (and largely overlapping) list of molecules is involved (Roberson et al., 1996; Sanes and Lichtman, 1999; Malenka and Bear, 2004). This parallel highlights the fact that AD is a disease of memory not just in terms of neuropsychology but also at the molecular level, and that addressing AD may be one of the most critical applications of basic knowledge about the molecular basis of synaptic plasticity.

Second, the molecular changes in AD are not unlimited and are, in fact, bounded by multiple levels of specificity. Many are restricted to specific anatomic structures (e.g., hippocampus vs. neocortex) or even to specific subregions (e.g., CA1 vs. CA3). It is increasingly apparent that this specificity extends to the level of individual cells (e.g., principal cells vs. interneurons), an observation with important implications. Aβ-induced activation of a neurotransmitter receptor on an excitatory principal neuron might result in overexcitation of its circuit, whereas activating the same receptor on inhibitory interneurons could shut the network down, (Palop et al., 2006) (**Figure 19**). Even at the molecular level, AD-related changes can be quite specific; among the important neurotrophic factors, BDNF seems to play an important role in AD, whereas in NT3 does not appear to be involved (Hock et al., 2000).

Finally, there is at least one great boon to the long list of molecules involved in AD: a surfeit of potential targets for treating the disease. Indeed, treatments aimed at many of the molecules discussed here are now in trials for AD (Jacobsen et al., 2005; Roberson and Mucke, 2006). In terms of the AD-associated molecules in the section titled 'Memory impairment by AD-related molecules,' diverse approaches are under study, including (1) reducing the production or speeding the removal of potentially toxic proteins, for example, with β- or γ-secretase inhibitors or via immune-mediated clearance (Citron, 2004; Weiner and Frenkel, 2006); (2) preventing unwanted post-translational processing or aggregation of Aβ, tau, and apoE into particularly toxic forms (Harris et al., 2003; Khlistunova et al., 2006; McLaurin et al., 2006); (3) restoring normal functions lost by AD-related modifications, such as the microtubule-stabilizing effect of tau (Zhang et al., 2005); and (4) in the case of apoE, forcing apoE4 to adopt more apoE3-like structure and function (Mahley et al., 2006). The plasticity-related molecules highlighted in the section titled 'Memory-related molecules in AD' may provide good complementary targets. Here, the goal is to restore plasticity, boost related memory mechanisms, and protect neurons against aberrant network

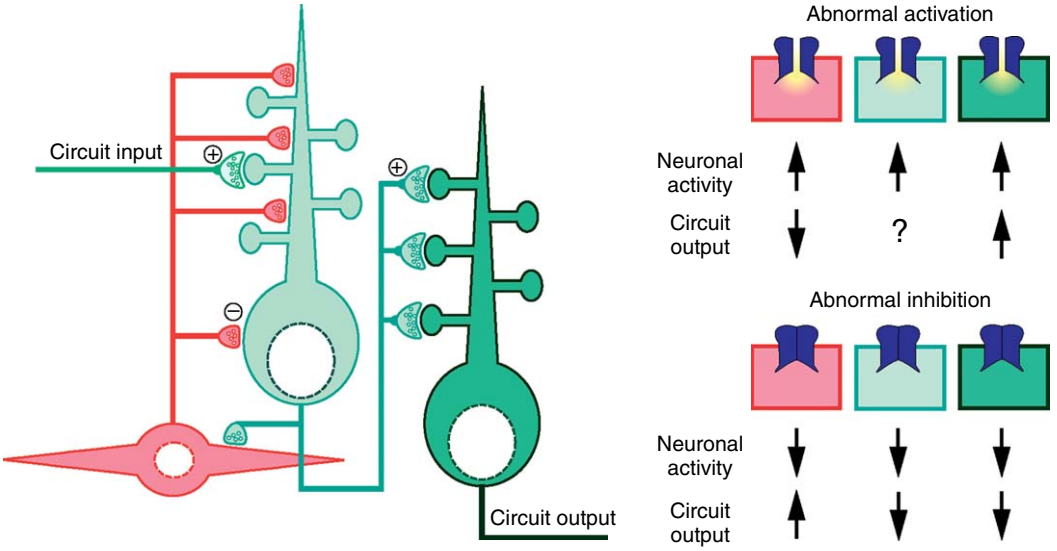


Figure 19 Consequences of the same molecular process on different cell types. The same molecular process can have a very different effect on circuit output depending on whether the cell it affects is an excitatory principal neuron (shades of green) or an inhibitory interneuron (red). Abnormal activation of an excitatory principal neuron will lead to overexcitation of the circuit, whereas abnormal activation of an inhibitory interneuron will shut the circuit down.

activities, even in the presence of pathogenic protein assemblies (Palop et al., 2006).

Thus, while complexity is certainly a feature of our current molecular understanding of AD, such issues pose exciting opportunities for neuroscientists working at the interface between AD and plasticity research, which is rapidly becoming one of the most active fronts in the battle against AD.

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4.16 Long-Term Potentiation: A Candidate Cellular Mechanism for Information Storage in the CNS

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In a very practical way, this chapter is a transition point for this volume. With this chapter, we transition from analyzing behavior to investigating cellular and molecular mechanisms for altering synaptic strength. We transition to attempting to understand mammalian memory by the reductionist approach of studying a simpler cellular phenomenon at the molecular level. Thus, this chapter will serve to place the many molecular details presented in other chapters into a broader context. In addition, wherever possible this chapter will be used as a launching point for further reading of other chapters in the volume by specifically citing other chapters at appropriate points along the way.

The particular circuits and neuronal connections that underlie most forms of mammalian learning and memory are mysterious at present, especially for hippocampus-dependent forms of learning. There is little understanding of the means by which complex memories are stored and recalled at the neural circuit level – this will be a very important avenue of future research. Thus in many ways the study of long-term potentiation (LTP) serves as a surrogate for studying hippocampus-dependent memory directly. LTP can only be viewed as a surrogate at present because very few studies are available directly implicating LTP (especially hippocampal LTP, which has been most widely studied) in defined memory behaviors. Even considering the vast number of published studies of LTP, we are left with a tentative causal link between LTP and memory *per se*.

Nevertheless, this chapter will focus on LTP. We will focus on it for three main reasons. First, it has been extensively studied and is the form of synaptic plasticity that is best understood at the molecular level. Second, it is a robust form of synaptic plasticity and worthy of investigation in its own right. Finally, it is a

specific candidate cellular mechanism for mediating certain forms of associative learning, spatial learning, and adaptive change in the central nervous system (CNS), in particular in the amygdala, hippocampus, and cerebral cortex, respectively.

4.16.1 Hebb's Postulate

Despite the various caveats concerning the specific role of LTP in hippocampus-dependent memory formation, there is a general hypothesis for memory storage that is available and broadly accepted. This hypothesis is the following:

> Memories are stored as alterations in the strength of synaptic connections between neurons in the CNS.

The significance of this general hypothesis should be emphasized – this is one of the few areas of contemporary cognitive research for which there is a unifying hypothesis.

This general hypothesis has a solid underlying rationale. Learning and memory manifest themselves as a change in an animal's behavior, and scientists capitalize on this to study these phenomena by observing and measuring changes in an animal's behavior in the wild or in experimental situations. However, all the behavior exhibited by an animal is a result of activity in the animal's nervous system. The nervous system comprises many kinds of cells, but the primary functional units of the nervous system are neurons. Because neurons are cells, all of an animal's behavioral repertoire is a manifestation of an underlying cellular phenomenon. By extension, changes in an animal's behavior such as occurs with learning must also be subserved by an underlying cellular change.

In general, the vast majority of the communication between neurons in the nervous system occurs at synapses. As synapses mediate the neuron–neuron communication that underlies an animal's behavior, changes in behavior are ultimately subserved by alterations in the nature, strength, or number of interneuronal synaptic contacts in the animal's nervous system. The capacity for alterations of synaptic connections between neurons is referred to as *synaptic plasticity*, and as described earlier, one of the great unifying theories to emerge from neuroscience research in the last century was that synaptic plasticity subserves learning and memory. LTP (of some sort at least) is the specific form of synaptic plasticity that is the leading candidate as a mechanism subserving behavior-modifying changes in synaptic strength that mediate higher-order learning and memory in mammals.

One of the pioneers in advancing the idea that changes in neuronal connectivity are a mechanism for memory was the Canadian psychologist Donald Hebb, who published his seminal formulation as what is now generally known as Hebb's postulate:

> When an axon of cell A . . . excites cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells so that A's efficiency as one of the cells firing B is increased. (Hebb, 1949)

Note the important contrast between Hebb's postulate and its popular contemporary formulation – one (Hebb's) specifies cell firing, and the other (the modern formulation) specifies synaptic change. These two phenomena are clearly different, and the current, exclusively synaptic, variant is incomplete. Changes in synapses are certainly important in information storage in the CNS, but we need to consider that the postsynaptic receptors sit in a membrane whose biophysical properties are carefully controlled. Regulation of membrane sodium channels, chloride channels, and potassium channels also contributes significantly to the net effect in the cell that any neurotransmitter-operated process can achieve.

Thus, limitations arise from ignoring potential long-term regulation of membrane biophysical properties. We need to consider that local changes in dendritic membrane excitability may be involved in cellular information processing and also that global changes in cellular excitability that alter the likelihood of the cell firing an action potential may also be a mechanism for information storage. These topics are addressed elsewhere in this volume (*See* Chapters 4.39, 4.40). Another potential mechanism involved in memory that involves the entire cell and not specific synapses is adult neurogenesis, the growth and functional integration of new neurons in the adult CNS. Neurogenesis will be addressed in other chapters (*See* Chapter 4.42). Finally, one can think of inhibition (e.g., GABAergic (GABA: gamma-aminobutyric acid) modulation) as operating above the level of the single synapse because it can control the likelihood of the cell firing an action potential. The role of inhibition in plasticity, and the plasticity of inhibition *per se*, are discussed in another chapter (*See* Chapter 4.18).

The possibility that global or cellwide alterations might be involved in memory is also relevant when considering global genomic (transcriptional) and

epigenomic changes, which affect the nucleus and thereby potentially the entire cell as well. These mechanisms are discussed elsewhere (*See* Chapters 4.27, 4.28, 4.42). One solution to the problem of global changes due to altered transcription is specific trafficking of the products of global changes in transcription. Various aspects of this are dealt with in other chapters (*See* Chapters 4.25, 4.29, 4.30, 4.33).

The idea of the involvement of processes such as excitability in memory, processes that encompass the entire cell, has been criticized as too limiting because with global changes in excitability, one loses the computational power of selectively altering the response at a single synaptic input (i.e., synapse specificity). However, we don't know how the neuron or the CNS computes a memory output. The fundamental unit of information storage may not be the synapse but the neuron. Future experiments will be necessary to resolve this issue, but it is nevertheless worthwhile to keep in mind the possibility that regulation of excitability, and regulation of neuronal properties cellwide, as well as the more typically considered alterations in synaptic connections, may play roles in memory storage.

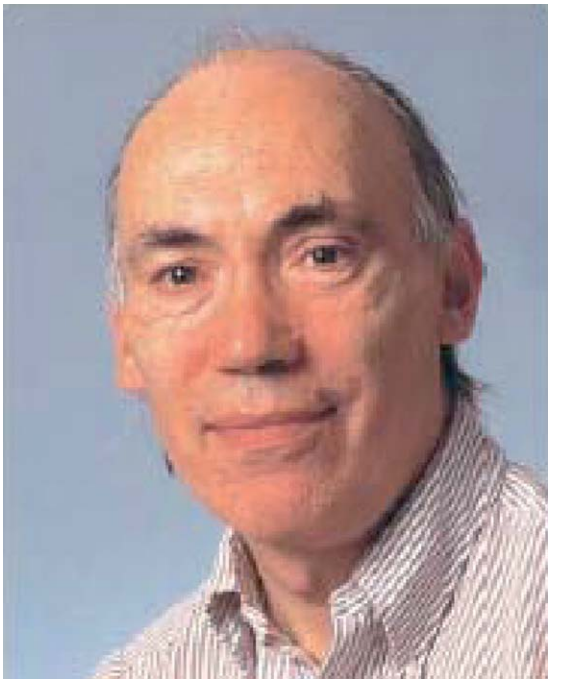


Figure 1 T. V. P. Bliss, *FRS*. Photo courtesy of Tim Bliss.

4.16.2 A Breakthrough Discovery – LTP in the Hippocampus

As a young postdoctoral researcher, Tim Bliss (**Figure 1**) set out to find a long-lasting form of synaptic plasticity in the hippocampus. By teaming up with Terje Lomo in Per Anderson's laboratory in Oslo, Bliss did just that. The seminal report by Bliss and Lomo in 1973, describing a phenomenon they termed long-term potentiation of synaptic transmission, set the stage for what is now over three decades of progress in understanding the basics of long-term synaptic alteration in the CNS.

In their experiments, Bliss and Lomo recorded synaptic responses in the dentate gyrus, stimulating the perforant path inputs from the entorhinal cortex (Bliss and Lomo, 1973). They used extracellular stimulating and recording electrodes implanted into the animal, and the basic experiment was begun by recording baseline synaptic transmission in this pathway. They discovered that a brief period of high-frequency (100-Hz 'tetanic') stimulation led to a robust increase in the strength of synaptic connections between the perforant path inputs from the entorhinal cortex onto the dentate granule neurons in the dentate gyrus (**Figure 2**). They also observed an increased likelihood of the cells firing action potentials in response to a constant synaptic input, a phenomenon they termed E-S (excitatory postsynaptic potential (EPSP)-to-spike) potentiation. These two phenomena together were termed LTP. LTP lasted many, many hours in this intact rabbit preparation. The appeal of LTP as an analog of memory was immediately apparent – it is a long-lasting change in neuronal function that is produced by a brief period of unique stimulus, exactly the sort of mechanism that had long been postulated to be involved in memory formation. This pioneering work of Bliss and Lomo set in motion a several-decades-long pursuit by numerous investigators geared toward understanding the attributes and mechanisms of LTP. Much of the progress in this area is described in the remaining chapters of this volume.

4.16.2.1 The Hippocampal Circuit and Measuring Synaptic Transmission in the Hippocampal Slice

Bliss and Lomo did their experiment using the intact rabbit, stimulating and recording in the anesthetized animal using implanted electrodes. In recent times, this preparation has been largely supplanted by the use of recordings from hippocampal slices maintained *in vitro* (**Figure 3**). Because most of the LTP

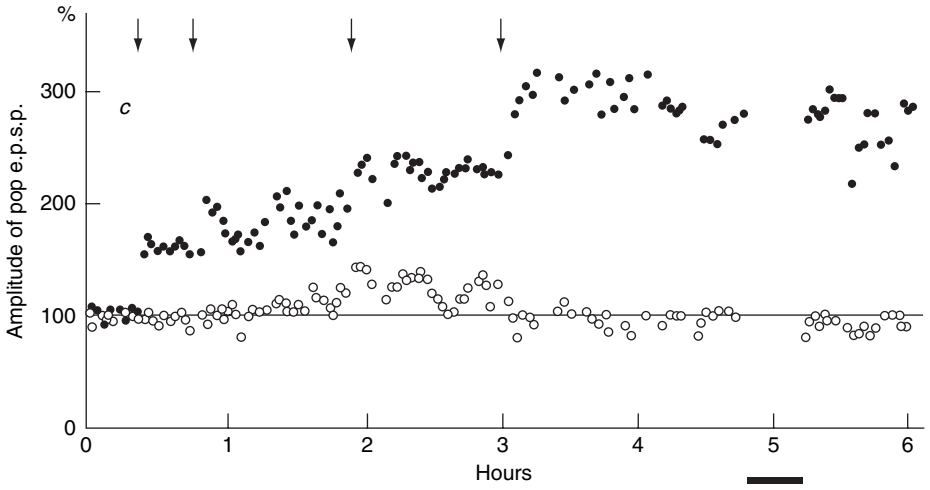


Figure 2 Bliss and Lomo's first published LTP experiment. As described in more detail in the text, in this pioneering work Tim Bliss and Terje Lomo demonstrated LTP of synaptic transmission. This specific experiment investigated synaptic transmission at perforant path inputs into the dentate gyrus (see **Figure 4**). Arrows indicate the delivery of high-frequency synaptic stimulation, resulting in LTP. Filled circles are responses from the tetanized pathways; open circles are a control pathway that did not receive tetanic stimulation. The bar, where no data points are available, indicates a period of time where Tim Bliss fell asleep. Data acquisition in this era involved the investigator directly measuring by hand synaptic responses from an oscilloscope screen. Moreover, it was not unusual for experiments to extend overnight due to the long amount of time involved in preparing the rabbit for the experiment, implanting the electrodes into the brain, and establishing a stable recording configuration. From Bliss TV and Lomo T (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J. Physiol.* 232: 331–356. Used with permission.

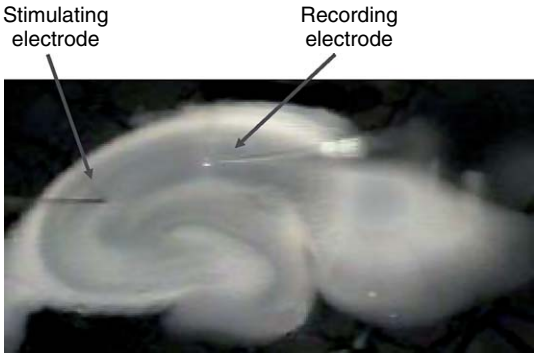


Figure 3 Electrodes in a living hippocampal slice. This photograph illustrates the appearance of a mouse hippocampal slice, maintained in a recording chamber. Responses in area CA1 are recorded using a saline-filled glass micropipette electrode (right) and a bipolar platinum stimulating electrode (left). See text and **Figure 4** for additional details.

experiments that will be described in the rest of the book come from this type of preparation, the next section will describe the hippocampal neuronal and synaptic circuit and give an overview of extracellular recording in a typical LTP experiment.

The main information processing circuit in the hippocampus is the relatively simple trisynaptic pathway, and much of this basic circuit is preserved in transverse slices across the long axis of the hippocampus (**Figure 4**). Various types of LTP can be induced at all three of these synaptic sites, and we will discuss later some mechanistic differences among the various types of LTP that can be induced. Most experiments on the basic attributes and mechanisms of LTP have been studies of the synaptic connections between axons from area CA3 pyramidal neurons that extend into area CA1. These are the synapses onto CA1 pyramidal neurons that are known as the Schaffer collateral inputs.

The main excitatory (i.e., glutamatergic) synaptic circuitry in the hippocampus, in overview, consists of three modules (see **Figure 4**) (van Groen and Wyss, 1990; Johnston and Amaral., 1998; Naber and Witter, 1998). Information enters the dentate gyrus of the hippocampal formation from cortical and subcortical structures via the perforant path inputs from the entorhinal cortex (**Figure 4**). These inputs make synaptic connections with the dentate granule cells of the dentate gyrus. After synapsing in the dentate gyrus, information is moved to area CA3 via the mossy fiber pathway, which consists of the axonal outputs of the dentate granule cells and their connections with pyramidal neurons in area CA3. After

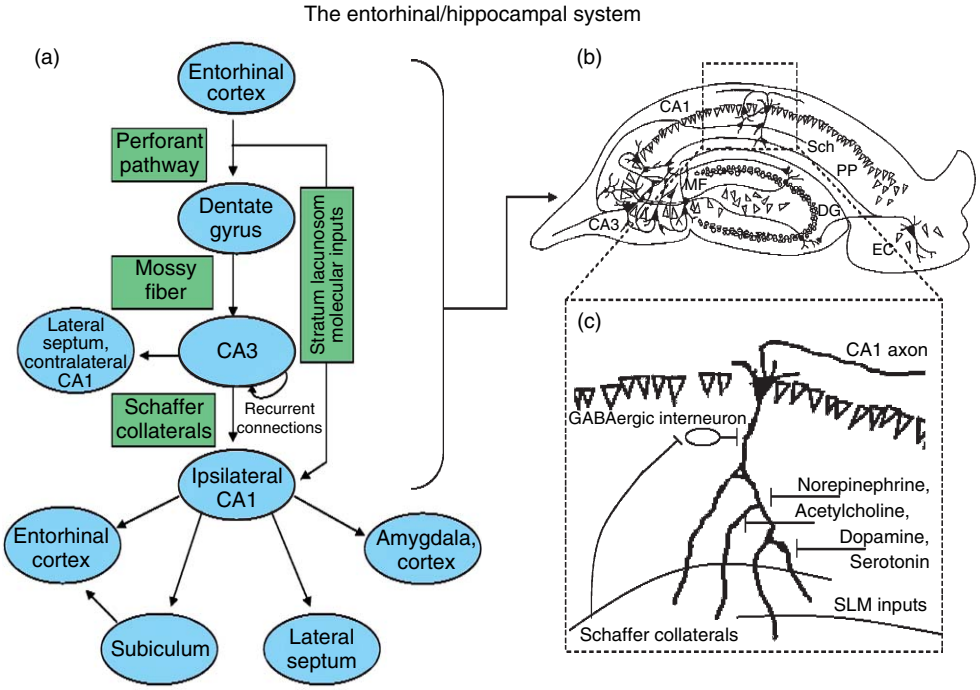


Figure 4 The entorhinal/hippocampal system. (a) This panel diagrams the principal inputs, outputs, and intrinsic connections. (b) In this panel, the central components of the circuit are delineated in a more anatomically correct fashion, illustrating the principal intrinsic connections of the dentate gyrus and hippocampus proper. (c) This is an expansion of area CA1 showing some of the synaptic inputs onto a single pyramidal neuron in area CA1. See text for additional details. Diagram by J. David Sweatt and Sarah E. Brown. Hippocampal diagram from Johnston D and Wu SM (1995) *Foundations of Cellular Neurophysiology*, p. 433. Cambridge, MA: MIT Press. Used with permission.

synapsing in area CA3, information is moved to area CA1 via the Schaffer collateral path, which consists largely of the axons of area CA3 pyramidal neurons along with other projections from area CA3 of the contralateral hippocampus as well. After synapsing in CA1, information exits the hippocampus via projections from CA1 pyramidal neurons and returns to subcortical and cortical structures.

The connections in this synaptic circuit are retained in a fairly impressive manner if one makes transverse slices of the hippocampus, as the inputs, 'trisynaptic circuit,' and outputs are laid out in a generally laminar fashion along the long axis of the hippocampal formation. This is a great advantage for *in vitro* electrophysiological experiments.

It is important to emphasize that the trisynaptic circuit outlined earlier is a great oversimplification, as there are a great many additional synaptic components of the hippocampus. For example, inhibitory GABAergic interneurons make synaptic connections with all of the principal excitatory neurons outlined earlier. These GABAergic inputs serve in both a feedforward and feedback fashion to control excitability. There are many recurrent and collateral excitatory connections between the excitatory pyramidal neurons as well, particularly in the area CA3 region. There is a direct projection from the entorhinal cortex to the distal regions of CA1 pyramidal neuron dendrites, a pathway known as the stratum lacunosum moleculare.

Finally, there are many modulatory projections into the hippocampus that make synaptic connections with the principal neurons (see **Figure 4**). These inputs are via long projection fibers from various anatomical nuclei in the brainstem region, and they are generally not directly excitatory or inhibitory, but rather serve to modulate synaptic connectivity in a fairly subtle way. There are four predominant extrinsic modulatory projections into the hippocampus. First, there are inputs of norepinephrine (NE)-containing fibers that project from the locus ceruleus. Second, there are dopamine (DA)-containing fibers that arise from the substantia nigra. There also are inputs using acetylcholine (ACh) from the medial septal nucleus and 5-hydroxytryptamine (5HT, serotonin) from the raphe nuclei.

4.16.2.2 LTP of Synaptic Responses

In a popular variation of the basic LTP experiment, extracellular field potential recordings in the dendritic regions of area CA1 are utilized to monitor synaptic transmission at Schaffer collateral synapses (see **Figure 5**). A bipolar stimulating electrode is placed in the *stratum radiatum* subfield of area CA1 and stimuli (typically constant current pulses ranging from 1 to 30 μA) are delivered. Stimuli delivered in this fashion stimulate the output axons of CA3 neurons that pass nearby, causing action potentials to propagate down these axons. Cellular responses to this stimulation are recorded using extracellular or intracellular electrophysiologic recording techniques.

The typical waveform in an extracellular recording consists of a fiber volley, which is an indication of the presynaptic action potential arriving at the recording site and the excitatory postsynaptic potential (EPSP) itself. The EPSP responses are a manifestation of synaptic activation (depolarization) in the CA1 pyramidal neurons. For measuring field (i.e., extracellularly recorded) EPSPs, the parameter typically measured is the initial slope of the EPSP waveform (see **Figure 5**). Absolute peak amplitude of EPSPs can also be measured, but the initial slope is the preferred index. This is because the initial slope is less subject to contamination from other sources of current flow in the slice. For example, currents are generated by feedforward inhibition due to GABAergic neuron activation. Also, if the cells fire action potentials, this also can contaminate later stages of the EPSP, even when one is recording from the dendritic region.

Extracellular field recordings measure responses from a population of neurons, so EPSPs recorded in this fashion are referred to as population EPSPs (pEPSPs). Note that pEPSPs are downward deflecting for stratum radiatum recordings (see **Figure 5**). If one is recording from the cell body layer (stratum pyramidale), the EPSP is an upward deflection, and if

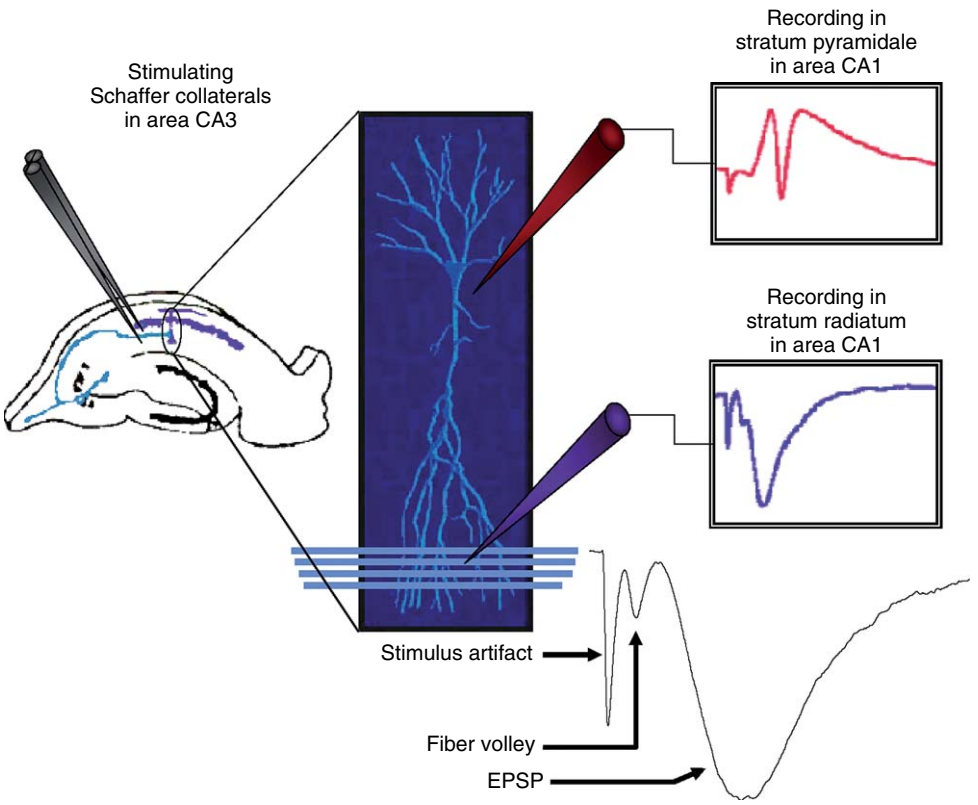


Figure 5 Recording configuration and typical physiologic responses in a hippocampal slice recording experiment. Electrode placements and responses from stratum pyramidale (cell body layer) and stratum radiatum (dendritic regions) are shown. In addition, the typical waveform of a population excitatory postsynaptic potential (EPSP) is illustrated, showing the stimulus artifact, fiber volley, and population EPSP. Figure and data by Joel Selcher.

the cells fire action potentials, the EPSP has superimposed on it a downward deflecting spike, the population spike. As mentioned earlier, for both stratum radiatum and stratum pyramidale recordings the EPSP slope measurements are taken as early as possible after the fiber volley to eliminate contamination by population spikes.

As a prelude to starting an LTP experiment, input-output (I/O) functions for stimulus intensity versus EPSP magnitude are recorded in response to increasing intensities of stimulation (see **Figure 6**). For the remainder of the experiment, the test stimulus intensity is set to elicit an EPSP that is approximately 35–50% of the maximum response

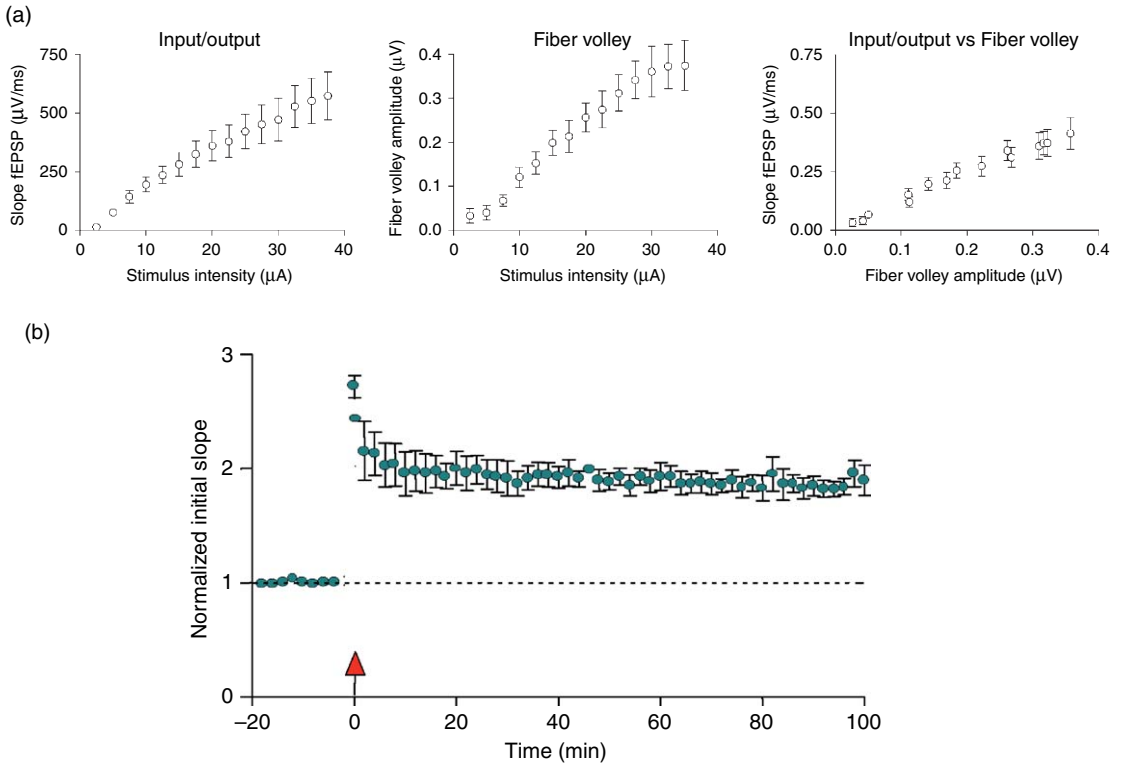


Figure 6 An input-output curve and typical LTP experiment. (a) The relationship of EPSP magnitude (field EPSP, EPSP) versus stimulus intensity (in microamperes), and the same data converted to an input-output relationship for EPSP versus fiber volley magnitude to allow an evaluation of postsynaptic response versus presynaptic response in the same hippocampal slice. (b) A typical high-frequency stimulation-induced potentiation of synaptic transmission in area CA1 of a rat hippocampal slice *in vitro*. The arrow indicates the delivery of 100-Hz (100 pulses/second) synaptic stimulation. Data courtesy of Ed Weeber and Coleen Atkins. In most pharmacologic experiments using physiologic recordings in hippocampal slice preparations, effects of drug application on baseline synaptic transmission can be evaluated by simply monitoring EPSPs before and after drug application, using a constant stimulus intensity. A more elaborate alternative is to produce input-output curves for EPSP initial slope (or magnitude) versus stimulus intensity for the presynaptic stimulus. These types of within-slice experiments are very straightforward, but in some experimental comparisons, this type of within-preparation design is not possible. For example, if one is comparing a wild type with a knockout animal, there of necessity must be a comparison across preparations. How does one evaluate if there is a difference in basal synaptic transmission in this situation? The principal confound is because although one has control over magnitude of the stimulus one delivers to the presynaptic fibers, differences in electrode placement, slice thickness, etc., from preparation to preparation cause variability in the magnitude of the synaptic response elicited by a constant stimulus amplitude. One commonly used approach to compare from one preparation (or animal strain) to the next is to quantitate the EPSP relative to the amplitude of the fiber volley in that same hippocampal slice. The rationale is that the fiber volley, which represents the action potentials firing in the presynaptic fibers, is a presynaptic physiologic response from within the same slice and that one can at least normalize the EPSP to a within-slice parameter. The underlying assumption is that the magnitude of the fiber volley is representative of the number of axons firing an action potential. Although not a perfect control, evaluating input-output relationships for fiber volley magnitude versus EPSP is a great improvement when making comparisons between different types of animals. If differences are observed, an increase in the fiber volley amplitude – EPSP slope relationship suggests an augmentation of synaptic transmission.

recorded during the I/O measurements. Baseline synaptic transmission at this constant test stimulus intensity is usually monitored for a period of 15–20 min to ensure a stable response.

Once the health of the hippocampal slice is confirmed as indicated by a stable baseline synaptic response, LTP can be induced using any one of a wide variety of different LTP induction protocols. Many popular variations include a single or repeated period of 1-s, 100-Hz stimulation (with delivery of the 100-Hz trains separated by 20 s or more) where stimulus intensity is at a level necessary for approximately half-maximal stimulation (see **Figure 6**). A variation is a strong induction protocol where LTP is induced with three pairs of 100-Hz, 1-s stimuli, where stimulus intensity is near that necessary for a maximal EPSP. This latter protocol gives robust LTP that lasts for essentially as long as one can keep the hippocampal slice alive. A final major variation is high-frequency stimulation patterned after the endogenous hippocampal theta rhythm; this will be described in more detail in a later section of this chapter.

4.16.2.3 Short-Term Plasticity: PTP and PPF

Two types of short-term plasticity are exhibited at hippocampal Schaffer collateral synapses and elsewhere that are activity dependent, just as is LTP. These are paired-pulse facilitation (PPF) and posttetanic potentiation (PTP). PPF is a form of short-term synaptic plasticity that is commonly held to be due to residual calcium augmenting neurotransmitter release presynaptically. When two single-stimulus pulses are applied with interpulse intervals ranging from 20 to 300 ms, the second EPSP produced is larger than the first (see **Figure 7**). This effect is referred to as PPF. The role of this type of synaptic plasticity in the behaving animal is unknown at this time; however, it clearly is a robust form of temporal integration of synaptic transmission and could be used in information processing behaviorally. The second form of short-term plasticity, PTP, is a large enhancement of synaptic efficacy observed after brief periods of high-frequency synaptic activity. For example, in experiments where LTP is induced with one or two 1-s, 100-Hz tetani, a large and transient increase in synaptic efficacy is produced immediately after high-frequency tetanus (see **Figure 7**). This is PTP. The mechanisms for PTP are unknown, but both PTP and PPF are *N*-methyl-D-aspartate (NMDA) receptor-independent phenomena. Both PPF and PTP, and some candidate molecular mechanisms that might underlie them, are discussed in another chapter (*See* Chapter 4.36) of this volume.

4.16.3 NMDA Receptor Dependence of LTP

In 1983, Graham Collingridge made the breakthrough discovery that induction of these tetanus-induced forms of LTP is blocked by a blockade of a specific subtype of glutamate receptor, the NMDA receptor (Collingridge et al., 1983). Collingridge's fascinating discovery was that the glutamate analog

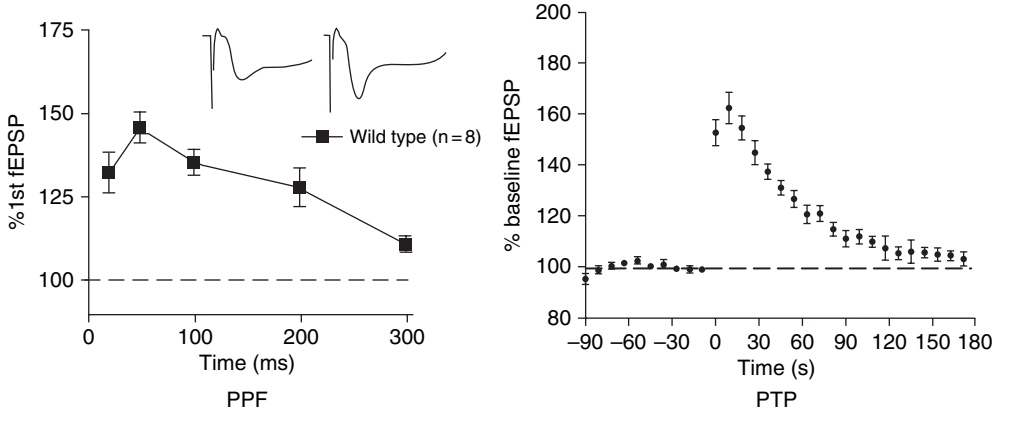


Figure 7 Paired-pulse facilitation (left) and posttetanic potentiation (right). See text for details. Data for both panels courtesy of Michal Levy. fEPSP, field EPSP.

aminophosphonovaleric acid (APV), an agent that selectively blocks the NMDA subtype of glutamate receptor, could block LTP induction while leaving baseline synaptic transmission entirely intact (Figure 8).

This was the first experiment to give a specific molecular insight into the mechanisms of LTP induction. The properties of the NMDA receptor that allow it to function in this unique role of triggering LTP are important, and we will return to a detailed analysis of regulation of the NMDA receptor later in this volume (*See* Chapter 4.20). For our purposes right now, pharmacologic blockers of NMDA receptor function have allowed the definition of different types of LTP that can be selectively induced with various physiologic stimulation protocols. For example, subsequent work has shown that an NMDA receptor-independent type of LTP can be induced in area CA1 and elsewhere in the hippocampus (mossy fibers to be precise), as well as in other parts of the CNS. We will return to a brief description of these types of LTP at the end of this chapter, but for now, we will continue to focus on NMDA receptor-dependent types of LTP.

Early studies of LTP used mostly high-frequency (100-Hz) stimulation in repeated 1-s-long trains as the LTP-inducing stimulation protocol. Although these protocols are still widely used to good effect, it is clear that such prolonged periods of high-frequency firing do not occur physiologically in the behaving animal. However, LTP can also be induced by stimulation protocols that are much more like naturally occurring neuronal firing patterns in the hippocampus. To date the forms of LTP induced by these types of stimulation have all been found to be NMDA receptor dependent in area CA1. Two popular variations of these protocols are based on the natural occurrence of an increased rate of hippocampal pyramidal neuron firing while a rat or mouse is exploring and learning about a new environment. Under these circumstances hippocampal pyramidal neurons fire bursts of action potentials at about 5 bursts/s (i.e., 5 Hz). This is the hippocampal 'theta' rhythm that has been described in the literature. One variation of LTP-inducing stimulation that mimics this pattern of firing is referred to as theta-frequency stimulation (TFS), which consists of 30 s of single stimuli delivered at 5 Hz. Another variation, theta-burst stimulation (TBS) consists of three trains of stimuli delivered at 20-s intervals, each train composed of ten stimulus bursts delivered at 5 Hz, with each burst consisting of four pulses at 100 Hz (see Figure 9). It is worth noting that these patterns of stimulation, which are based on naturally occurring firing patterns *in vivo*, lead to LTP in hippocampal slice preparations as well.

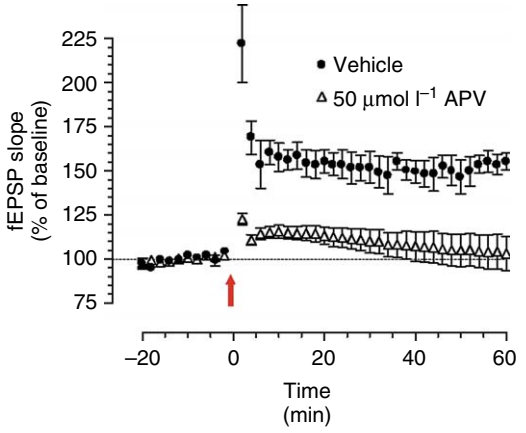


Figure 8 APV block of LTP. These data are from recordings *in vitro* from mouse hippocampal slices, demonstrating the NMDA receptor dependence of tetanus-induced LTP. Identical high-frequency synaptic stimulation was delivered in control (filled circles) and NMDA receptor antagonist (APV, open triangles) treated slices. fEPSP, field EPSP. Data courtesy of Joel Selcher.

4.16.3.1 Pairing LTP

Of course, much more sophisticated electrophysiologic techniques than extracellular recording can be used to monitor synaptic function. Intracellular recording and patch clamp techniques that measure electrophysiologic responses in single neurons have also been used widely in studies of LTP. These types of recording techniques perturb the cell that is being recorded from and lead to 'run-down' of the postsynaptic response in the cell impaled by the electrode. This limits the duration of the LTP experiment to however long the cell stays alive – somewhere in the range of 60 to 90 min for an accomplished physiologist. Regardless, in these recording configurations one can induce synaptic potentiation using tetanic stimulation or theta-pattern stimulation and measure LTP as an increase in postsynaptic currents through glutamate-gated ion channels or as an increase in postsynaptic depolarization when monitoring the membrane potential.

Control of the postsynaptic neuron's membrane potential with cellular recording techniques also allows for some sophisticated variations of the LTP induction paradigm. In one particularly important

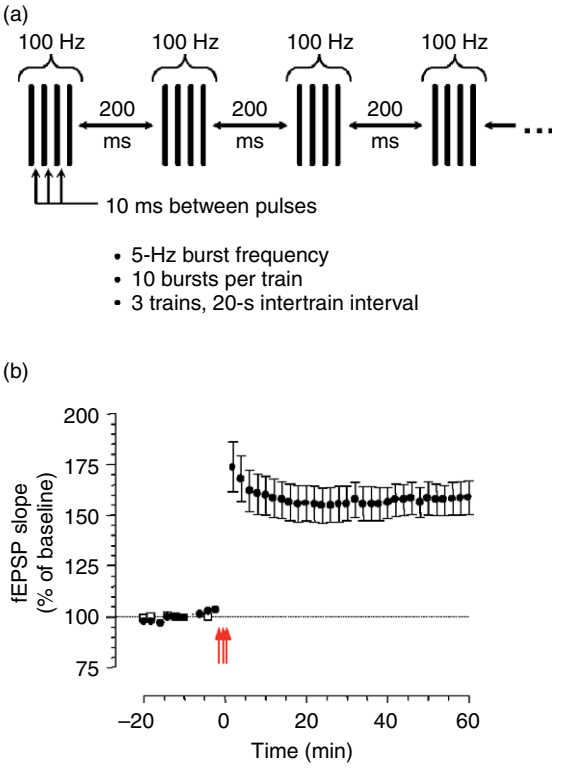


Figure 9 LTP triggered by theta-burst stimulation (TBS) in the mouse hippocampus. (a) Schematic depicting TBS. This LTP induction paradigm consists of three trains of 10 high-frequency bursts delivered at 5 Hz. (b) LTP induced with TBS (TBS-LTP) in hippocampal area CA1. The three red arrows represent the three TBS trains. fEPSP, field EPSP.

series of experiments, it was discovered that LTP can be induced by pairing repeated single presynaptic stimuli with postsynaptic membrane depolarization, so-called pairing LTP (Wigstrom and Gustafsson, 1986) (**Figure 10**).

The basis for pairing LTP comes from one of the fundamental properties of the NMDA receptor (**Figure 11**). The NMDA receptor is both a glutamate-gated channel and a voltage-dependent one. The simultaneous presence of glutamate and a depolarized membrane is necessary and sufficient (when the coagonist glycine is present) to gate the channel. Pairing synaptic stimulation with membrane depolarization provided via the recording electrode (plus the low levels of glycine always normally present) opens the NMDA receptor channel and leads to the induction of LTP.

How does the NMDA receptor trigger LTP? The NMDA receptor is a calcium channel, and its gating leads to elevated intracellular calcium in the postsynaptic neuron. This calcium influx triggers LTP, and indeed many other chapters in this volume deal with the various processes this calcium influx triggers. It is important to remember that it is not necessarily the case that every calcium molecule involved in LTP induction actually comes through the NMDA receptor. Calcium influx through membrane calcium channels and calcium released from intracellular stores may also be involved.

The gating of the NMDA receptor/channel involves a voltage-dependent Mg^{++} block of the channel pore. Depolarization of the membrane in which the NMDA receptor resides is necessary to drive the divalent Mg^{++} cation out of the pore, which then allows calcium ions to flow through. Thus, the simultaneous occurrence of both glutamate in the synapse and a depolarized postsynaptic membrane are necessary to open the channel and allow LTP-triggering calcium into the postsynaptic cell.

These properties, glutamate dependence *and* voltage dependence, of the NMDA receptor allow it to function as a coincidence detector. This is a critical aspect of NMDA receptor regulation, and this allows for a unique contribution of the NMDA receptor to information processing at the molecular level. Using the NMDA receptor, the neuron can trigger a unique event, calcium influx, specifically when a particular synapse is both active presynaptically (glutamate is present in the synapse) and postsynaptically (when the membrane is depolarized).

This confers a computational property of associativity on the synapse. This attribute is nicely illustrated by 'pairing' LTP, as described earlier, where low-frequency synaptic activity paired with postsynaptic depolarization can lead to LTP. The associative property of the NMDA receptor allows for many other types of sophisticated information processing as well, however. For example, activation of a weak input to a neuron can induce potentiation, provided a strong input to the same neuron is activated at the same time (Barrionuevo and Brown, 1983). These particular features of LTP induction have stimulated a great deal of interest, as they are reminiscent of classical conditioning, with depolarization and synaptic input roughly corresponding to unconditioned and conditioned stimuli, respectively.

The associative nature of NMDA receptor activation allows for synapse specificity of LTP induction as well, which has been shown to occur experimentally. If one pairs postsynaptic depolarization with activity at one set of synaptic inputs to a cell, while leaving a second input silent or active only during

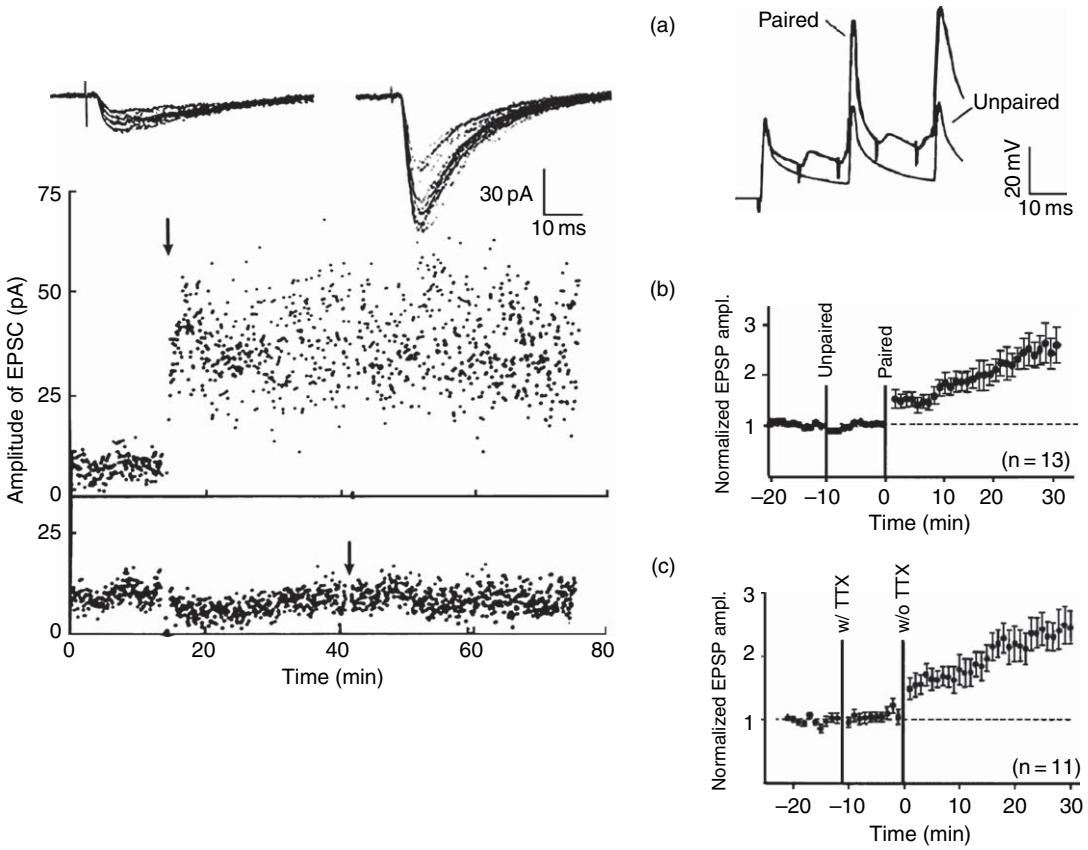


Figure 10 Pairing LTP. (Left panel) LTP of synaptic transmission induced by pairing postsynaptic depolarization with synaptic activity. The upper panels illustrate postsynaptic currents (EPSCs) recorded directly from the postsynaptic neuron using voltage-clamp techniques. The data shown are a pairing LTP experiment (upper) and control, nonpaired pathway (lower). In the pairing LTP experiment, hippocampal CA1 pyramidal neurons were depolarized from −70 mV to 0 mV while the paired pathway was stimulated at 2 Hz 40 times. Control received no stimulation during depolarization. From Malinow R and Tsien RW (1990) Presynaptic enhancement shown by whole-cell recordings of long-term potentiation in hippocampal slices. *Nature* 346: 177–180. (Right panels) Pairing small EPSPs with back-propagating dendritic action potentials induces LTP. Inset (a): Subthreshold EPSPs paired with back-propagating action potentials increase dendritic action potential amplitude. Voltage-clamp recording at approximately 240 μm from soma, that is, in the dendritic tree of the neuron (see **Figure 12**). Action potentials were evoked by 2-ms current injections through a somatic whole-cell electrode at 20-ms intervals. Alone, action potential amplitude was small (unpaired). Paired with EPSPs (5 stimuli at 100 Hz), the action potential amplitude increased greatly (paired). Inset (b): Grouped data showing normalized EPSP amplitude after unpaired and paired stimulation. The pairing protocol shown in (a) was repeated 5 times at 5 Hz at 15-s intervals for a total of 2 times. Inset (c): A similar pairing protocol was given with and without applying the sodium channel blocker tetrodotoxin (TTX, to block action potential propagation) to the proximal apical dendrites to prevent back-propagating action potentials from reaching the synaptic input sites. LTP was induced only when action potentials fully back-propagated into the dendrites. Reproduced with permission from Magee JC and Johnston D (1997) A synaptically controlled, associative signal for Hebbian plasticity in hippocampal neurons. *Science* 275: 209–213.

periods at which the postsynaptic membrane is near the resting potential, then selective potentiation of the paired input pathway occurs.

Similarly, in field stimulation experiments LTP is restricted to tetanized pathways – even inputs convergent on the same dendritic region of the postsynaptic neuron are not potentiated if they receive only baseline synaptic transmission in the absence of synaptic activity sufficient to adequately depolarize the postsynaptic neuron (Anderson et al., 1977). This last point illustrates the basis for LTP cooperativity. LTP induction in extracellular stimulation experiments requires cooperative interaction of afferent fibers, which in essence means there is an intensity threshold for triggering LTP induction. Sufficient total synaptic activation by the input fibers must be

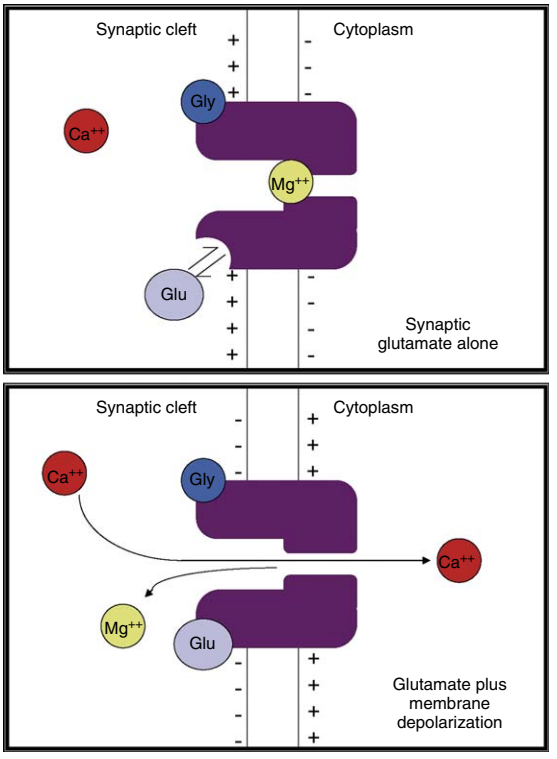


Figure 11 Coincidence detection by the NMDA receptor. The simultaneous presence of glutamate and membrane depolarization is necessary for relieving Mg^{++} blockade and allowing calcium influx. Figure by J. David Sweatt and Sarah E. Brown.

achieved such that the postsynaptic membrane is adequately depolarized to allow opening of the NMDA receptor (McNaughton et al., 1978).

4.16.3.2 Dendritic Action Potentials

In the context of the functioning hippocampal neuron *in vivo*, the associative nature of NMDA receptor activation means that a given neuron must reach a critical level of depolarization for LTP to occur at any of its synapses. Specifically, in the physiologic context the hippocampal pyramidal neuron generally must reach the threshold for firing an action potential, although there are some interesting alternatives to this that we will discuss later in this chapter and elsewhere in this volume (*See* Chapter 4.39). Although action potentials are, of course, triggered in the active zone of the cell body, hippocampal pyramidal neurons along with many other types of CNS neurons can actively propagate action potentials into the dendritic regions: the so-called back-propagating action potential (Magee and Johnston, 1997) (*See* **Figure 12** and Chapters 4.35 and 4.39). These dendritic action potentials are just like action potentials propagated down axons in that they are carried predominantly by voltage-dependent ion channels such as sodium channels. The penetration of the back-propagating action potential into the dendritic region provides a wave of membrane depolarization that allows for the opening of the voltage-dependent NMDA receptor/ion channels. *Active* propagation of the action potential is necessary because the biophysical properties of the dendritic membrane dampen the passive propagation of membrane depolarization, thus an active process such as action potential propagation is required. As a generalization, in many instances in the intact cell, back-propagating action potentials are what allow sufficient depolarization to reach hippocampal pyramidal neuron synapses to open NMDA receptors. In an ironic twist, this has brought us back to a more literal reading of Hebb's postulate, where, as we discussed at the beginning of this chapter, Hebb actually specified *firing* of the postsynaptic neuron as being necessary for the strengthening of its connections.

In fact, the timing of the arrival of a dendritic action potential with synaptic glutamate input appears to play an important part in precise, timing-dependent triggering of synaptic plasticity in the hippocampus (Magee and Johnston, 1997) (*See* **Figures 10** and **13**). It has been observed that a critical timing window is involved vis-à-vis back-propagating action potentials: glutamate arrival in the synaptic cleft must slightly precede the back-propagating action potential for the NMDA receptor to be effectively opened. This timing dependence arises in part due to the time required for glutamate to bind to and open the NMDA receptor. The duration of an action potential is, of course, quite short, so in essence the glutamate must be there first and already be bound to the receptor for full activation to occur. (Additional factors are also involved; *See* Bi and Poo, 1998; Kamondi et al., 1998; Linden, 1999; and Johnston et al., 2000 for a discussion.)

This order-of-paring specificity allows for a precision of information processing – not only must the membrane be depolarized but also as a practical matter, the cell must fire an action potential. Moreover, the timing of the back-propagating action potential arriving at a synapse must be appropriate. It is easy to imagine how the nervous system could capitalize on these properties to allow for forming precise timing-dependent associations between two events.

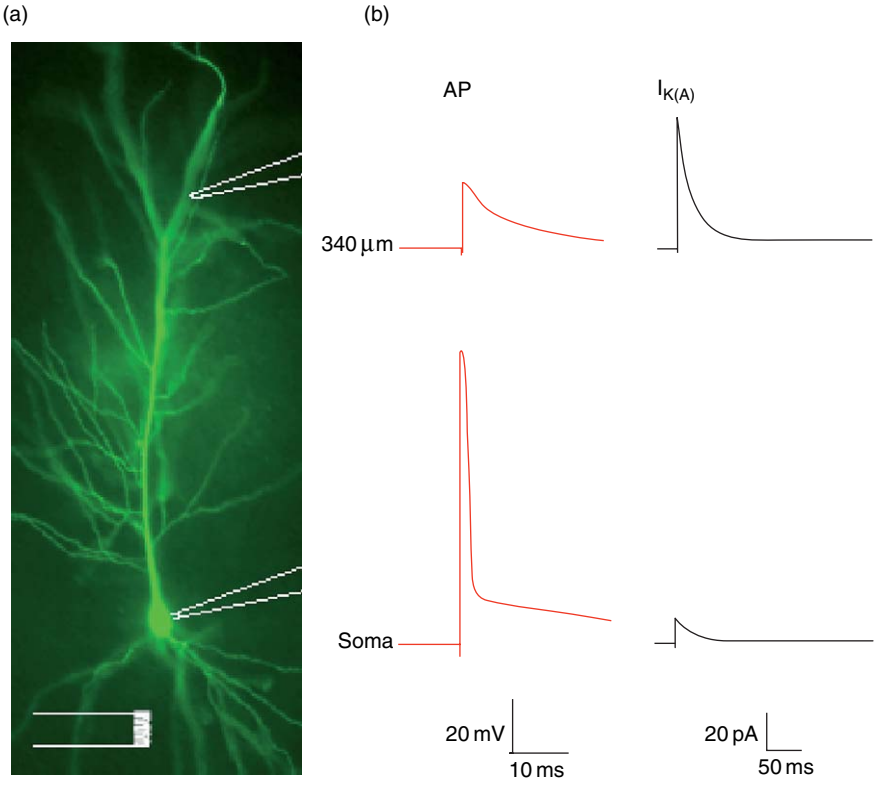


Figure 12 Back-propagating action potentials in dendrites of CA1 pyramidal neurons. (a) Indicates the recording setup, with a bipolar stimulating electrode used to trigger action potentials at the cell body region (lower left), a recording electrode in the cell soma to monitor firing of an action potential, and a recording electrode in the dendrites (upper right) to monitor propagation of the action potential into the distal dendritic region. (b) Traces in (b) indicate the data recorded from the soma (lower) and dendritic (upper) electrodes. The left-hand traces from (b) (labeled AP) indicate the membrane depolarization achieved at the soma and dendrite when an action potential is triggered and propagates into the dendritic region. Note that the dendritic action potential is of lower magnitude and broader due to the effects of dendritic membrane biophysical properties as the action potential propagates down the dendrite. The right half of (b) shows current flow through 'A-type' voltage-dependent potassium currents observed in the soma and dendrites. The density of A-type potassium currents increases dramatically as one progresses outward from the soma into the dendritic regions, as illustrated by the much larger potassium current observed in the distal dendritic electrode. These voltage-dependent potassium channels are key regulators of the likelihood of back-propagating action potentials reaching various parts of the dendritic tree. Data and figure reproduced from Yuan LL, Adams JP, Swank M, Sweatt JD, and Johnston D (2002) Protein kinase modulation of dendritic K+ channels in hippocampus involves a mitogen-activated protein kinase pathway. *J. Neurosci.* 22: 4860–4868, with permission.

One twist to the order-of-pairing specificity is that if the order is reversed and the action potential arrives before the EPSP, then synaptic depression is produced. The mechanisms for this attribute are under investigation at present. One hypothesis is that the backward pairing by various potential mechanisms leads to a lower level of calcium influx, which produces synaptic depression (*See* following discussion and Chapter 4.17).

In other chapters we will discuss in more detail the molecular mechanisms by which local effects regulating membrane depolarization within specific dendritic branches or dendritic subregions may be achieved. Moreover, we will discuss the signal transduction mechanisms by which modulatory neurotransmitter systems can regulate the likelihood of action potential back propagation through controlling dendritic potassium channels, and we will discuss how this might allowing for sophisticated information processing through an interplay of action potential propagation, glutamate release, and neuromodulation (*See* Chapter 4.39). All of these things become possible because the dendritic membrane in which the NMDA receptors reside is not passive, but contains voltage-dependent ion channels. Thus controlling the postsynaptic membrane biophysical properties can be a critical determinant for regulating the triggering of synaptic change.

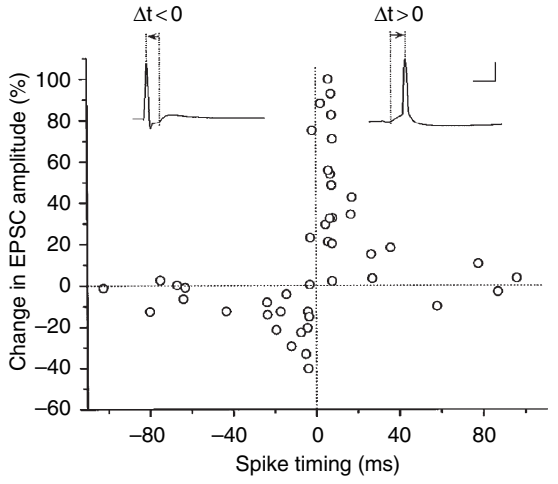


Figure 13 The timing of back-propagating action potentials with synaptic activity determines whether synaptic strength is altered, and in which direction. Precise timing of the arrival of a back-propagating action potential (a 'spike') with synaptic glutamate determines the effect of paired depolarization and synaptic activity. A narrow window when the arrival of the synaptic EPSP immediately precedes or follows the arrival of the back-propagating action potential determines whether synaptic strength is increased, is decreased, or remains the same. See text for additional discussion. EPSC, excitatory postsynaptic current. Figure adapted from Bi GQ and Poo MM (1998) Synaptic modifications in cultured hippocampal neurons: Dependence on spike timing, synaptic strength, and postsynaptic cell type. *J. Neurosci.* 18: 10464–10472, with permission.

4.16.4 NMDA Receptor-Independent LTP

Although the vast majority of studies of LTP and its molecular mechanisms have investigated NMDA receptor-dependent processes, as mentioned earlier there also are several types of NMDA receptor-independent LTP. The next section will briefly describe a few different types of NMDA receptor-independent LTP as background material and to highlight them as important areas of investigation.

4.16.4.1 200-Hz LTP

NMDA receptor-independent LTP can be induced at the Schaffer collateral synapses in area CA1, the same synapses discussed thus far. This allows for somewhat of a comparison and contrast of two different types of LTP at the same synapse. A protocol that elicits NMDA receptor-independent LTP in area CA1 is the use of four 0.5-s, 200-Hz stimuli separated by 5 s (Grover and Teyler, 1990). LTP induced with this stimulation protocol is insensitive to NMDA receptor-selective antagonists such as APV (*See* **Figure 14**). It is interesting that simply doubling the rate of tetanic stimulation from 100 Hz to 200 Hz appears to shift activity-dependent mechanisms for synaptic potentiation into NMDA receptor independence. At the simplest level of thinking, this indicates that there is some unique type of temporal integration going on at the higher frequency stimulation that allows for superseding the necessity for NMDA receptor activation. What might the 200-Hz stimulation be uniquely stimulating? One appealing hypothesis arises from the observation that 200-Hz LTP is blocked by blockers of voltage-sensitive calcium channels. Thus, the current working model is that 200-Hz stimulation elicits sufficiently large and sufficiently prolonged membrane depolarization, resulting in the opening of voltage-dependent calcium channels, to trigger elevation of postsynaptic calcium sufficient to trigger LTP synaptic potentiation. One observation consistent with this hypothesis is that injection of postsynaptic calcium chelators blocks 200-Hz stimulation-induced LTP.

4.16.4.2 TEA LTP

NMDA receptor-independent LTP in area CA1 can also be induced using tetraethylammonium (TEA^+) ion application, a form of LTP that is referred to as LTP_k (Aniksztejn and Ben-Ari, 1991; Powell et al., 1994). TEA^+ is a nonspecific potassium channel blocker, the application of which greatly increases membrane excitability. Like 200-Hz LTP, LTP_k is insensitive to NMDA receptor antagonists and is blocked by a blockade of voltage-sensitive calcium channels. Moreover, LTP_k is also blocked by postsynaptic calcium chelator injection as well. The induction of LTP_k is dependent on synaptic activity, as AMPA receptor antagonists block its induction. Similar to 200-Hz LTP, the current model for TEA LTP is that synaptic depolarization via alpha-amino-3-hydroxy-5-methyl-4 isoxazole propionic acid (AMPA) receptor activation, augmented by the hyperexcitable membrane due to K^+ channel blockade, leads to a relatively large and prolonged membrane depolarization. This leads to the triggering of LTP through postsynaptic calcium influx.

4.16.4.3 Mossy Fiber LTP in Area CA3

The predominant model system for studying NMDA receptor-independent LTP is not the Schaffer collateral synapses, but rather the mossy fiber inputs into area CA3 pyramidal neurons. Considerable excitement

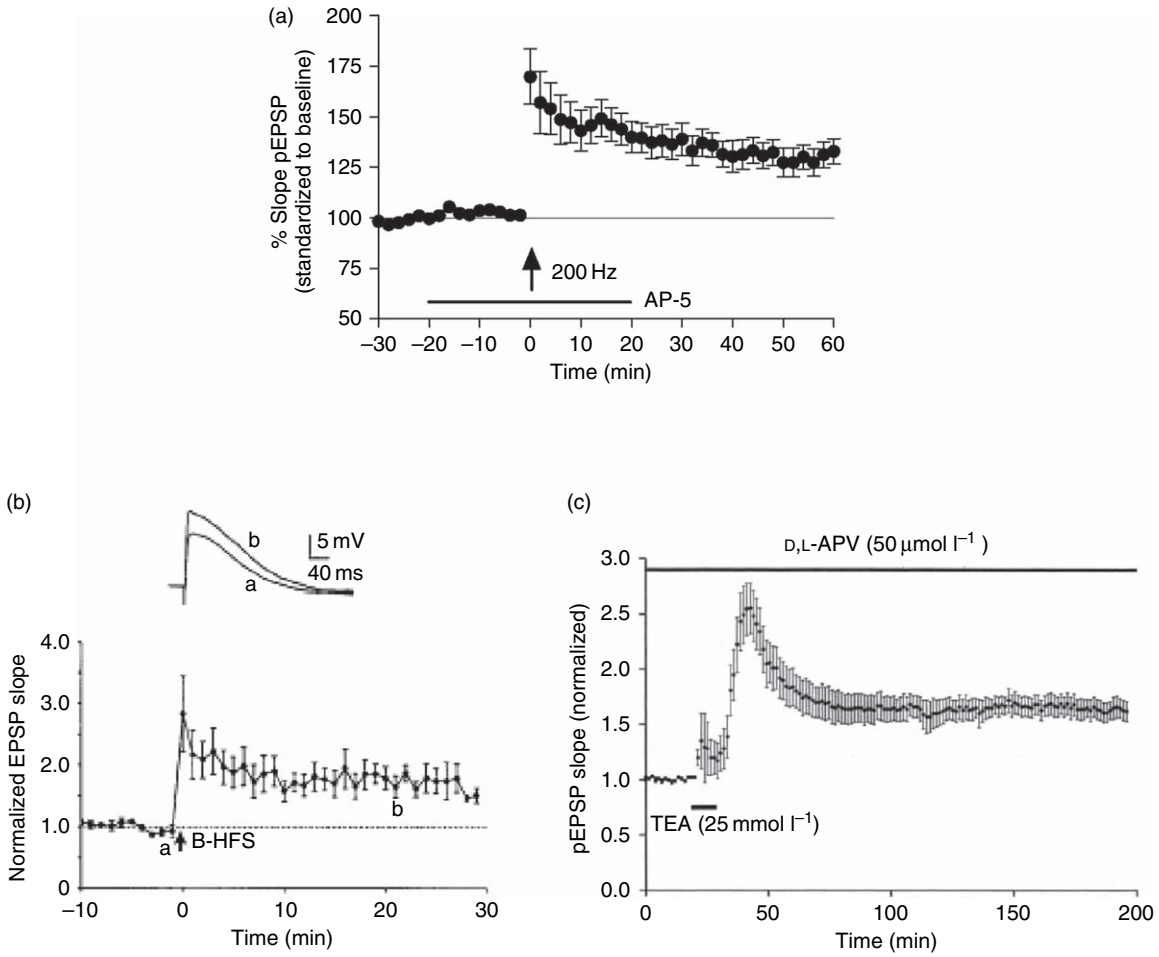


Figure 14 Examples of NMDA receptor-independent LTP. (a) 200-Hz stimulation in area CA1 elicits LTP even in the presence of the NMDA receptor antagonist APV. pEPSP, population EPSP. Data courtesy of Ed Weeber. (b) LTP at mossy fiber inputs into area CA3 is also NMDA receptor independent – the potentiation shown occurred in the presence of blockers of the NMDA receptor. Data courtesy of Rick Gray. From Kapur A, Yeckel MF, Gray R, and Johnston D (1998) L-Type calcium channels are required for one form of hippocampal mossy fiber LTP. *J. Neurophysiol.* 79: 2181–2190; used with permission. (c) Application of the K channel blocker tetra-ethyl ammonium (TEA) also elicits NMDA receptor-independent LTP in area CA1. Data courtesy of Craig Powell (Ph.D. thesis, Baylor College of Medicine, p. 50).

accompanied the discovery of NMDA receptor-independent LTP at these synapses by Harris and Cotman (1986). The mossy fiber synapses are unique, large synapses with unusual presynaptic specializations, and there has been much interest in comparing the attributes and mechanisms of induction of mossy fiber LTP (MF-LTP) with those of NMDA receptor-dependent LTP in area CA1.

However, subsequent progress in investigating the mechanistic differences between these two types of LTP has been relatively slow for several reasons. First, the experiments are technically difficult physiologically – typically area CA3 is the first part of the hippocampal slice preparation to die *in vitro*. The local circuitry in area CA3 is complex, with many recurrent excitatory connections between neurons there: synapses that also are plastic and exhibit NMDA receptor-dependent LTP. Most problematic has been that there has been an ongoing controversy about the necessity of postsynaptic events, especially elevations of postsynaptic calcium, for the induction of MF-LTP. There are basically two schools of thought on MF-LTP. One line of thinking is that MF-LTP is entirely presynaptic in its induction and expression (Zalutsky and Nicoll, 1990). A second line of thinking is that MF-LTP has a requirement for postsynaptic signal transduction events for its induction (*See*, e.g., Kapur et al., 1998; Yeckel et al., 1999). Based on the available

literature, it is difficult to come up with a definitive answer to the locus and mechanisms of induction of MF-LTP. For the purposes of this chapter, which focuses on NMDA receptor-dependent forms of LTP, I will simply note that this has been an area of controversy. Evidence supporting a presynaptic locus for MF-LTP is discussed in more detail in another chapter (*See* Chapter 4.36).

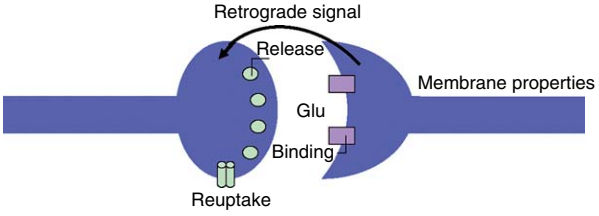
4.16.5 A Role for Calcium Influx in NMDA Receptor-Dependent LTP

In contrast to the story with MF-LTP, NMDA receptor-dependent LTP at Schaffer collateral synapses has achieved a broad consensus of a necessity for elevations of postsynaptic calcium for triggering LTP (Lynch et al., 1983). In fact, this is one of the few areas of LTP research in which there is almost universal agreement.

The case for a role for elevated postsynaptic calcium in triggering LTP is quite clear-cut and solid. It is well established and has been reviewed adequately a sufficient number of times (Johnston et al., 1992; Nicoll and Malenka, 1995; Chittajallu et al., 1998), so this material will only be presented in overview here. A principal line of evidence is that injection of calcium chelators postsynaptically blocks the induction of LTP. Also, inhibitors of a variety of calcium-activated enzymes also block LTP induction, including when they are specifically introduced into the postsynaptic neuron. Fluorescent imaging experiments using calcium-sensitive indicators have clearly demonstrated that postsynaptic calcium is elevated with LTP-inducing stimulation. Finally, elevating postsynaptic calcium is sufficient to cause synaptic potentiation (although there has been some controversy on this point). Thus the hypothesis of a role for postsynaptic calcium elevation in triggering LTP has met the three classic criteria (block, measure, mimic) necessary for 'proving' a hypothesis (Sweatt, 2003), and this idea is on a solid experimental footing.

4.16.6 Presynaptic versus Postsynaptic Mechanisms

One of the most intensely studied and least satisfactorily resolved aspects of LTP concerns the locus of LTP maintenance and expression. One component of LTP is an increase in the EPSP, which could arise from increasing glutamate concentrations in the synapse or by increasing the responsiveness to glutamate by the postsynaptic cell (**Figure 15**). The 'pre' versus 'post' debate is whether the relevant changes reside presynaptically, manifest as an increase in neurotransmitter release or similar phenomenon, or whether they reside postsynaptically as a change in glutamate receptor responsiveness. Over the last 15 years or so there have been numerous experiments performed to try to address this question, and as of yet there is no clear consensus answer. Popularity of the 'pre' hypothesis versus the 'post' hypothesis has waxed and waned, and this oscillation may continue for some time yet. The next few paragraphs will summarize a few representative findings to provide background on these issues. Reading the recent papers by Choi et al. (2000), Bolshakov et al. (1997), and Nicoll and Malenka (1999) will provide a feel for the nature of the ongoing debate.



Presynaptic = Altered
- Neurotransmitter amount in vesicles
- Number of vesicles released
- Kinetics of release
- Glutamate reuptake
- Probability of vesicle fusion

Postsynaptic = Altered
- Number of AMPA receptors
- Insertion of AMPA receptors
- Ion flow through AMPA channels
- Membrane electrical properties

Additional possibilities include changes in number of total synaptic connections between two cells

Figure 15 Potential sites of synaptic modification in LTP. Figure by J. David Sweatt and Sarah E. Brown.

In some of the earliest studies to begin to get at LTP mechanistically, it became clear that infusing compounds into the postsynaptic cell led to a block of LTP. A few of these studies involving calcium chelators were described in the last section, and some studies investigating protein kinases are described in other chapters of this volume (*See* Chapters 4.21, 4.22, and 4.23). If compounds that are limited in their distribution to the postsynaptic compartment block LTP, the most parsimonious hypothesis is that LTP resides postsynaptically.

However, shortly thereafter, evidence began to accumulate suggesting that presynaptic changes were involved in LTP expression as well. For example, various types of 'quantal' analysis that had been successfully applied at the neuromuscular junction to dissect presynaptic changes from postsynaptic changes suggested that LTP is associated with changes presynaptically. In a series of investigations, several laboratories used whole-cell recordings of synaptic transmission in hippocampal slices and found an increase in the probability of release, a strong indicator of presynaptic changes in classic quantal analysis (Dolphin et al., 1982; Bekkers and Stevens, 1990; Malinow and Tsien, 1990; Malinow, 1991; Malgaroli et al., 1995; Zakharenko et al., 2001). These findings fit nicely with earlier studies from Tim Bliss's laboratory suggesting an increase in glutamate release in LTP as well (Dolphin et al., 1982). Given findings supporting postsynaptic locus on the one hand and presynaptic locus on the other, why not just hypothesize that there are changes both presynaptically and postsynaptically? The rub came in that some of the quantal analysis results seemed to exclude postsynaptic changes as occurring.

These findings in the early 1990s ushered in an exciting phase of LTP research that was important independent of the pre versus post debate *per se*. If there are changes presynaptically but these changes are triggered by events originating in the postsynaptic cell, as the earlier inhibitor-perfusion experiments had indicated, then the existence of a *retrograde messenger* is implied. A retrograde messenger is a compound generated in the postsynaptic compartment that diffuses back to and signals changes in the presynaptic compartment – the opposite (retrograde) direction from normal synaptic transmission. Moreover, if the compound is generated intracellularly in the postsynaptic neuron then the compound must be able to traverse the postsynaptic membrane somehow. The data supporting presynaptic changes in LTP implied the existence of such a signaling system, and this hypothesis launched a number of interesting and important experiments to determine what types of molecules might serve such a role – some of these are highlighted in **Figure 16** and described in other chapters (*See* Chapters 4.21, 4.36, and 4.37).

However, in the mid-1990s the pre/post pendulum began to swing back in the opposite direction, toward the postsynaptic side. Several groups found evidence for postsynaptic changes that could account for the apparently presynaptic changes identified by quantal analysis studies. Specifically, evidence was generated for what are termed *silent synapses* (see **Figure 17**). These are synapses that contain NMDA receptors but no AMPA receptors – they are capable of synaptic plasticity mediated by NMDA receptor activation but are physiologically silent in terms of baseline synaptic transmission. Silent synapses are rendered active by NMDA receptor-triggered

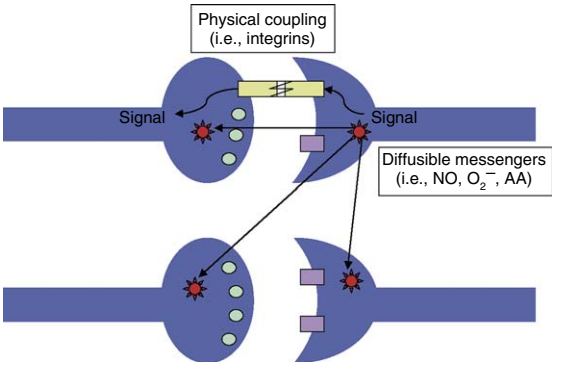


Figure 16 Potential mechanisms for retrograde signaling. Figure by J. David Sweatt and Sarah E. Brown.

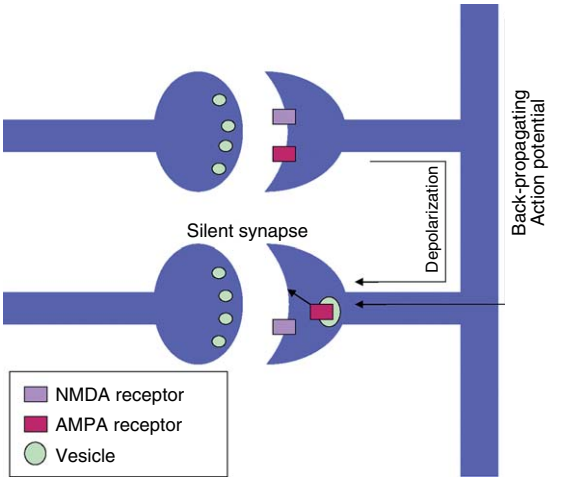


Figure 17 A simplified model of silent synapses. Figure by J. David Sweatt and Sarah E. Brown.

activation of latent AMPA receptors postsynaptically. Such uncovering of silent AMPA receptors could involve membrane insertion or posttranslational activation of already-inserted receptors. Activation of silent synapses is a postsynaptic mechanism that could explain the effects (decreased failure rate, for example) in quantal analysis experiments that implied presynaptic changes. Thus, there is now an argument that all of LTP physiology and biochemistry could be postsynaptic.

This model for conversion of silent synapses into active synapses by AMPA receptor insertion is an entirely postsynaptic phenomenon. However, there has been a variation of this idea proposed, which has been referred to as a whispering synapse. A whispering synapse has both AMPA and NMDA receptors in it, but because of a number of hypothetical factors such as glutamate affinity differences between NMDA and AMPA receptors, kinetics of glutamate elevation in the synapse, or spatial localization of the receptors, the AMPA receptors are silent. A whispering synapse is converted to being fully active by a presynaptic mechanism. An increase in glutamate release presynaptically, resulting in an elevation of glutamate levels in the synapse, then allows the effective activation of preexisting AMPA receptors with baseline synaptic transmission. By this mechanism, a synapse that was previously silent with respect to baseline synaptic transmission is rendered detectably active. However, this alternative mechanism requires no change in the postsynaptic compartment whatsoever.

Like the retrograde messenger hypothesis, the silent synapses hypothesis has also led to a number of important and interesting experiments that warrant attention aside from the pre versus post debate. Specifically, these experiments have focused new attention on the importance of considering the postsynaptic compartment in a cell-biological context. Mechanisms of receptor insertion, trafficking, and turnover that had been studied in nonneuronal cells are now beginning to get the attention they deserve in neurons as well. Other chapters address these issues in greater detail (*See* Chapters 4.30, 4.31, and 4.36). Like retrograde signaling, experiments arising from investigating mechanisms for activation of silent synapses have led to important 'spin-off' studies that are important independent of the precipitating issue of pre versus post.

Given the variety of evidence described so far, should one conclude that LTP resides presynaptically, postsynaptically, or both? Although there is not yet an unambiguous consensus in the pre versus post debate, overall the available literature indicates that changes are occurring in both the presynaptic and postsynaptic compartments. Two different types of approaches are briefly mentioned here, but these types of experiments are described in much more detail in other chapters in this volume (*See* Chapters 4.34, 4.35, 4.36, 4.38). First, a number of experiments using sophisticated imaging techniques have found LTP to be associated with presynaptic changes such as increased vesicle recycling and increased presynaptic membrane turnover (see, for example, Malgaroli et al., 1995; Zakharenko et al., 2001). Also, direct biochemical measurements of the phosphorylation of proteins selectively localized to the presynaptic compartment have shown LTP-associated changes. Conceptually similar experiments looking at phosphorylation of postsynaptic proteins have found the same thing. (These types of experiments are described in other chapters (*See* Chapters 4.30, 4.31, 4.32). Thus, imaging and biochemistry studies have fairly clearly illustrated that sustained biochemical changes are happening in both the presynaptic and postsynaptic cell.

This conclusion and indeed all of the pre versus post experiments have a very important caveat to keep in mind. In trying to reach a consensus conclusion, one is making a comparison across a wide spectrum of different types of experiments and different preparations. For example, one is comparing results with cultured cells versus hippocampal slices. One is trying to compare results for different types of LTP, LTP induced using pairing versus tetanic stimulation protocols. One likely is looking at different stages of LTP in comparing results from different experimental time points. Finally, in these experiments the various investigators are using material from different developmental stages in the animal, where the neurons under study are in different stages of their differentiation pathway. These considerations are a good reason to exercise caution in interpreting the experiments at this point, and indeed these issues may be contributing greatly to the apparent incompatibility of the results obtained in different labs. Another chapter of this volume presents a nice discussion of these various issues (*See* Chapter 4.30).

4.16.7 LTP Can Include an Increased Action Potential Firing Component

Another caveat to keep in mind is that the preceding discussion deals only with mechanisms contributing to increases in synaptic strength. The increased EPSP is typically measured in field recording

experiments as an increase in the initial slope of the EPSP (or EPSP magnitude), and as was discussed earlier, a second component of LTP is referred to as EPSP-spike (E-S) potentiation. As was already mentioned, E-S potentiation was identified by Bliss and Lomo in the first published report of LTP (1973) and is defined as an increase in population spike amplitude that cannot be attributed to an increase in synaptic transmission (i.e., initial EPSP slope in field recordings). Thus, E-S potentiation is a term used to refer to the postsynaptic cell having an increased probability of firing an action potential at a constant strength of synaptic input.

E-S potentiation at Schaffer collateral synapses can be observed using recordings in stratum pyramidale, as is illustrated in **Figure 18**. In this example, Eric Roberson generated I/O curves for the initial slope of the EPSP and the population spike amplitude, using various stimulus intensities, before and after LTP induction. E-S potentiation is manifest as an increase in population spike amplitude, even when responses are normalized to EPSP slope. Roberson's research also found that the probability of induction and magnitude of EPSP-spike (E-S) potentiation in area CA1 is more variable than LTP of synaptic transmission. A similar greater variability in E-S potentiation was observed by Bliss and Lomo in their original report as well.

What is the mechanism for this long-term increase in the likelihood of firing an action potential? One possibility is that there are changes in the intrinsic excitability of the postsynaptic neuron. Particularly appealing is the idea that long-term downregulation of dendritic potassium channel function could cause a persisting increase in cellular excitability and action potential firing. Although investigations of this hypothesis are still at an early stage, some recent work has suggested that E-S potentiation has a component due to intrinsic changes in the postsynaptic neuron. This idea is discussed further in another chapter (*See* Chapter 4.40).

Progress in testing this hypothesis has been slow due to the technically difficult nature of the experiments.

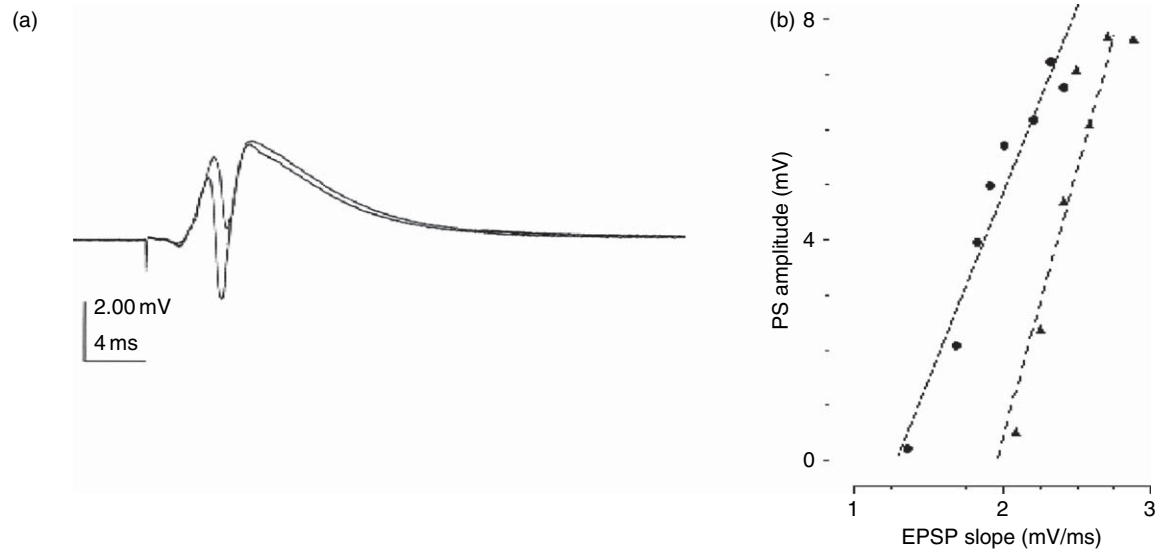


Figure 18 EPSP-spike (E-S) potentiation in area CA1. Extracellular recordings were made in the cell body layer of area CA1 (using stimulation of the Schaffer collateral inputs), and input-output (I/O) curves were performed using a range of 5 to 45 μA constant current stimulation. Initial slopes of the EPSP and population spike (PS) amplitude were then determined from the tracings and the data plotted as PS amplitude versus EPSP slope. (a) Superimposed representative tracings for before and 75 min after tetanic stimulation, showing the increased PS amplitude after tetanic stimulation. (b) Plots are shown for pretetanus (triangles) and 75 min posttetanus (circles). In this experiment five 100-Hz tetani were delivered. E-S coupling was assayed in hippocampal slices by taking a second set of I/O measurements after the induction of LTP. The baseline I/O curve and the poststimulation I/O curve were then compared to assess whether a change in excitability has occurred over the course of the experiment. Although in the illustration both EPSP slope and PS amplitude were measured from the same waveform, recording from the cell body layer, the preferred approach is to record EPSPs in the dendritic region and simultaneously record spikes independently from the cell body layer. This minimizes cross contamination of the PS changes in the EPSP measurements and vice versa. Data and figure courtesy of Erik Roberson.

Most patch-clamp physiologic studies of LTP have utilized recordings from the cell body, which are not capable of detecting changes in channels localized to the dendrites due to technical limitations. Thus, testing the idea of changes in dendritic excitability as a mechanism contributing to E-S potentiation requires dendritic patch-clamp recording, which at present only a few laboratories do routinely.

However, a more thoroughly investigated mechanism for E-S potentiation is based on alterations in feed-forward inhibitory connections onto pyramidal neurons in area CA1 (see **Figure 19**). These mechanisms for altered excitability and, indeed, plasticity of inhibitory synapses are discussed in detail in another chapter in this volume (*See* Chapter 4.18). However, the next few paragraphs present an overview of this area.

A number of different types of neurons in the hippocampus are called interneurons (or intrinsic neurons) because their inputs and outputs are restricted to local areas of the hippocampus itself. In other words, they only communicate with other neurons nearby in the hippocampus. Most of these neurons in area CA1 use the inhibitory neurotransmitter GABA, and their actions are to inhibit firing of CA1 pyramidal neurons. Different GABAergic interneurons make connections in all the dendritic regions of CA1 pyramidal neurons as well as the initial segment of the axon where the action potential originates. A single GABAergic interneuron may contact a thousand pyramidal neurons; thus, the effects of altered interneuron function are not generally limited to a single follower cell.

Interneurons in area CA1 receive glutamatergic Schaffer collateral projections just as the pyramidal neurons do – in fact, the inputs to the interneurons are branches of the same axons impinging the pyramidal neurons. Glutamate release at these interneuron synapses activates the interneurons and causes downstream release of GABA onto the pyramidal neurons. This inhibitory action is of course slightly delayed at the level of the single cell that receives input from the same Schaffer collateral axon that is activating the GABAergic interneuron, because there is an extra synaptic connection involved.

How does this local circuit contribute to E-S potentiation? Two different groups have shown that the same stimulation that produces LTP at the Schaffer collateral pyramidal neuron synapses simultaneously produces a decreased efficacy of coupling (long-term depression, LTD) of the Schaffer collateral interneuron synapses (McMahon and Kauer, 1997; Lu et al., 2000). Thus, although the excitatory input to the pyramidal neuron is being enhanced, the feed-forward inhibitory GABA input is diminished. This causes a net increase in excitability and increased likelihood of firing an action potential, added on top of the increased EPSP due to the normal LTP mechanisms. This is, of course, the definition of E-S potentiation.

There are a couple of interesting properties for this LTD at the Schaffer collateral interneuron synapse. First, it is NMDA receptor dependent just like LTP. This explains why one does not see E-S potentiation independent of synaptic potentiation in

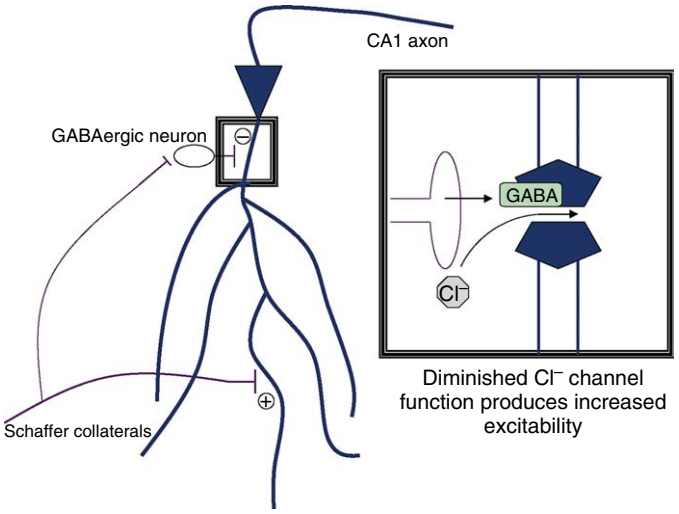


Figure 19 The GABAergic interneuron model of ES potentiation. One potential mechanism for E-S potentiation is diminution of inhibitory feed-forward inhibition through GABAergic interneurons in area CA1. Specific possible sites for this effect include LTD of the Schaffer collateral inputs onto GABAergic neurons, or synaptic depression of the interneuron-CA1 pyramidal neuron synapse. Figure by J. David Sweatt and Sarah E. Brown.

experiments where APV is infused onto the slice. Second, and more interesting, the LTD is not specific to the activated synapse – other Schaffer collateral inputs onto the same interneuron are also depressed (McMahon and Kauer, 1997). Therefore, there is decreased feed-forward inhibition across all the inputs (and outputs of course) for the whole interneuron. The interneuron has a diminished response to all its inputs and, therefore, decreased feedforward inhibition to all its outputs. Thus, the interneuron LTD appears to be serving to modulate the behavior of an entire small local circuit of neuronal connections. The precise role this interesting attribute plays in hippocampal information processing is unclear at present, but it is under study.

4.16.8 Temporal Integration Is a Key Factor in LTP Induction

At one level, it is a statement of the obvious to say that LTP induction depends on temporal integration. After all, the characteristic that distinguishes LTP induction protocols from baseline stimulation is that during an LTP induction protocol, stimulation is delivered at a higher rate. It obviously is the case that if the only attribute that is different is that the synapse is seeing activity at 100 pulses per second rather than once per 20 seconds, then unique timing-dependent processes are triggering LTP, which is simply a restatement of one definition of temporal integration. But what are the unique events that are happening physiologically with high-frequency stimulation? Stated briefly, the answer to this question is that temporal integration is occurring such that the cell is reaching a threshold of depolarization to fire an action potential (Scharfman and Sarvey, 1985; Johnston et al., 1999; Linden, 1999). This action potential firing then leads to membrane depolarization to allow opening of NMDA receptors. The following paragraphs will describe two different ways in which this can happen.

The first mechanism can be illustrated by considering what happens during the 1-s period of 100-Hz tetanus. Such closely spaced stimulation means that postsynaptic depolarization from the first EPSP carries over into the second stimulation, and so on, and so on, 96 more times. Stated more precisely, the postsynaptic membrane potential does not recover to the original resting potential before an additional depolarizing EPSP is triggered, and temporal summation of postsynaptic depolarization occurs. The summed depolarization eventually reaches threshold for the cell to fire an action potential. This is one of the classic examples of neuronal temporal integration, and of course, such a process is not limited to hippocampal pyramidal neurons. One unique aspect of this in hippocampal neurons, and probably other cortical neurons as well, is that triggering of the action potential is used to generate a back-propagating action potential into the dendrites, which is involved in depolarizing the NMDA receptor and triggering synaptic plasticity.

A second example comes from considering LTP induced by theta-pattern stimulation. With this type of LTP induction protocol, delivered at the slower 5 Hz (once/200 ms) rate, temporal integration is similarly involved but occurs via a different route. After all, 200 ms is long enough for the postsynaptic membrane potential to completely recover before the next wave of depolarization, so temporal integration of the sort described earlier is inadequate as an explanation. Joel Selcher investigated this question by examining the physiologic events occurring during the period of TFS. For illustrative purposes, his results with TFS stimulation will be discussed, although he and others observed similar effects with TBS as well.

These experiments used TFS consisting of 30 s of 5-Hz stimulation. This stimulation paradigm evokes stable LTP as described earlier and illustrated in **Figure 20**. Population spikes were assessed during the TFS period, utilizing a dual-recording electrode technique. The stimulating electrode remained in hippocampal area CA3 and activated Schaffer collateral fibers innervating area CA1. One recording electrode was positioned in stratum radiatum of area CA1 to record synaptic responses, field EPSPs (**Figure 20(b)**). Another electrode was placed in stratum pyramidale, the cell body layer, to record action potential firing in response to the same input. For each single stimulus, the initial slope of the EPSP recorded in stratum radiatum and the amplitude of the population spike recorded in stratum pyramidale were measured throughout the period of 5-Hz stimulation.

TFS resulted in a short-lived increase in action potential firing during the 30 seconds of 5-Hz stimulation (see **Figures 20(c)** and **20(d)**). For roughly the first 20 s of the stimulation, the amplitude of the population spike increased dramatically. Meanwhile, over this same time period, the EPSP slope recorded in stratum radiatum gradually declined. Therefore, the ratio of the population spike amplitude to the EPSP slope increased over time, indicating an increased likelihood of action potential firing over

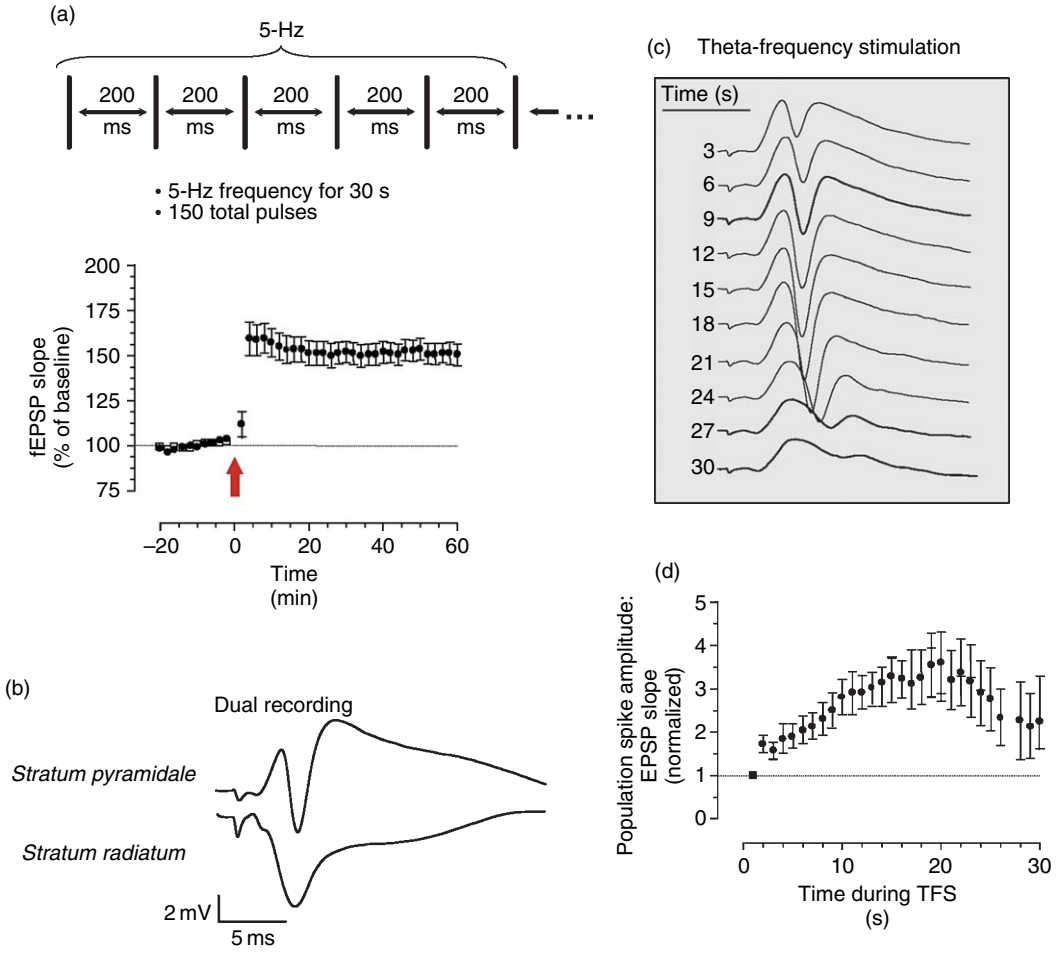


Figure 20 Increased action potential firing over the course of theta-frequency stimulation (TFS). (a) The TFS protocol and TFS-induced LTP in mouse hippocampal slices. (b) Electrode placement configuration for recording EPSP and population spikes simultaneously during TFS. (c) Representative traces in response to TFS from a hippocampal slice. Note the difference in the population spike between the first and 18th stimulation of the stimulation paradigm. (d) Quantitation of increased spike amplitude during TFS. Population spike counts recorded in stratum pyramidale of hippocampal area CA1 during theta-burst stimulation is plotted versus burst number during TFS. Slices showed a progressive increase in spike generation during the first two-thirds of TFS. Data and figures courtesy of Joel Selcher.

the short time course of the TFS (**Figure 20(d)**). Once again, for TFS as for 100-Hz tetanic stimulation, some temporal integration process is taking place to cause action potential firing during the period of LTP-inducing stimulation.

The mechanism for this temporal integration is not clear at present: clearly temporal summation of the sort operating in 100-Hz stimulation is not sufficient to explain it. However, a variety of previous studies have suggested that for LTP induced by TFS, there is an important role for attenuation of feed-forward GABAergic inhibition onto pyramidal neurons (Davies et al., 1991; Mott and Lewis, 1991; Chapman et al., 1998) (**Figure 21**). One current hypothesis is that short-term synaptic depression in the GABAergic local circuit during TFS, due to stimulation of presynaptic GABA-B autoreceptors, leads to a loss of GABA-mediated inhibition, increased excitability, and increased firing of action potentials during the period of TFS.

4.16.9 LTP Can Be Divided into Phases

Contemporary models divide very long-lasting LTP (i.e., LTP lasting in the range of 5 to 6 h) into at least three phases. LTP comprising all three phases can be

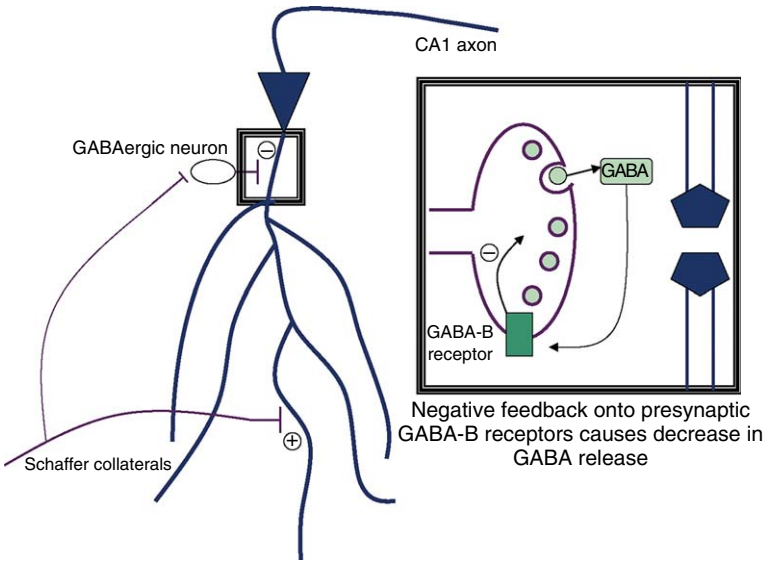


Figure 21 GABA-B receptors in temporal integration with TBS. This figure presents one model for the increased excitability that occurs during TBS, based on autoinhibition at GABAergic inputs onto CA1 pyramidal neurons during the period of stimulation. Figure by J. David Sweatt and Sarah E. Brown.

induced with repeated trains of high-frequency stimulation in area CA1 (see **Figure 22**), and the phases are expressed sequentially over time to constitute what we call 'LTP.' Late LTP (L-LTP) is hypothesized to be dependent for its induction on changes in gene expression, and this phase of LTP lasts many hours (see also Winder et al., 1998). Early LTP (E-LTP) is likely subserved by persistently activated protein kinases, as we will discuss in other chapters (*See* Chapters 4.22 and 4.23), and starts at around 30 min or less posttetanus and is over in about 2–3 h. The first stage of LTP, generally referred to as short-term potentiation (STP), is independent of protein kinase activity for its induction and lasts about 30 min. I prefer to refer to the first stage of LTP as initial LTP to emphasize that it is a persistent form of NMDA receptor-dependent synaptic plasticity that is induced by LTP-inducing tetanic stimulation and is a prelude to E-LTP and L-LTP (Roberson et al., 1996). The mechanisms for initial LTP (aka STP) are essentially a complete mystery at present.

Readers may note some degree of ambiguity in the times specified for each phase of LTP. This is in part because the phases are very descriptive, and different labs often use slightly different conditions for their LTP experiments. For example, for technical reasons most L-LTP experiments are performed at room temperature, or 27–28°C, because it is much easier to maintain a healthy slice for many hours at these lower

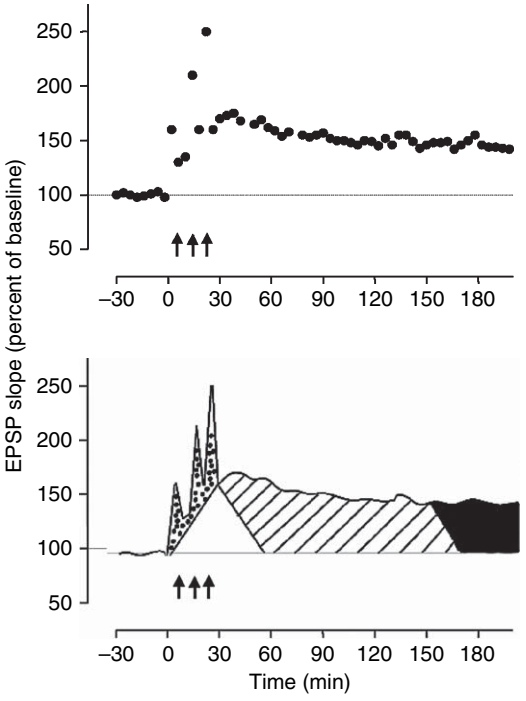


Figure 22 Immediate, early, and late LTP. The upper panel is real data from a late-phase LTP experiment, courtesy of Eric Roberson. The lower panel is a cartoon adaptation of the same data approximating the initial, early, and late stages of LTP. Adapted from Roberson ED, English JD, and Sweatt JD (1996) A biochemist's view of long-term potentiation. *Learn. Mem.* 3: 1–24, with permission.

temperatures. Many E-LTP experiments, especially those involving direct biochemical measurements, are performed at 32–35°C. Comparing studies done at different temperatures is complicated by the pronounced temperature dependence of essentially all chemical reactions. A doubling of reaction rate for a change from room temperature to 32°C is fairly common for biochemical reactions. For these many reasons, it is difficult to try to compare experiments done at one temperature to experiments done at another. 'Late' LTP may start at 3 h at room temperature, start at 1.5 h at 32°C, and start at 45 min *in vivo*.

4.16.9.1 E-LTP and L-LTP – Types versus Phases

E-LTP and L-LTP refer to different *temporal* phases of LTP. These phases are subserved by different *maintenance* mechanisms of different time courses and durations. These two phases of LTP, E-LTP and L-LTP, are not exclusive of each other. In fact, depending on the LTP induction protocol used, E-LTP can be ongoing while L-LTP is developing, and one supplants the other over time. This has certain theoretical implications that are discussed in more detail in Roberson et al. (1996). These definitions are important as we transition to molecular mechanisms in other chapters of this volume (*See* Chapters 4.20, 4.21, 4.22, 4.23, 4.25, 4.27, 4.28, 4.29, 4.30, 4.31, 4.32, 4.33, 4.34, and 4.36). These definitions contain an underlying assumption about the biochemistry of LTP that is an organizing principle for the rest of this volume, that is, that different phases of LTP are subserved by distinct molecular mechanisms.

However, the terms E-LTP and L-LTP have been used in a slightly different fashion as well, in particular as popularized by the Kandel laboratory (*See* Winder et al., 1998). The Kandel laboratory and others use a terminology that divides the NMDA receptor-dependent form of LTP in area CA1 into E-LTP and L-LTP as well. E-LTP and L-LTP in this terminology refer to what one can characterize as two subtypes of LTP – a transient form (typically lasting 1–2 h) and a long-lasting form (lasting at least 5 h or more). The latter form of LTP is characterized by its dependence on intact protein synthesis, and the induction of this form of LTP requires delivery of multiple tetanic stimuli. E-LTP in this alternative nomenclature is induced by fewer tetanic stimuli and is protein synthesis independent. In this usage, E-LTP and L-LTP are defined as different *types* of LTP, not as temporal phases of LTP. Thus, one must keep in mind that two slightly different variations in the use of the terms E-LTP and L-LTP exist in the literature.

Before turning to a discussion of some implications of LTP having phases, a final set of three terms must be introduced – three terms widely used in the LTP literature. These terms arose from pharmacological inhibitor studies of LTP, and these types of studies will be reviewed in a moment. However, first we will simply introduce the terms.

Induction refers to the transient events serving to trigger the formation of LTP. *Maintenance*, or more specifically a maintenance mechanism, refers to the persisting biochemical signal that lasts in the cell. This persisting biochemical signal acts on an effector, for example, a glutamate receptor or the presynaptic release machinery, resulting in the *expression* of LTP.

It is important to keep in mind that, depending on the design of the experiment, induction, maintenance, and expression could be differentially inhibited (see **Figure 23**). The simplest type of experiment does not do this – for example, imagine if one applies an enzyme inhibitor (or knocks out a gene) before, during, and after the period of LTP-inducing high-frequency stimulation, this manipulation may block LTP. However, this does not distinguish whether the missing activity is required for the induction, the expression, or the maintenance of LTP. To distinguish among these possibilities, imagine instead applying the inhibitor selectively at different time points during the experiment. If inhibitor is applied only during the tetanus and then washed out and it blocks the generation of LTP, one can conclude that the enzyme is necessary for LTP induction. If the inhibitor is applied after the tetanus and it reverses the potentiation, it may be blocking either the maintenance or expression of LTP, as was nicely illustrated in an early experiment by Malinow et al. (1988), where they applied a protein kinase inhibitor after LTP induction. In this experiment, transient application of a kinase inhibitor after tetanus blocked synaptic potentiation, but the potentiation recovered after removal of the inhibitor. This is a blockade of LTP *expression*. However, if the kinase inhibitor had caused the potentiation to be lost irreversibly, the inhibitor would then by definition have blocked the *maintenance* of LTP.

Finally, it is important to synthesize the concepts of induction, maintenance, and expression with the concept of phases. Simply stated, three phases of LTP (initial-, E-, and L-LTP) times three distinct

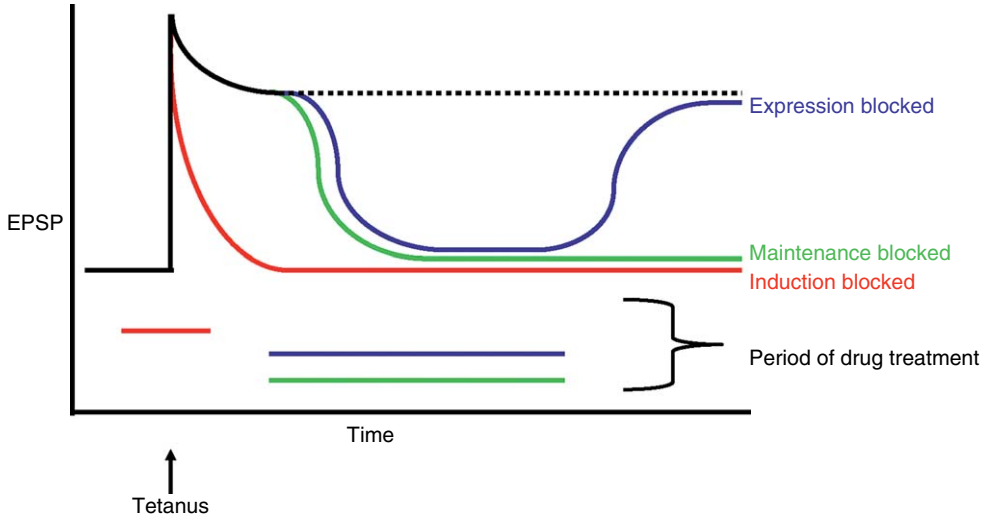


Figure 23 Induction, maintenance, and expression of LTP. This schematic illustrates the different experimental approaches to dissecting effects on the biochemical mechanisms subserving LTP induction, maintenance, or expression. See text for additional details.

underlying mechanisms for each phase (induction, maintenance, and expression) give nine separate categories into which any particular molecular mechanism contributing to LTP may fit (see Figure 24). Added to this is the complexity that one phase could be largely presynaptic and another largely postsynaptic. Interesting implications begin to arise from thinking about LTP this way: How is it that the different mechanisms for the different phases interact with each other? Is the maintenance mechanism for one phase the induction mechanism for the next, or do the mechanisms for the phases operate independently? If the maintenance and expression mechanisms for the phases are independent, how does the magnitude of LTP stay constant as the shorter-lasting phase decays? How does the mechanism for L-LTP know where to stop, so that the magnitude of L-LTP is the same as the magnitude that E-LTP had attained? Roberson et al. (1996) discusses some hypothetical answers to these questions. It also is important to keep in mind that in many ways the same considerations apply to memory itself. If memory is encoded as some complex set of molecular changes, how is it that fidelity of memory maintained as short-term memory fades into long-term memory, for example? Although we will not arrive at an answer to these many questions, it is instructive to begin to formulate a hypothetical framework for their discussion.

4.16.10 Spine Anatomy and Biochemical Compartmentalization

So far we have discussed the synapse in largely abstract terms related mostly to its function. However, the synapse is also a physical entity, and the structural attributes of this entity confer some interesting properties (reviewed in Chapter 4.34 of this volume). This brief section will describe certain physical aspects of the synapse that will be important to consider before moving on to other chapters of this volume, which discuss details about the molecular mechanisms for LTP. In brief, three points are highlighted in this

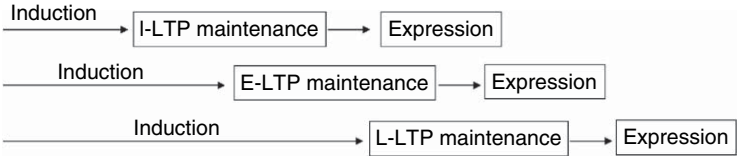


Figure 24 Mechanisms of induction, maintenance, and expression. This diagram highlights the importance of considering that each different phase of LTP may have separate and parallel induction, maintenance, and expression mechanisms. Figure by J. David Sweatt and Sarah E. Brown.

section. First, most synapses in the CNS and almost all excitatory synapses in the hippocampus are at specialized structures called *dendritic spines*. Second, spines are small, well-circumscribed biochemical compartments that localize proteins and signal molecules to a specific postsynaptic compartment. Third, spines are, of course, contiguous with dendrites and thus continuously sense the local dendritic membrane potential.

A picture of part of the dendritic region of an area CA1 pyramidal neuron is shown in **Figure 25**. The fuzzy appearance of the CA1 dendritic tree in this picture is due to the abundance of small dendritic spines protruding at right angles to the dendritic shaft. Almost all (about 95%) of the Schaffer collateral synapses we have been discussing in the abstract are actually physically present at spines. Most spines have a fairly simple, elongated, mushroomlike (i.e., chicken drumstick) shape, although there is clearly great diversity of their morphology. For example, a low percentage (about 2%) of CA1 pyramidal neuron spines are bifurcated and actually have two synapses on them. Spines have an actin-based cytoskeleton, and most have both smooth endoplasmic reticulums that can contribute to local calcium release and polyribosomes, where local protein synthesis occurs. In hippocampal pyramidal neurons, microtubules and mitochondria are limited to the dendritic shaft.

A distinguishing feature of the area of synaptic contact at the spine is the postsynaptic density, or PSD. This is a highly compact biochemical structure containing scaffolding proteins, receptors, and signal transduction components. The calcium/calmodulin-sensitive protein kinase CaMKII is particularly enriched at the PSD, as is a structural protein called PSD-95, a name that is based on its molecular weight. These molecules are discussed in much more detail elsewhere (*See* Chapters 4.20, 4.23, and 4.32).

The dendritic spine membrane surrounds the PSD and the area immediately below it and thus circumscribes a discrete biochemical compartment. The spine neck, however, is open to the dendritic shaft so there is still considerable diffusion of soluble spine contents (such as calcium and second messengers) into the local dendritic region. Nevertheless, on short time scales the spine compartment may serve to effectively localize signaling molecules to a specific synapse. Moreover, molecules tethered to the PSD by scaffolding proteins and the like probably have fairly limited diffusion because the spine compartment will make them tend to rebind at the same PSD as they unbind and rebind. Thus, this spine morphology is likely to be an important component for achieving synaptic specificity in LTP and other forms of synaptic plasticity.

The compartmentalization of molecules by the dendritic spine is not generally paralleled by an electrical compartmentalization. At one point, a popular line of thinking was that the shape and properties of the spine neck might regulate the capacity of electrical signals to get to and from the spine head compartment. This idea is no longer considered tenable, and as a first approximation, we can assume that the spine membrane potential reflects the local

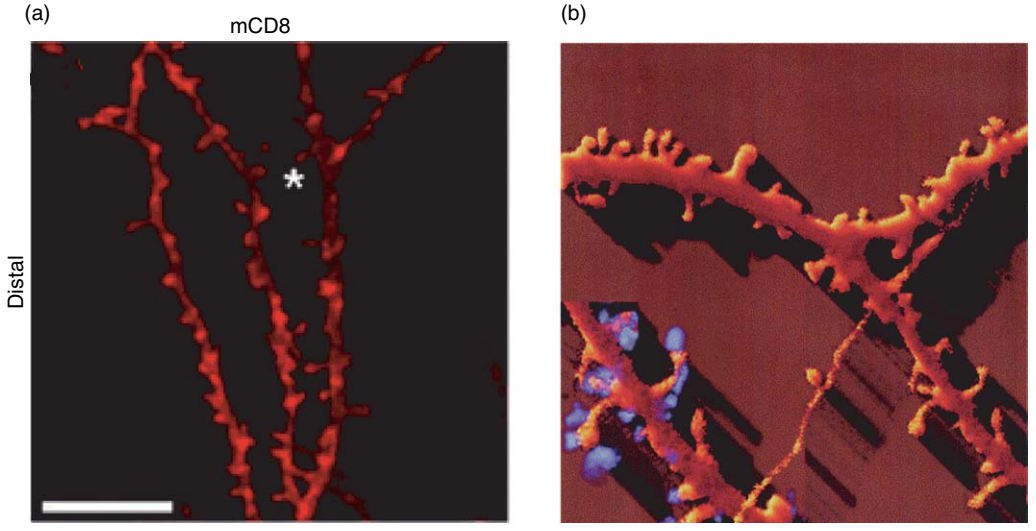


Figure 25 Dendrites with spines in a hippocampal pyramidal neuron. This figure illustrates the presence and shapes of dendritic spines on pyramidal neurons in the hippocampus. The spines are the small mushroom-shaped lateral projections containing synaptic contacts. (a) Courtesy of Liqun Lou, Stanford University. (b) Courtesy of E. Korkotian, The Weizmann Institute.

dendritic shaft membrane potential. However, it is likely that electrical compartmentalization does occur in dendrites, but this is at the level of the various dendritic branches as well as a component contributed by their overall distance from the soma (Johnston and Wu, 1995). This introduces the fascinating possibility that local generation and restricted propagation of action potentials within a specific dendritic subregion might be used as a mechanism for generating dendritic branch-specific plasticity.

4.16.11 LTP Outside the Hippocampus

The abundance of literature dedicated to studying LTP in the hippocampus might lead a newcomer to the field to suppose that LTP is somehow restricted to these synapses. However, plasticity of synaptic function, including phenomena such as LTP and LTD, is the rule rather than the exception for most forebrain synapses. LTP outside the hippocampus has been mostly studied in the cerebral cortex and the amygdala. The likely functional roles for LTP at these other sites are quite diverse, but two specific examples are worth highlighting. LTP-like processes in the cerebral cortex play a role in activity-dependent development of the visual system and other sensory systems. LTP in the amygdala has received prominent attention as a mechanism contributing to cued fear conditioning. The role of LTP in amygdala-dependent fear conditioning, in fact, is the area for which the strongest case can be made for a direct demonstration of a behavioral role for LTP. This will be discussed in more detail elsewhere (*See* Chapter 4.11). It is important to keep in mind through the rest of this volume that cortical LTP and amygdalar LTP probably exhibit some mechanistic differences from the NMDA receptor-dependent LTP that we will be focusing on. However, the molecular similarities are likely to greatly outweigh the differences (Schafe et al., 2000).

4.16.12 Modulation of LTP Induction

In one sense, the hippocampal slice is a denervated preparation. In the intact animal, the hippocampus receives numerous input fibers that provide modulatory inputs of the neurotransmitters DA, NE, 5HT, and ACh. Functionally these inputs are largely lost as a necessity of physically preparing the hippocampal slice for the experiment. However, these lost modulatory inputs can be partially reconstituted by directly applying the neurotransmitters (or more commonly pharmacologic substitutes) to the slice preparation *in vitro*. This approach has been used quite successfully to gain insights into the physiologic mechanisms and functional roles of these inputs in the intact brain.

NE, DA, and ACh-mimicking compounds can all modulate the induction of LTP at Schaffer collateral synapses. Specifically, agents acting at various subtypes of receptors for these compounds can increase the likelihood of LTP induction and the magnitude of LTP that is induced. Several examples of this type of modulation experiment are shown in **Figure 26**. In one example (panel A), 5-Hz stimulation of Schaffer collateral synapses, for 3 min, gives essentially no potentiation. Coapplication of isoproterenol, a beta-adrenergic receptor agonist that mimics endogenous NE, converts a nonpotentiating signal into a potentiating one (Thomas et al., 1996). Under other conditions beta-adrenergic agonists can augment the *magnitude* of LTP induced as well, if different physiologic stimulation protocols are used that evoke modest LTP. Similar types of effects can be observed for activation of various subtypes of receptors for ACh and DA (see Yuan et al., 2002).

One known site of action of neuromodulators is regulation of back-propagating action potentials in pyramidal neuron dendrites. All of these agents, which modulate LTP induction, can modulate the magnitude of back-propagating action potentials (see **Figure 26(b)**). The augmentation of back-propagating action potentials is a means by which these neurotransmitters can enhance membrane depolarization and thereby enhance NMDA receptor opening.

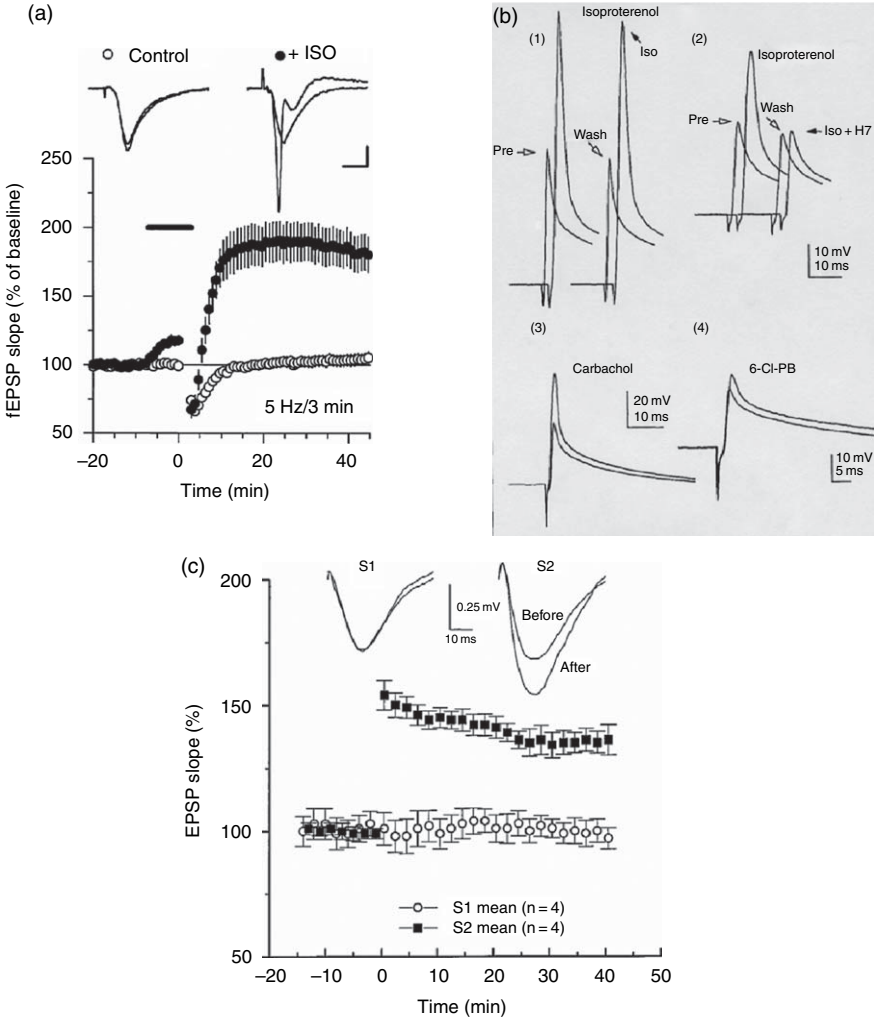
The growth factor BDNF (brain-derived neurotrophic factor) can also modulate the induction of LTP by a number of mechanisms, at least one of which is presynaptic (Gottschalk et al., 1998; Lu and Chow, 1999; Xu et al., 2000; **Figure 26(c)**). BDNF, acting through its cell-surface receptor TrkB, acts on presynaptic terminals to selectively facilitate neurotransmitter release during high-frequency stimulation. This is an interesting example of modulation of LTP induction that is activity dependent but localized to the presynaptic compartment. The mechanisms controlling the levels of BDNF in the adult hippocampus are not entirely clear at this point, but it is fairly well established that hippocampal BDNF levels can be regulated by a variety of neuronal activity-dependent processes and indeed in response to environmental signals impinging on the behaving animal.

4.16.13 Depotentiation and LTD

If synapses can be potentiated and this potentiation is very long-lasting, over time the synapses will be driven to their maximum synaptic strength. In this condition, there is no longer synaptic plasticity and no further capacity for that synapse to participate in synaptic-plasticity-dependent processes. Worse yet, over the lifetime of an animal, synapses will by random chance experience LTP-inducing conditions (presynaptic activity coincident with a postsynaptic action potential, for example) numerous times. If LTP is irreversible, ultimately every synapse will be maximally potentiated – obviously not a desirable condition vis-à-vis memory storage.

Consideration of this conundrum raises two implications. First, synapses that are involved in lifelong memory storage must be rendered essentially aplastic. In order to have good fidelity of memory storage over the lifetime of an animal, a synapse involved in permanent memory storage must be rendered immutable to a change in synaptic strength due to the random occurrence of what would normally be LTP-inducing stimulation.

But what about synapses like those in the hippocampus that are not sites of memory storage, but rather whose plasticity is part of the active processing of forming new long-term memories? To retain their plasticity and hence their capacity to contribute to memory formation, their potentiation must be reversible. Schaffer collateral synapses can undergo activity-dependent reversal of LTP, a phenomenon termed depotentiation (see **Figure 27**). Another activity-dependent way to decrease synaptic strength



is LTD, the mirror image of LTP. LTD is a long-lasting decrease of synaptic strength below baseline. Using a logic similar to that of the first paragraph of this section, the phenomenon of dedepression of synaptic transmission is implied, although this has not been widely studied at this point.

As a practical matter it is often difficult to separate depotentiation from LTD experimentally. For example, a 'baseline' response in hippocampal slices or *in vivo* likely is a mixture of basal synaptic activity and activity at previously potentiated synapses. Moreover, for the most part the stimulation protocols used to induce depotentiation are variations of the protocols used to induce LTD. Nevertheless, mechanistic investigations have made clear that depotentiation and LTD use different mechanisms (Lee et al., 1998; Lee et al., 2000) and thus must be considered as distinct processes. This is addressed elsewhere in this volume (*See* Chapter 4.31).

Physiologic LTD (and depotentiation) induction protocols generally involve variations of repetitive 1-Hz stimulation (Lee et al., 1998; Kemp et al., 2000). A common protocol is to deliver 900 stimuli at 1 Hz, but there also are LTD protocols that use random small variations in frequency in the 1-Hz region and variations that use paired-pulse stimuli delivered at 1 Hz. Synaptic depression appears to be fairly robust *in vivo*, but is quite difficult to induce in hippocampal slices from adult animals. LTD *in vitro* is almost always studied using slices from immature animals, or cultured immature neurons, and it is possible that LTD as it is currently studied *in vitro* is largely a manifestation of what is normally a developmental mechanism.

One ironic aspect of the LTP/LTD story is that both phenomena at Schaffer collateral synapses can be blocked by NMDA receptor antagonists. This suggests that calcium influx triggers both processes, and indeed current models of LTD induction hypothesize that

Figure 26 Neuromodulation of LTP induction. (a) Modulation of LTP induction by the beta-adrenergic agonist isoproterenol (ISO). Activity-dependent β-adrenergic modulation of low-frequency stimulation-induced LTP in the hippocampus CA1 region. In control experiments (no ISO), 3 min of 5-Hz stimulation (delivered at time = 0, open symbols, n = 26) had no lasting effect on synaptic transmission (45 min after 5-Hz stimulation, field EPSPs (fEPSPs) were not significantly different from pre–5-Hz baseline, $t(25) = 1.01$). However, 3 min of 5-Hz stimulation delivered at the end of a 10-min application of 1.0 mmol l^{-1} ISO (indicated by the bar) induced LTP (closed symbols, $p < 0.01$ compared with baseline). The traces are fEPSPs recorded during baseline and 45 min after 5-Hz stimulation in the presence and absence (control) of ISO. Calibration bars are 2.0 mV and 5.0 ms. Reproduced from Thomas MJ, Moody TD, Makhinson M, and O'Dell TJ (1996) Activity-dependent beta-adrenergic modulation of low frequency stimulation induced LTP in the hippocampal CA1 region. *Neuron* 17: 475–482, with permission. (b) One potential mechanism for neuromodulation is regulation of back-propagating action potentials in CA1 dendrites. The data shown illustrate amplification of dendritic action potentials by isoproterenol (1) and its susceptibility to inhibition by the protein kinase inhibitor H7 (2). The traces shown are from dendritic patch-clamp recordings from hippocampal pyramidal neurons. Muscarinic agonist (carbachol, (3)) and the dopamine receptor agonist 6-Cl-PB also can give various degrees of action-potential modulation as well. (1) Bath application of 1 μmol l^{-1} isoproterenol resulted in a 104% increase in amplitude, from 41 mV ('Pre') to 84 mV, of an antidromically initiated action potential recorded 220 μm from the soma. Wash-out of isoproterenol amplitude (38 mV; 'wash'). With a second application of isoproterenol (dark arrow labeled 'Iso'), the amplitude again increased twofold to 80 mV. (2) In a different recording 300 μmol l^{-1} H-7, a generic kinase inhibitor, was included in the control sine during the wash-out of isoproterenol. The subsequent second application of isoproterenol failed to lead to a second increase in amplitude (dark arrow labeled 'Iso + H7'). (3) In a distal recording (300 μM), 1 μmol l^{-1} carbachol increased the action potential amplitude by 81%, from 27–60 mV. In the carbachol experiments, cells were held hyperpolarized to –80 mV to remove Na+ channel inactivation. (4) One of the 6 out of 10 recordings where 6-Cl-PB led to an increase in amplitude. In a recording 220 μm from the soma, 10 μmol l^{-1} 6-Cl-PB increased dendritic action potential amplitude by 26%, from 21 to 26.5 mV. The cells were held at –70 mV in all 6-Cl-PB experiments. Adapted from Johnston D, Hoffman DA, Colbert CM, and Magee JC (1999) Regulation of back-propagating action potentials in hippocampal neurons. *Curr. Opin. Neurobiol.* 9: 288–292, with permission. (c) BDNF also modulates LTP induction in response to theta-frequency type stimulation. Two stimulating electrodes were positioned on either side of a single recording electrode to stimulate two different groups of afferents converging in the same dendritic field in CA1. Stimulation was applied to Schaffer collaterals alternately at low frequency (1 per min). After a period of baseline recording, LTP was induced with a theta-burst stimulation applied at time 0 only to one pathway (S1, filled squares). Simultaneous recording of an independent pathway (S2, open circles) showed no change in its synaptic strength after the theta burst was delivered to S1. BDNF (closed squares) selectively facilitates the induction of LTP in the tetanized pathway without affecting the synaptic efficacy of the untetanized pathway. EPSPs were recorded in the CA1 area of BDNF-treated slices. Synaptic efficacy (initial slope of field EPSPs) is expressed as a percentage of baseline value recorded during the 20 min before the tetanus. Representative traces of field EPSPs from S1 and S2 pathways were taken 10 min before and 40 min after the theta-burst stimulation. Adapted from Gottschalk W, Pozzo-Miller LD, Figurov A, and Lu B (1998) Presynaptic modulation of synaptic transmission and plasticity by brain-derived neurotrophic factor in the developing hippocampus. *J. Neurosci.* 18: 6830–6839, with permission.

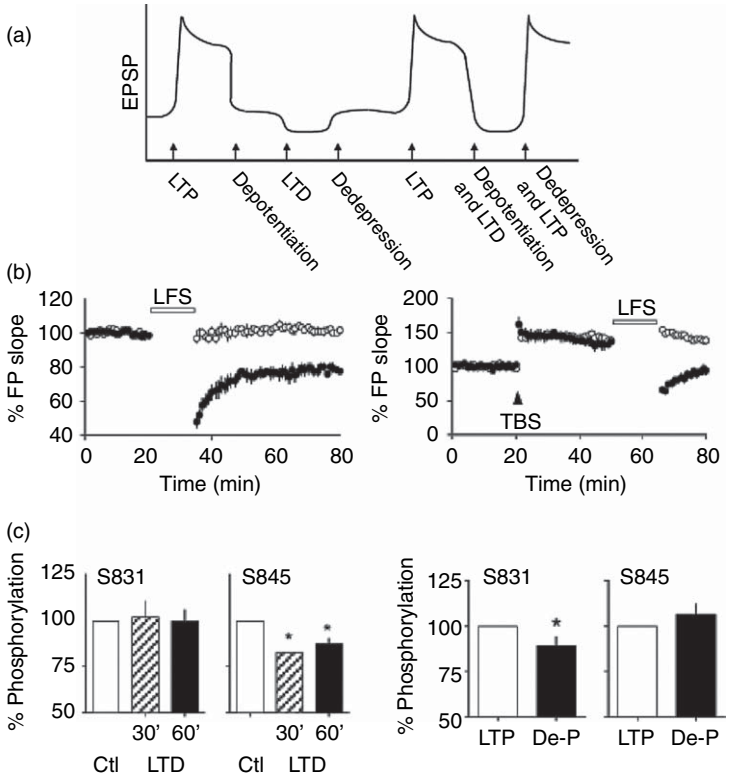


Figure 27 Depotentiation and LTD. (a) Schematic illustrating LTP, depotentiation, LTD, dedepression, and combinations of them. Figure by J. David Sweatt and Sarah E. Brown. (b) LTD and depotentiation in hippocampal neurons. Simultaneous recording of slices receiving baseline stimulation (control, open circles) and 1-Hz stimulation (closed circles). FP, Field potential. Regulation of distinct AMPA receptor phosphorylation sites during bidirectional synaptic plasticity. (c) Homosynaptic LTD in CA1 is associated with dephosphorylation of GluR1 at a PKA site (ser845). Depotentiation gives dephosphorylation at a CaMKII/PKC site (ser831). Adapted from Lee HK, Barbarosie M, Kameyama K, Bear MF, and Huganir RL (2000) Regulation of distinct AMPA receptor phosphorylation sites during bidirectional synaptic plasticity. *Nature* 405: 955–959, with permission from Elsevier.

LTD is caused by an influx of calcium that achieves a lower level than that needed for LTP induction. This lower level of calcium is hypothesized to selectively activate protein phosphatases and, by this mechanism, to lower synaptic efficacy.

Another very different type of LTD is cerebellar LTD. Cerebellar LTD occurs at synapses onto Purkinje neurons in the cerebellar cortex. Cerebellar LTD is a very interesting phenomenon because its behavioral role is much better understood than the hippocampal plasticity phenomena we are discussing throughout this book. Among other things, cerebellar LTD is involved in associative eye-blink conditioning, a cerebellum-dependent classical conditioning paradigm. Considerable progress has been made in investigating the roles and mechanisms of cerebellar LTD, as will be described in another chapter (*See* Chapter 4.17).

4.16.14 Summary

Like learning, LTP can be defined as a long-lasting change in output in response to a transient input. The persistence of this effect has been demonstrated to extend many hours *in vitro* and several weeks *in vivo*. We do not know how LTP relates to memory, and there is evidence for and against the hypothesis that hippocampal LTP is involved in memory. Regardless, it is the best-understood example of long-lasting synaptic plasticity in the mammalian CNS, and it is a model for how long-lasting memory-associated changes are likely to occur in the CNS. One premise of many chapters of this volume is that understanding LTP will yield valid insights into the mechanisms of plasticity that underlie learning and memory in the brain. The bona fide changes in neuronal connections that occur *in vivo* may or may not be identical to LTP as

it is presently studied in the laboratory, but this does not diminish its utility as a cellular model system for studying lasting neuronal change in the mammalian CNS.

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4.17 LTD – Synaptic Depression and Memory Storage

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4.17.1 Introduction

A widely held assumption among neuroscientists is that experience is capable of persistently modifying the properties of synapses, and that this use-dependent modification is central to both neuronal memory storage and the refinement of connections in brain development. This general idea was initially voiced by Sechenov and Cajal and was later formalized by Hebb (1949) in his famous synaptic modification postulate:

> When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased. (Hebb, 1949)

However, it was not until many years later that an electrophysiological model system emerged that appeared to embody this idea of activity-dependent synaptic memories in the mammalian brain. Bliss and Lomo (1973) showed that brief, high-frequency

stimulation of a population of axons, the perforant path projection to the hippocampal dentate gyrus, produced an increase in the strength of these synapses which could last for hours. This phenomenon, called long-term potentiation (LTP), has since been seen to last for days to weeks in chronic preparations. The duration of LTP, together with its initial discovery in the hippocampus, a brain region known from behavioral studies to be important for the storage of declarative memory, produced a surge of interest in LTP as a putative cellular model system for memory. This interest was only increased when it became clear that under certain conditions LTP could display some of the formal properties of learning such as specificity (LTP is confined to activated synapses) and associativity (weak stimulation of an input to a postsynaptic cell will only induce LTP when paired with a neighboring strong input to that same cell).

While the first studies of LTP relied upon field potential recording in the intact hippocampus, this phenomenon has subsequently been observed in almost every type of glutamatergic synapse in the brain and has been extensively studied in reduced preparations such as brain slices and cultures of embryonic neurons. At the same time that LTP was gradually 'escaping' from the hippocampus, it was becoming clear that it was not the only form of use-dependent synaptic modification. The converse phenomenon, long-term depression (LTD) was also initially observed in the hippocampus before being found in other brain regions. At present, it appears likely that there are no synapses that express only LTP or LTD. In most synapses, LTP and LTD are typically evoked by brief, strong stimulation and sustained, weak stimulation, respectively. The direction of change in synaptic strength (LTP vs. LTD) is believed to be determined, at most types of synapses, by the amount of postsynaptic activity (as indexed by Ca influx) which occurs during induction: a small amount of postsynaptic Ca influx results in LTD, while a larger amount results in LTP (see Linden, 1999; Zucker, 1999, for review).

If LTP in the mature brain truly functions to underlie memory storage, then what is the function of LTD? One proposal has been that LTD is a "neuronal substrate of forgetting" (Tsumoto, 1993). While there is no definitive evidence to dispute this view, there is no definitive support for it either. A potentially more useful construct is to consider that information is likely to be stored in the brain, at least in part, as an array of synaptic weights. If these synapses are driven to their maximal or minimal strengths, then those elements of the array become limited in their ability to contribute to subsequent plasticity. Thus, neural circuits containing synapses that can actively both increase and decrease their strength are at a distinct computational advantage.

Experience-dependent refinement of connections during brain development can also potentially benefit from having both LTP and LTD mechanisms. Synapses which undergo strong, correlated activity can be strengthened and thereby retained, while synapses which have weak uncorrelated activity can be weakened and ultimately removed. Like memory storage, one could imagine that developmental refinement of connections could proceed using either LTD or LTP alone, but the presence of both allows for faster and more flexible change.

In this article we will not attempt to provide a comprehensive overview of LTD at the many synapses in the brain where it has been studied. Rather, we will focus on the two best-understood forms (LTD at the hippocampal Schaffer collateral/commissural-CA1 pyramidal cell synapse and LTD at the cerebellar parallel fiber–Purkinje cell synapse) as case studies to examine both the cellular processes which underlie LTD and its larger role in behavior and development.

4.17.2 LTD of the Hippocampal Schaffer Collateral-CA1 Synapse

The Schaffer collateral-CA1 synapse is widely used as a model synapse for the study of LTP and of synaptic plasticity in general. The hippocampus is not necessarily the brain area of choice when it comes to relating synaptic gain changes to their behavioral consequences, because it is many synapses from the sensory periphery. Nevertheless, the hippocampus does store certain types of information, and it is reasonable to expect that synaptic plasticity participates in this process (Riedel et al., 1999). Moreover, available evidence suggests that what has been learned about synaptic plasticity in the hippocampus is also widely applicable to synapses elsewhere in the brain. For example, it seems that synaptic plasticity at glutamatergic synapses onto some (but not all) neocortical pyramidal cells operates using similar rules for induction as its counterpart at CA1 hippocampal synapses.

Although the original discovery of LTP (in the dentate gyrus, by Terje Lømo) was accidental (the unexpected outcome of experiments designed to study synaptic responses during repetitive stimulation), it was quickly embraced as a potential synaptic

mechanism for memory (Bliss and Lomo, 1973). Excitement grew in the mid-1980s, when the properties of LTP in CA1 were shown to satisfy the requirements of Hebb's famous postulate that active synapses strengthen when their activity correlates specifically with a strong postsynaptic response (Wigstrom and Gustafsson, 1985; Kelso et al., 1986; Malinow and Miller, 1986; Sastry et al., 1986). However, theoreticians had concluded years before that 'Hebbian' synaptic modifications alone were not likely to be sufficient to account for memory storage; the efficient storage of information by synapses requires bidirectional synaptic modifications; i.e., LTD as well as LTP. Thus, the search for homosynaptic LTD in the hippocampus was theoretically motivated; it was not an accident (the reasons that it was not stumbled on accidentally will become clear in the discussion below). The theoretical suggestion was that synapses should depress when their activity *fails* to correlate with a strong postsynaptic response. To realize this situation experimentally, induction of LTD was attempted in CA1 using prolonged trains of presynaptic stimulation, delivered at frequencies (0.5–10 Hz) that fail to evoke a strong postsynaptic response (Dudek and Bear, 1992). Trains of low-frequency stimulation (LFS) are now the standard protocol for induction of homosynaptic LTD in CA1 and at synapses throughout the forebrain.

When discussing induction and expression mechanisms of LTD, it is important to note that LFS induces at least two, and possibly three (Berretta and Cherubini, 1998), mechanistically distinct forms of LTD in CA1, whose discovery solved previously existing contradictions (see Bear and Abraham, 1996). One form depends on the activation of *N*-methyl-D-aspartate receptors (NMDARs); another depends on the activation of group 1 metabotropic glutamate receptors (mGluRs), which are postsynaptic glutamate receptors coupled to phosphoinositide metabolism. These two forms will be described separately here.

4.17.3 Theoretical Framework

The bidirectional modification of excitatory synaptic transmission is not just an abstract theoretical construct. We need to understand mechanisms of bidirectional synaptic plasticity because direct experimental observations have shown, repeatedly, that synapses in the cerebral cortex are, in fact, bidirectionally modifiable. The value added by a theoretical structure is that it helps to make sense of what bidirectional synaptic plasticity accomplishes with respect to information storage and provides insight into how it might be implemented.

Neurons throughout the cerebral cortex, including area CA1 of the hippocampus, have stimulus-selective receptive fields. Chronic recording from cortical neurons has shown that as something new is learned, stimulus selectivity changes – some synaptic inputs potentiate and others depress. In CA1, for example, neurons show selectivity for positions in space, and this selectivity shifts rapidly as animals learn a new spatial environment (Breese et al., 1989; Wilson and McNaughton, 1993). What does a stable shift in selectivity tell us about memory? Neural network theory suggests that the selectivity shift reflects the creation of new neural representations. The memory is encoded by changing the pattern of synaptic weights across the network of neurons (Bear, 1996).

Now consider what happens when more new information is learned: stimulus selectivity (i.e., the pattern of synaptic weights) shifts further. An implication of this finding is that previously encoded memories can remain stable, even as the pattern of synaptic strengths is again modified to create new representations. According to this way of thinking, memory requires the episodic (if not continual) bidirectional modification of synaptic transmission to fine-tune the patterns of synaptic weights in the neural network. It is important to emphasize, of course, that in the absence of new learned information, synaptic weights must remain stable. Passive decay of synaptic weight (that is, back to an initial value that might be larger or smaller) leads to a loss of the stored representations.

The bidirectional modification of synaptic transmission obviously requires that individual synapses on neurons be capable of some form of LTP and some form of LTD. However, every theory of memory storage that assumes bidirectional synaptic modification places an important constraint on the mechanisms of LTP and LTD: reversibility. Consider the problem that would arise if the LTP and LTD mechanisms were distinct and irreversible. While it is true that synaptic weights could be fine-tuned initially by simple summation of the two independent processes, eventually saturation would occur as the synapses underwent rounds of bidirectional modification (see **Figure 6**). This problem does not occur if LTP and LTD are inverse processes mechanistically.

Now we come to the question of what distinguishes stimulation conditions that yield synaptic potentiation from those that yield synaptic depression. To specifically encode memory, synaptic modifications must depend on the presynaptic activation of the synapses bringing information into the network. In other words, the modifications must be 'homosynaptic.' The variables that determine the polarity or sign of the modification, in principle, could be the absolute amount of presynaptic activity, the concurrent level and timing of postsynaptic activity, or some combination of these variables. There are many abstract theoretical 'learning rules' based on these variables, but the most useful are those that attempt to account for what has actually been observed experimentally. One very influential proposal was made by Bienenstock, Cooper, and Munro (1982) in what is known as the BCM theory. In order to account for the development and plasticity of neuronal stimulus selectivity, they proposed that active synapses are potentiated when the total postsynaptic response exceeds a critical value, called the 'modification threshold' (qm), and that active synapses are depressed when the total postsynaptic response is greater than zero but less than qm. In addition, it was proposed that the value of qm varies as a function of the average integrated postsynaptic activity.

Once the requirements for LTP induction in CA1 had been elucidated, a specific physiological basis for the BCM theory became apparent. The proposal was made (1) that the term qm corresponds to the critical level of postsynaptic depolarization at which the Ca flux through the NMDAR exceeds the threshold for inducing LTP; (2) that LTD should be a consequence of presynaptic activity that consistently fails to evoke a postsynaptic Ca response large enough to induce LTP; and (3) that the postsynaptic threshold for LTP should vary depending on the stimulation history of the postsynaptic neuron (Bear et al., 1987). These hypotheses have now all been validated experimentally.

The BCM theory motivated the search for LTD using LFS (Dudek and Bear, 1992). The rationale was to provide a high level of presynaptic activity that did not evoke a large postsynaptic response. Critical variables for LTD induction in rat hippocampal slices proved to be the stimulation strength (it could not be so strong as to elicit orthodromic action potentials), healthy inhibition, stimulation frequency (<10 Hz), the number of stimuli (typically hundreds), and the age of the animal (greater magnitude before 35 days of age). LTD was found to be a very reliable phenomenon when the appropriate conditions were met. Even so, there was concern that LTD might be an artifact. First, there was heightened skepticism because several previous reports of LTD induction using different protocols had proven difficult to replicate in the hippocampus; second, the same LFS protocol that was found to induce LTD had previously been reported by others to be ineffective in altering baseline synaptic transmission; and third, synaptic depression could easily be dismissed as a pathological change rather than a form of synaptic plasticity. Most of these concerns faded when it was found that at least one form of LTD depended specifically upon activation of NMDARs and a rise in postsynaptic Ca, that the same synapses that showed LTD could subsequently be potentiated, and that LTD could be elicited *in vivo* (Dudek and Bear, 1992, 1993; Mulkey and Malenka, 1992; Thiels et al., 1994; Heynen et al., 1996; Debanne et al., 1997; Manahan-Vaughan, 1997).

It is now apparent that multiple forms of LTD exist in CA1, possibly at the same synapses. Remarkably, however, all forms of LTD can be elicited using variations of the LFS protocol, specifically under conditions that fail to evoke a large postsynaptic response. Thus, the hypothesis of homosynaptic LTD, inspired by the BCM theory, has been amply confirmed. As we will discuss further, it is also noteworthy that the NMDAR-dependent form of LTD appears to be the functional inverse of LTP, thus satisfying the theoretical requirement that synaptic modifications be both bidirectional and reversible. Thus, at least in theory, the mechanisms of LTD as well as LTP can contribute to the receptive field plasticity underlying memory storage in the hippocampus and elsewhere.

4.17.4 NMDAR-Dependent LTD

4.17.4.1 Induction by Calcium

Induction of homosynaptic LTD in CA1 using the standard 1-Hz LFS protocol *in vivo*, and under most experimental conditions *in vitro*, is blocked by NMDAR antagonists (Dudek and Bear, 1992; Mulkey and Malenka, 1992; Heynen et al., 1996; Manahan-Vaughan, 1997). Although this form of LTD shares with LTP a dependence upon NMDA receptor activation and a rise in postsynaptic Ca ion concentration, there is a systematic difference in the

type of stimulation that yields the two types of synaptic modification. This difference is easily demonstrated simply by varying the frequency of tetanic stimulation. In rat CA1, for example, 900 pulses at 0.5–3 Hz typically yields LTD, whereas the same amount of stimulation at frequencies greater than 10 Hz yields LTP (**Figure 1(a)**; Dudek and Bear, 1992). The different consequences of stimulation at different frequencies have been attributed to systematic differences in the postsynaptic Ca currents through the postsynaptic NMDA receptors. Indeed, it is now well established that the critical variables are postsynaptic depolarization and Ca entry, not stimulation frequency *per se*. For example, while 1-Hz stimulation normally produces LTD, postsynaptic hyperpolarization during conditioning prevents any change, and depolarization leads to induction of LTP (Mulkey and Malenka, 1992). Likewise, while high-frequency stimulation normally produces LTP, it produces LTD instead if delivered in the presence of subsaturating concentrations of an NMDA receptor antagonist (Cummings et al., 1996; **Figure 1(b)**, **(c)**).

The appropriate activation of postsynaptic NMDARs appears to be sufficient to induce LTD; presynaptic activity is not necessary. This conclusion is supported by the observation that photolysis of caged extracellular glutamate (Kandler et al., 1998; Dodt et al., 1999) and brief bath application of NMDA (Lee et al., 1998; Kamal et al., 1999) induce LTD without concurrent presynaptic stimulation. In agreement with the idea that Ca passing through NMDARs is the trigger for LTD, photolysis of caged Ca in the postsynaptic neuron can also induce synaptic depression (Neveu and Zucker, 1996). This finding is important, as it indicates that LTD can be induced by Ca entry through the NMDAR without the need to invoke any other Ca-independent signaling or triggering process. Curiously, however, a modest, brief elevation in Ca concentration ([Ca]) was found to induce LTP and LTD with equal probability. Subsequent analysis suggests that LTD is most reliably induced (and LTP is never induced) by a modest ($\sim$0.7 µmol l^{-1}) but prolonged ($\sim$60 s) rise in [Ca]. LTP, in contrast, is most reliably induced by a large ($\sim$10 µmol l^{-1}) and brief ($\sim$3 s) increase in [Ca] (Yang et al., 1999). Although these findings are all consistent with the proposal that LTD and LTP are triggered by distinct Ca responses during NMDAR activation (Bear et al., 1987; Lisman, 1989; Artola and Singer, 1993; Bear and Malenka, 1994) and with imaging studies that confirmed

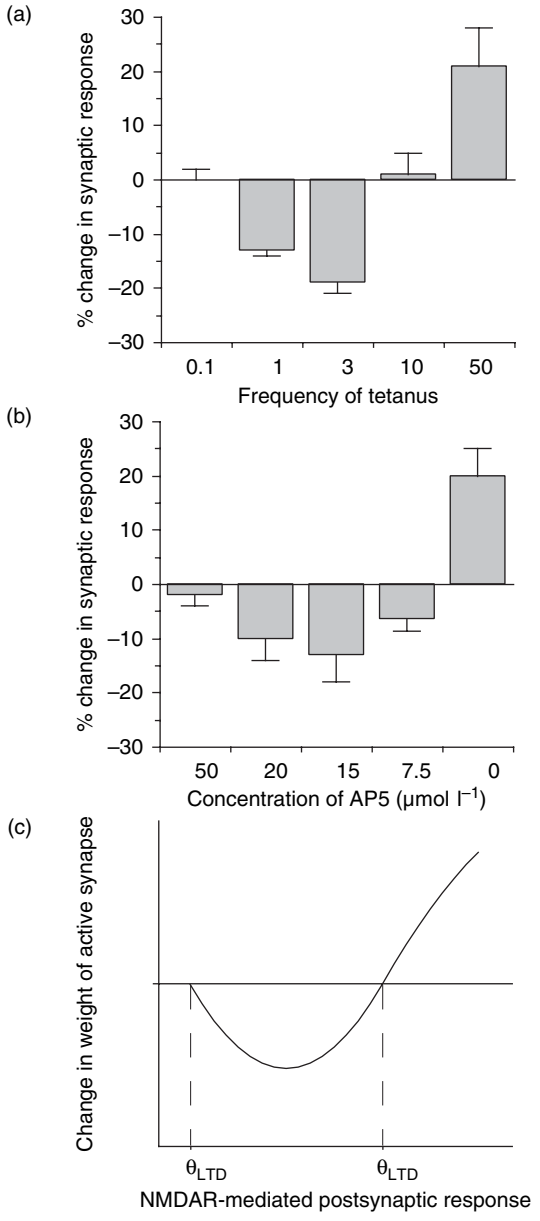


Figure 1 Induction of NMDAR-dependent bidirectional plasticity of the Schaffer collateral synapse in CA1. (a) Summary of the effects of a 900-pulse tetanus delivered at different frequencies (replotted from Dudek SM and Bear MF (1992) Homosynaptic long-term depression in area CA1 of hippocampus and effects of NMDA receptor blockade. *Proc. Natl. Acad. Sci. USA* 89: 4363–4367, with permission from the National Academy of Sciences, USA). (b) Summary of the effects of a 600-pulse, 20-Hz tetanus in different concentrations of the NMDA receptor antagonist AP5 (replotted from Cummings JA, Mulkey RM, Nicoll RA, and Malenka RC (1996) Ca signalling requirements for long-term depression in the hippocampus. *Neuron* 16: 825–833, with permission from Elsevier). (c) A synaptic 'learning rule' based on data such as those given in (a) and (b). This learning rule is formally similar to that proposed in the BCM theory.

that LTP-inducing stimuli elicit larger Ca transients than LTD-inducing stimuli (Hansel et al., 1997; Cormier et al., 2001), they argue against a simple relationship between synaptic modification and Ca level. The dynamics of the Ca response are also important determinants of the polarity of synaptic modification.

Perhaps not surprisingly, under certain circumstances voltage-gated Ca channels may also contribute to the Ca signal that triggers LTD (Christie et al., 1996). However, this role appears to be supplementary, since NMDAR activation is still required to observe homosynaptic LTD under these conditions, and Ca channel activation is not always necessary (Selig et al., 1995b). Nonetheless, the modulation of synaptic plasticity by active dendritic Ca conductances can be striking. Markram et al. (1997) made the remarkable observation that a back-propagating dendritic action potential, precisely timed to occur a few milliseconds before a synaptically evoked excitatory postsynaptic potential (EPSP), could promote induction of LTD in neocortical pyramidal neurons by stimuli that otherwise are ineffective. Similar findings have been reported in hippocampal cultures (Bi and Poo, 1998). The relative timing of coincident pre- and postsynaptic activity determines the amplitude of Ca transients in dendritic spines: Ca transients are larger when an EPSP precedes an action potential (AP) than when it follows it (Koester and Sakmann, 1998). As the former condition favors LTP induction (Markram et al., 1997), these results demonstrate that, under physiological conditions, larger Ca signals are indeed associated with LTP induction, whereas lower Ca signals more likely result in the induction of LTD.

An appealing hypothesis for this observation is that voltage-gated Ca influx inactivates NMDARs, thus causing a relatively lower Ca transient upon subsequent synaptic activation (Linden, 1999; Zucker, 1999). From a computational perspective, the observation that LTP results from EPSPs followed by APs, whereas LTD results from activation in the reverse order, provides a perfect correlate of Hebbian (and anti-Hebbian) concepts. APs following EPSPs suggest that the activated input repetitively and successfully contributed sufficient depolarization so that the spike threshold was reached, which is a Hebbian requirement for strengthening of that synapse. On the other hand, EPSPs following APs can be interpreted as random, uncorrelated presynaptic activity, leading to weakening of those synaptic inputs.

To summarize what has been learned to date, LTD is induced by an elevation of Ca that is constrained, apparently, by three variables: (1) proximity to the postsynaptic membrane, (2) peak concentration, and (3) duration. Synaptic stimulation causes LTD when it yields the appropriate response within the 'box' defined by these parameters. While LFS at 1 Hz is usually effective, under different experimental conditions different protocols may be required (e.g., Thiels et al., 1994; Debanne et al., 1994). Ca entry through the NMDA receptor is sufficient to induce LTD, but this can also be supplemented by other Ca sources (Christie et al., 1996; Reyes and Stanton, 1996).

4.17.5 The Role of Calcium-Dependent Enzymatic Reactions

A key component in the theory of synaptic memory formation is that synaptic efficacy is controlled by the phosphorylation state of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs) which can mediate biophysical changes at the individual receptor level and/or can modify the insertion/internalization balance of AMPARs (for review see Song and Huganir, 2002). Induction of LTP requires activation of Ca-dependent serine-threonine protein kinases in the postsynaptic neuron. Key molecules in hippocampal LTP are Ca/calmodulin-dependent protein kinase II (CaMKII; Malenka et al., 1989; Malinow et al., 1989; Silva et al., 1992; Pettit et al., 1994; Lledo et al., 1995) and protein kinase C (PKC), whose ζ isoform is upregulated during LTP maintenance (Hrabetova and Sacktor, 1996). Lisman (1989) proposed that LTD might result from activation of a protein phosphatase cascade, leading to dephosphorylation of the same synaptic proteins that are involved in LTP. Subsequent experiments by Mulkey and coworkers confirmed an essential role for postsynaptic protein phosphatase 1 (PP1) and calcineurin (PP2B) in the induction of LTD with LFS (Mulkey et al., 1993, 1994).

The basic working hypothesis continues to be that LTD results from dephosphorylation of postsynaptic PP1 substrates and that CaMKII and PP1 act as a kinase/phosphatase switch, regulating the phosphorylation state of AMPARs and their auxiliary proteins (Lisman and Zhabotinsky, 2001; Malleret et al., 2001). As will be discussed, there is now direct evidence for dephosphorylation of synaptic proteins following

LTD induction protocols (Lee et al., 1998, 2000, 2003; Ramakers et al., 1999). Moreover, there is evidence that PP1 activity is persistently increased by LTD-inducing stimulation (Thiels et al., 1998) and that peptides that inhibit PP1 binding to target proteins block LTD induction (Morishita et al., 2001). PP1 is regulated by the protein inhibitor-1 (I-1). When I-1 is phosphorylated by protein kinase A (PKA), PP1 is inactive. Dephosphorylation of I-1 by PP2B releases PP1 from inhibition (Cohen, 1989; Nairn and Shenolikar, 1992). PP2B is activated by Ca/calmodulin and, therefore, is believed to be key for translating an increase in [Ca] into LTD (**Figure 2**).

Before moving on to consider expression mechanisms, it should be noted that there are also data suggesting that Ca/calmodulin triggers LTD by activating nitric oxide synthase (Izumi and Zorumski, 1993; Gage et al., 1997). The proposed mechanism of nitric oxide action is the retrograde activation of a second messenger cascade in the presynaptic terminal involving soluble guanylyl cyclase and cyclic guanosine monophosphate (cGMP)-dependent protein kinase (Reyes et al., 1999). This scenario is not universally agreed upon, however, since others report no effect of nitric oxide inhibitors on LTD (Cummings et al., 1994). At the present time, it appears that this mechanism lies in parallel with

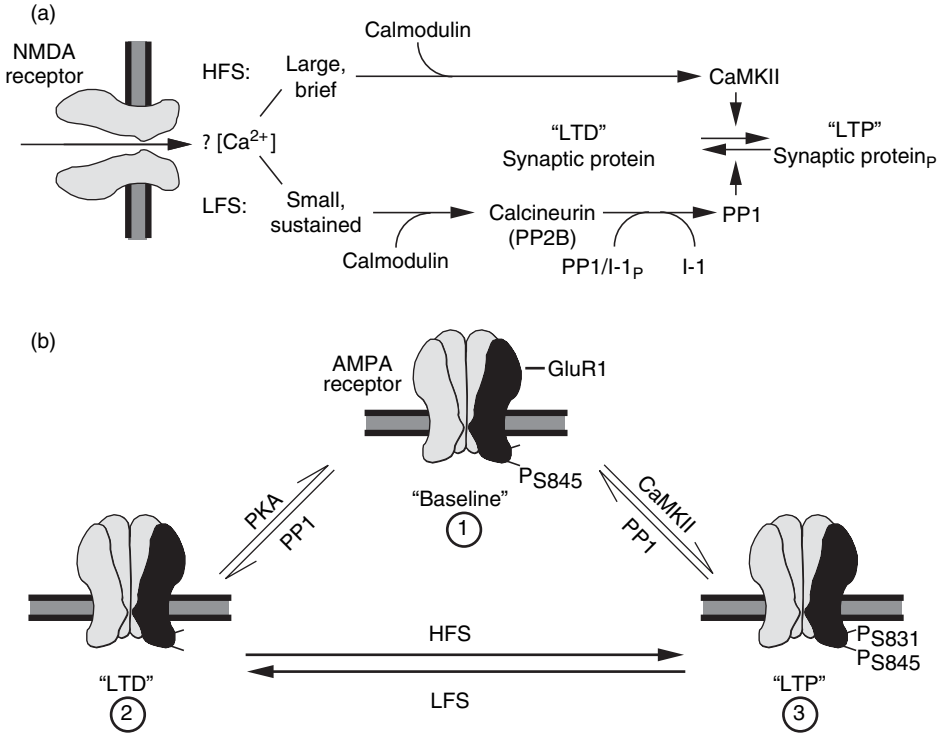


Figure 2 Molecular models for bidirectional plasticity of the Schaffer collateral synapse in CA1. (a) Induction. Strong NMDA receptor activation resulting from high-frequency stimulation (HFS) causes a large, brief rise in intracellular calcium. This calcium signal triggers activation of CaMKII and the induction of LTP by phosphorylation of synaptic proteins. Weak NMDA receptor activation resulting from low-frequency stimulation (LFS) produces a smaller but more sustained rise in intracellular calcium. This calcium signal selectively activates calcineurin which dephosphorylates inhibitor-1 (I-1). Dephosphorylation of I-1 relieves protein phosphatase 1 (PP1) from inhibition. LTD results from dephosphorylation of the same synaptic proteins involved in LTP. (b) Expression. (1) Under baseline conditions, the GluR1 subunit of AMPA receptors is highly phosphorylated at ser-845, a protein kinase A (PKA) substrate. (2) LFS causes dephosphorylation of ser-845 and LTD. (3) From the baseline state, HFS causes phosphorylation of ser-831, a CaMKII substrate, and LTP. According to this model, LTD and LTP result from bidirectional modifications of AMPA receptor phosphorylation, but at different sites. Thus, dedepression and LTP are not formally equivalent, nor are depotentiation and LTD. (4) The fourth possible state, in which ser-831 is phosphorylated and ser-845 is not, has not been observed experimentally. Adapted from Kameyama K, Lee HK, Bear MF, and Huganir RL (1998) Involvement of a postsynaptic protein kinase A substrate in the expression of homosynaptic long-term depression. *Neuron* 21: 1163–1175, with permission from Elsevier.

that involving postsynaptic phosphatase activation. It is plausible that nitric oxide signaling is involved in the mGluR-dependent form of LTD (discussed in the following section), but this remains to be examined explicitly.

4.17.5.1 Expression Mechanisms

It now seems very clear that a modification of postsynaptic glutamate sensitivity is a major expression mechanism for the NMDAR-dependent form of LTD. As mentioned earlier, liberating caged glutamate in CA1, under conditions where synaptic transmission is blocked, results in LTD of the glutatmate-evoked currents. This LTD is restricted spatially to the site of glutamate release and depends upon NMDAR activation and postsynaptic protein phosphatase activity (Kandler et al., 1998). The possibility remains that this form of LTD is actually expressed at extrasynaptic glutamate receptors and therefore could be mechanistically distinct from LFS-induced LTD. However, very similar findings have recently been obtained in rat neocortex where, in addition, it was shown that synaptically induced LTD results in a decrease in sensitivity to laser-stimulated photolysis of caged glutamate. Moreover, LFS-induced LTD occluded further synaptic depression by glutamate pulses (Dodt et al., 1999). The close similarities between CA1 and neocortical LTD (Kirkwood et al., 1993; Kirkwood and Bear, 1994) suggest that these findings may apply generally to NMDAR-dependent LTD in the cerebral cortex, at least in some layers.

A very interesting picture has emerged recently to account for decreased glutamate sensitivity following LTD. The model of bidirectional synaptic modification through reversible changes in the phosphorylation of postsynaptic substrates (Lisman, 1989; Bear and Malenka, 1994) begged the question of which synaptic phosphoproteins are involved. Now there is direct evidence that LTD is associated with dephosphorylation of AMPAR subunits, the consequence of which is known to be depression of glutamate-evoked currents. In addition, there are converging lines of evidence that LTD is associated with the removal of glutamate receptors from the postsynaptic membrane (for review see Song and Huganir, 2002; Collingridge et al., 2004). We shall discuss each of these mechanisms, in turn.

AMPARs are heteromeric complexes assembled from four homologous subunits (GluR1–4) in various combinations (Seeburg, 1993; Hollmann and Heinemann, 1994). The large majority of AMPARs in the hippocampus contain both GluR1 and GluR2 subunits (Wenthold et al., 1996). Hippocampal LTP largely rests on modifications of the GluR1 subunits, which determine trafficking behavior in GluR1/GluR2 heteromers (Song and Huganir, 2002). The GluR1 subunit is highly regulated by protein phosphorylation and contains identified phosphorylation sites on the intracellular carboxy-terminal domain. Serine-831 is phosphorylated by CaMKII and PKC, while serine-845 is phosphorylated by PKA (Roche et al., 1996; Barria et al., 1997a). Phosphorylation of either of these sites has been shown to potentiate AMPAR function through distinct biophysical mechanisms (Derkach et al., 1999). Phosphorylation site-specific antibodies have been used to measure the phosphorylation state of receptors *in situ* (Mammen et al., 1997). The PKA site shows higher basal phosphorylation than the CaMKII/PKC site (Lee et al., 1998).

To investigate the changes in AMPAR phosphorylation that occur following synaptic plasticity, Lee et al. (1998) devised a method to induce LTD chemically in hippocampus slices. The rationale behind this approach was to increase the probability of detecting biochemical changes by maximizing the number of affected synapses in the slice. They showed that brief bath application of NMDA induces synaptic depression (called chem-LTD) that shares a common expression mechanism with LFS-induced LTD. Biochemical analysis showed a selective dephosphorylation of serine-845 (the PKA site) following induction of chem-LTD. Phosphorylation of serine-831 (the CaMKII/PKC site), in contrast, was not altered by the treatment. This result was unexpected, since serine-831 is the site phosphorylated during LTP (Barria et al., 1997b). Thus, these findings contradict the simple notion that LTD and LTP reflect bidirectional changes in the phosphorylation of the same site. By refining the biochemical detection method, these findings have now been confirmed using synaptically induced LTD and LTP (Lee et al., 2000). LFS delivered to naive synapses causes dephosphorylation of the PKA site and LTD. Conversely, theta-burst stimulation (TBS) delivered to naive synapses causes phosphorylation of the CaMKII/PKC site and LTP. Both types of synaptic change are reversible, however. Thus, LFS delivered after prior induction of LTP causes dephosphorylation of the CaMKII/PKC site and depotentiation of the synaptic response. TBS delivered after prior induction of LTD causes phosphorylation of the

PKA site and de-depression of the synaptic response (**Figure 2**; Kameyama et al., 1998). This PKA-mediated recovery from depression is associated with AMPA receptor reinsertion (Ehlers, 2000).

Despite the progress that has been made in characterizing the phosphorylation events underlyling LTP and LTD induction, some important aspects still remain unsolved. While the CaMKII/PKC phosphorylation site serine-831 is phosphorylated during LTP (Lee et al., 2000) and this phosphorylation event leads to an increase in receptor conductance (Derkach et al., 1999), serine-831 phosphorylation does not affect receptor trafficking (Hayashi et al., 2000). A current hypothesis is that synaptic targeting of GluR1/GluR2 receptors ultimately requires CaMKII-mediated phosphorylation of a family of small transmembrane AMPAR regulatory proteins (TARPs), such as stargazin (for review see Nicoll et al., 2006). Phosphorylation at the PKA site serine-845 seems to act as a 'priming' step for GluR1 membrane insertion (Esteban et al., 2003). As described above, dephosphorylation at serine-845 occurs during LTD (Lee et al., 2000), and indeed promotes internalization of AMPARs (Lee et al., 2003). PKC also plays a role in the delivery of GluR1 subunits to synapses. In a recent study, it was demonstrated that a PKC (all isoforms)-mediated phosphorylation at serine-818 is required for GluR1 insertion and LTP (Boehm et al., 2006). Moreover, it has been shown that the constitutively active PKC isoform PKMζ promotes AMPAR membrane insertion and might be crucial for maintaining increased receptor numbers (Ling et al., 2006). Taken together, the available data suggest that all three GluR1 phosphorylation sites discussed here, namely serine-818, -831, and -845, are involved in the induction of LTP, which likely sets constraints for LTP-inducing stimuli, but also opens up routes to modify the probability for GluR1 insertion and LTP.

While the available evidence for the role of GluR1 insertion in LTP is compelling and the remaining questions mostly focus on the underlying phosphorylation steps, the events underlying hippocampal LTD induction are less clear. As pointed out earlier, it has been shown that dephosphorylation of the GluR1 subunit at serine-845 is involved in LTD and GluR1 endocytosis. In mutant mice, in which phosphorylation at serine-831 and -845 is prevented by mutations to alanine ('phospho-free mice') NMDAR-dependent LTD is blocked (Lee et al., 2003). This finding is consistent with earlier reports demonstrating that inhibition of PP2B blocks GluR1 internalization, which has been interpreted as showing that phosphatase activity is required for GluR1 endocytosis and LTD (Beattie et al., 2000). However, a recently promoted hypothesis gains weight, which states that LTP is mediated by membrane delivery of AMPA receptors with long cytoplasmic termini (i.e., GluR1-, GluR2L-, GluR4-containing AMPARs), whereas LTD is mediated by the endocytosis of AMPA receptors with short cytoplasmic termini (GluR2-containing AMPARs) (for review see Malinow, 2003). The refilling of receptor 'pools' required to maintain plasticity would then occur through a slow exchange of AMPARs (McCormack et al., 2006). Time will tell how these findings can be reconciled. However, there is indeed plenty of evidence showing that GluR2 subunits can cycle in and out of the membrane as well.

The carboxy terminus of GluR2 binds to N-ethylmaleimide-sensitive factor (NSF), a protein previously shown to play an essential role in membrane fusion events (Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998; Lüscher et al., 1999; Noel et al., 1999). NSF-GluR2 binding promotes GluR2 insertion at hippocampal synapses (Lüscher et al., 1999). Under physiological conditions, NSF is activated by NO-mediated S-nitrosylation, which enables NSF to bind to GluR2, thus promoting GluR2 surface expression (Huang et al., 2005). Conversely, GluR2 phosphorylation at serine-880 promotes a clathrin-mediated GluR2 endocytosis (Man et al., 2000), which leads to LTD (Seidenman et al., 2003). GluR2 endocytosis involves unbinding of GluR2 from the glutamate receptor-interacting protein GRIP1 (Dong et al., 1997) and binding to protein interacting with C-kinase 1 (PICK1) (Xia et al., 1999; Chung et al., 2000).

The pool of NSF-regulated AMPARs appears to be required for expression of LTD, because LFS has no effect after these receptors are internalized (Lüscher et al., 1999; Luthi et al., 1999). Three lines of evidence suggest that AMPAR internalization is an expression mechanism for LTD. First, in hippocampal slices, prior saturation of LTD renders the AMPARs at the depressed synapses (but not at other synapses on the same neuron) insensitive to inhibitors of the NSF-GluR2 interaction (Luthi et al., 1999). Second, in hippocampal cell culture, field stimulation at 5 Hz causes an NMDAR-dependent depression of spontaneous miniature excitatory postsynaptic current (EPSC) amplitudes and the loss of surface-expressed GluR1 (Carroll et al., 1999). Third, in the adult hippocampus *in vivo*, there is an NMDAR-dependent loss

of GluR1 and GluR2 from the synaptoneurosomal biochemical fraction following induction of LTD (Heynen et al., 2000).

In keeping with the evidence cited, the magnitude of the postsynaptic response to quantal release of glutamate (the quantal size) is decreased after LTD (Oliet et al., 1996). However, in addition, a robust finding is that the number of quantal responses to synaptic stimulation (the quantal content) is also decreased (Stevens and Wang, 1994; Goda and Stevens, 1996; Oliet et al., 1996; Carroll et al., 1999). According to traditional assumptions, decreased quantal content reflects the failure of neurotransmitter release in response to a presynaptic action potential. It is interesting, therefore, that a decrease in quantal content is also a consequence of disrupting the NSF-GluR2 interaction (Luthi et al., 1999). Presumably this results from the total loss of AMPARs from some synapses. Thus, although presynaptic changes may also occur following LFS (Ramakers et al., 1999), there is apparently no need to invoke a presynaptic mechanism to account for the key properties of NMDAR-dependent LTD of AMPAR-mediated responses.

Another robust finding is that responses to activation of NMDARs are also depressed following LTD (Xiao et al., 1994, 1995; Selig et al., 1995a). While these observations are consistent with the parallel loss of AMPA receptors and NMDA receptors from synaptoneurosomes following LTD *in vivo* (Heynen et al., 2000), they could also reflect a component of LTD expression that is presynaptic. The GluR internalization in response to intracellular manipulations of the NSF-GluR2 interaction (Luthi et al., 1999), or of clathrin-mediated endocytosis (Lüscher et al., 1999; Man et al., 2000; Wang and Linden, 2000), has been restricted to AMPARs only. Thus, the mechanism for NMDAR regulation is apparently distinct from that for AMPAR regulation.

4.17.6 Modulation of LTD

As discussed, induction of LTD depends on postsynaptic phosphatase activation by Ca passing through NMDAR channels. Thus, it comes as no surprise that LTD is subject to modulation by factors that alter the Ca flux in response to synaptic stimulation and by factors that alter the intracellular enzymatic response to a change in Ca concentration.

An example of the first type of modulation is the effect of altered inhibition. Under some experimental conditions, reduced inhibition may be required to allow the NMDAR activation that is necessary to induce LTD (Wagner and Alger, 1995). However, under other conditions, reduced inhibition may suppress LTD in response to LFS at certain frequencies by facilitating induction of LTP instead (Steele and Mauk, 1999). Similarly, the conditions required for LTD induction depend on the properties of postsynaptic NMDARs. For example, overexpression of the NR2B subunit, which leads to a prolongation of NMDAR-mediated synaptic currents, changes the frequency-response function to promote LTP and suppress LTD across a range of stimulation frequencies (Tang et al., 1999). Modulation of inhibition and NMDAR subunit composition are physiologically relevant, as these parameters change during development and are regulated by activity.

The intracellular response to a change in Ca depends on the availability of Ca binding proteins, such as calmodulin, and on the location, concentration, and activity of the kinases and phosphatases that regulate synaptic strength. Mutations that alter these parameters have been shown to enhance (Mayford et al., 1995) or disrupt (Brandon et al., 1995; Qi et al., 1996; Migaud et al., 1998) LTD. PKA seems to play a pivotal role in the intracellular regulation of LTD. According to the current model for LTD induction, activation of PKA would be expected to inhibit LTD by preventing the activation of PP1 (via I-1 phosphorylation) and by maintaining AMPAR phosphorylation at a high level. Regulation of LTD via PKA is also physiologically relevant. For example, there is evidence that PKA activation in response to stimulation of noradrenergic b-receptors shifts the frequency response function to favor LTP over LTD (Blitzer et al., 1995, 1998; Thomas et al., 1996; Katsuki et al., 1997). Conversely, activation of muscarinic acetylcholine receptors facilitates LTD (Kirkwood et al., 1999), possibly by PKC-mediated inhibition of adenylyl cyclase (Stanton, 1995; Nouranifar et al., 1998).

Other variables that impact LTD are postnatal age and the behavioral state of the animal. Although the mechanism remains to be determined, it is well established that the magnitude and reliability of LTD decline with increasing age (Dudek and Bear, 1993; Errington et al., 1995; Wagner and Alger, 1995a; Kamal et al., 1998), supporting the idea that this mechanism plays an important role in the refinement of circuits during critical periods of development (Rittenhouse et al., 1999). However, the existence of LTD in the adult hippocampus has been the subject of some controversy, with some labs reporting

success (Thiels et al., 1994; Heynen et al., 1996; Manahan-Vaughan, 1997) and others reporting failure (Errington et al., 1995; Doyle et al., 1997; Staubli and Scafidi, 1997) to observe LTD *in vivo*. Resolution of this controversy may now be at hand. First, it has been established that there are rat strain differences in the expression of LTD (Manahan-Vaughan and Braunewell, 1999). Second, and most importantly, it has been shown that LTD is powerfully modulated by the behavioral state of the animal. When LTD-resistant animals are exposed to mild stress (Kim et al., 1994; Xu et al., 1997), or even simply to a novel environment (Manahan-Vaughan and Braunewell, 1999), there is a striking facilitation of LTD. The stress effect may be mediated by glucocorticoids (Coussens et al., 1997; Xu et al., 1998b); the mechanism for the novelty effect is unknown, although modulation by acetylcholine has been suggested (Bear, 1999). Whatever the mechanism, the results show that brain-state is a crucial variable that must be controlled during studies of LTD in adults.

The effect of novelty exposure on LTD is particularly interesting. When LFS is delivered as animals explore a novel environment, the resulting LTD lasts for weeks regardless of the strain of rat. However, the facilitation of synaptic plasticity is so marked that the usual 1-Hz tetanus is no longer required to induce LTD. The low-frequency electrical stimulation normally used to monitor synaptic transmission (5 pulses given at 0.1 Hz every 5 min for 15 min) is enough to significantly depress synaptic transmission for several hours if it is delivered during novelty exposure. An exciting possibility is that the pattern of electrical stimulation imposed on the brain during the novel experience is incorporated into the memory of that experience, and this memory is stored as LTD of the synapses that were active at that time. Indeed, recordings from neurons in the temporal lobes have consistently revealed that a cellular correlate of recognition memory is a diminished response to the learned stimulus (Xiang and Brown, 1998). Perhaps this reduced response, and the memory trace, is accounted for by the mechanisms of homosynaptic LTD.

A final type of LTD modulation was suggested by the BCM theory. The idea was that the value of qm (the LTD-LTP crossover point) should vary depending on the history of the integrated postsynaptic response (Bear et al., 1987; Bear, 1995). After periods of strong postsynaptic activity, the modification threshold slides to promote LTD over LTP; after periods of postsynaptic inactivity, the threshold adjusts to promote LTP over LTD. In this way, the properties of synaptic plasticity adjust to keep the network of modifiable synapses within a useful dynamic range. There is now compelling evidence from a number of systems that the stimulation requirements for induction of LTD are indeed altered by prior postsynaptic activity (Kirkwood et al., 1996; Holland and Wagner, 1998; Wang and Wagner, 1999). The mechanisms for this plasticity of synaptic plasticity, or 'metaplasticity' (Abraham and Bear, 1996), remain to be determined, but the obvious candidates are clear from this discussion of LTD modulation. Changes in inhibition (Huang et al., 1999b; Steele and Mauk, 1999), NMDAR properties (Quinlan et al., 1999), and the balance of postsynaptic kinases and phosphatases (Mayford et al., 1995; Migaud et al., 1998) have all been proposed as mechanisms for the sliding modification threshold of the BCM theory.

4.17.7 mGluR-Dependent LTD

4.17.7.1 Induction

In addition to activating ionotropic receptors, glutamate stimulates G-protein coupled mGluRs. There are three classes of mGluR, defined by their pharmacology and coupling to second messenger pathways (Pin and Bockaert, 1995). Group 1 mGluRs (designated mGluR1 and mGluR5) stimulate phosphoinositide (PI) turnover via activation of phospholipase C (PLC). It is of historical interest to note that, based on theoretical considerations, the proposal was made that PI-coupled mGluRs play a role in triggering synaptic depression in the cerebral cortex (Dudek and Bear, 1989), and that the protocol of using LFS to induce homosynaptic LTD was designed originally with the aim of testing this hypothesis (Dudek and Bear, 1992). Although this early work implicated NMDARs instead, it was not long before a role for mGluRs was suggested for LTD. In particular, Bolshakov and Siegelbaum (1994) found that the mGluR antagonist α-methyl-4-carboxyphenylglycine (MCPG) prevents homosynaptic LTD in response to LFS in slices from very young rats (postnatal day 3–7). LTD in this preparation also required a rise in intracellular [Ca] and activation of voltage-gated Ca channels during LFS, but not activation of NMDARs.

Confusion about mGluR involvement in LTD persisted for a number of years due to some failures to replicate (Selig et al., 1995b), exacerbated by the finding that MCPG is actually a very weak antagonist of the action of glutamate at mGluR5 (Brabet et al., 1995; Huber et al., 1998). Fortunately, the smoke has now

cleared. First, it is now clear that the activation of mGluRs necessary to induce LTD is often not achieved using the usual 1- to 5-Hz stimulus trains. Protocols that work reliably are those that enhance glutamate release during conditioning stimulation, such as delivering prolonged trains of paired pulses (Kemp and Bashir, 1997a), or by antagonizing the adenosine inhibition of glutamate release (de Mendonca et al., 1997; Kemp and Bashir, 1997b). These protocols produce LTD of large magnitude with a component that cannot be blocked with NMDAR antagonists. Second, the use of new, potent mGluR antagonists and genetically altered mice has established that the NMDAR-independent LTD requires activation of mGluR5, and, conversely, that induction of NMDAR-dependent LTD does not (Bortolotto et al., 1999; Huber et al., 2001; Sawtell et al., 1999).

It has now been established that activation of group 1 mGluRs induces LTD by a mechanism that is entirely distinct from that engaged by NMDAR activation (Oliet et al., 1997). This mGluR-dependent LTD (mGluR-LTD) can be induced by synaptic stimulation in the presence of NMDAR antagonists, or by simple pharmacological activation of mGluRs using the group 1 mGluR-selective agonist DHPG ((RS)-3,5-dihydroxyphenylglycine) (Fitzjohn et al., 1999; Huber et al., 2001). Remarkably, the specific group 1 mGluRs involved differ for chemically and synaptically induced LTD. Whereas DHPG-induced LTD involves mGluR1 and mGluR5 activation, synaptically induced LTD is only mGluR5-dependent (Volk et al., 2006). The requirement of voltage-gated Ca entry for induction of mGluR-dependent LTD (mGluR-LTD) by synaptic stimulation has been confirmed, although the type of channel (L- or T-type) apparently varies depending on the circumstances (Bolshakov and Siegelbaum, 1994; Oliet et al., 1997; Otani and Connor, 1998). In addition, synaptically induced mGluR-LTD requires activation of postsynaptic PLC (Reyes and Stanton, 1998) and PKC (Bolshakov and Siegelbaum, 1994; Oliet et al., 1997; Otani and Connor, 1998). Unlike the NMDAR-dependent LTD (NMDAR-LTD), mGluR-LTD is not affected by inhibition of postsynaptic PP1 (Oliet et al., 1997).

Biochemical experiments suggest that activation of group 1 mGluRs in synaptoneurosomes stimulates, in a PKC-dependent manner, the aggregation of ribosomes and mRNA, and the synthesis of the fragile X mental retardation protein (Weiler and Greenough, 1993; Weiler et al., 1997). Thus, it is of considerable interest that mGluR-LTD is prevented by manipulations that interfere with protein synthesis. Huber et al. (2001) have shown that induction of mGluR-LTD is prevented by the postsynaptic inhibition of mRNA translation during conditioning stimulation. Because the LTD is homosynaptic and occurs even when the dendrites are isolated from their cell bodies, a requirement for rapid, synapse-specific synthesis of proteins from preexisting mRNA is strongly suggested. These findings are consistent with a number of converging lines of evidence suggesting a major role for mRNA translation in the mechanisms of mGluR5 action (Merlin et al., 1998; Raymond et al., 2000).

The discovery of polyribosomes at the base of dendritic spines has long invited speculation that synaptic activity regulates the protein composition, and therefore function, of synapses in the brain (Steward et al., 1988). Available data now indicate that mGluR5 activation triggers synapse-specific mRNA translation, and that one functional consequence is LTD (**Figure 3**). The obvious questions to be examined next concern the mechanism of translation regulation, the identity of the essential transcripts, and the mechanism that couples new protein synthesis to a change in synaptic function. The mGluR-LTD model should prove extremely valuable for answering these questions.

4.17.7.2 Expression

At the present time, more is known about how mGluR-LTD is *not* expressed than about how it is expressed. Specifically, mGluR-LTD is not expressed via the same mechanism as NMDAR-LTD. This conclusion is supported by the finding that the two forms of LTD are additive and do not mutually occlude one another. Moreover, while NMDAR-LTD is reversed by induction of LTP (and vice versa), mGluR-LTD is not (Oliet et al., 1997; Fitzjohn et al., 1999; Huber et al., 2001). It has been reported that after mGluR-LTD the quantal content, but not the quantal size, is reduced (Bolshakov and Siegelbaum, 1994; Oliet et al., 1997), consistent with a presynaptic expression side. However, this observation could also be explained by an all-or-none loss of postsynaptic AMPARs at individual synapses (all-or-none because a graded decrease would be reflected in a decrease in quantal size). Studies on the phosphorylation state of AMPARs in mGluR-LTD could not provide a consistent view on the expression side of mGluR-LTD so far. Whereas one study reported that

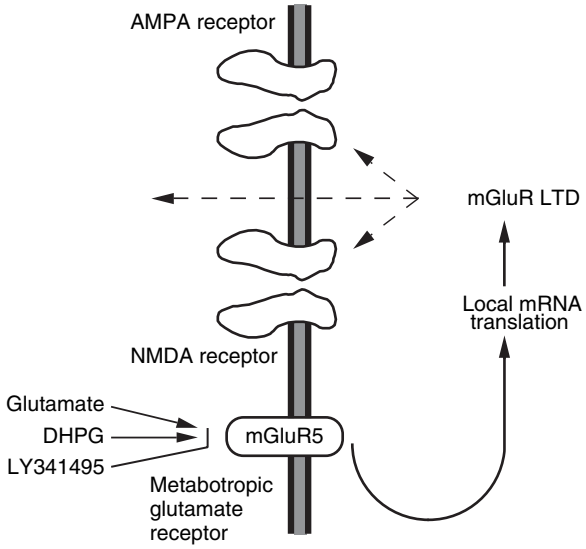


Figure 3 Model for metabotropic glutamate receptor-dependent LTD (mGluR-LTD) in CA1. Activating mGluR5 during synaptic stimulation, or by the selective agonist DHPG, triggers LTD that can be prevented by the selective mGluR antagonist LY341495. Synaptically evoked mGluR-LTD requires local translation of preexisting mRNA.

mGluR-LTD, unlike NMDAR-LTD, is not associated with a dephosphorylation of AMPA receptors (Huber et al., 2001), another found that in DHPG-induced LTD the activation of tyrosine phosphatases leads to a tyrosine dephosphorylation of AMPARs and their subsequent endocytosis (Moult et al., 2006). A possible explanation for this discrepancy might be that a developmental switch occurs between the second and the third postnatal week from a pre- to a postsynaptic expression side of mGluR-LTD (Nosyreva and Huber, 2005).

4.17.8 Depotentiation

The term 'depotentiation' refers to the reversal of previously established LTP, which can be elicited by variations of the LFS protocol. Confusion has arisen because the same term has been used to describe two phenomena. One type of depotentiation, of course, is homosynaptic LTD of synapses from a potentiated baseline, which can be induced at any time following LTP induction and utilizes the same mechanisms discussed above. The second type of depotentiation refers to the disruption of LTP that occurs when LFS is delivered within a relatively brief time window immediately following LTP induction. This time-sensitive depotentiation apparently is caused by interference with the transient intracellular biochemical reactions that are required to 'fix' LTP in the period that follows strong NMDAR activation.

4.17.8.1 Time-Sensitive Depotentiation

High-frequency synaptic stimulation (HFS) typically induces LTP in CA1. However, establishment of stable LTP is prevented if the HFS is followed by certain types of synaptic stimulation including, but not restricted to, LFS (Hesse and Teyler, 1976; Barrionuevo et al., 1980; Arai et al., 1990; Staubli and Lynch, 1990; Fujii et al., 1991; Barr et al., 1995; Holscher et al., 1997). This retrograde disruption of LTP is time dependent. LFS within 5 min of HFS can completely prevent LTP; however, the same stimulation may have no effect when delivered 1 h after HFS.

A clear picture of the mechanism for time-sensitive depotentiation (TS-DP) has finally emerged (**Figure 4**). Although the upstream regulation varies depending on the type of stimulation used, the critical downstream requirement for TS-DP is activation of postsynaptic PP1 during the sensitive period (O'Dell and Kandel, 1994; Staubli and Chun, 1996; Huang et al., 1999a). This period coincides with the time when LTP can be disrupted by inhibition of protein kinases (Huber et al., 1995). Thus, the data indicate that stable establishment of LTP requires the active serine-threonine phosphorylation of synaptic substrates for a defined time period. If this phosphorylation is prevented or reversed, so is LTP.

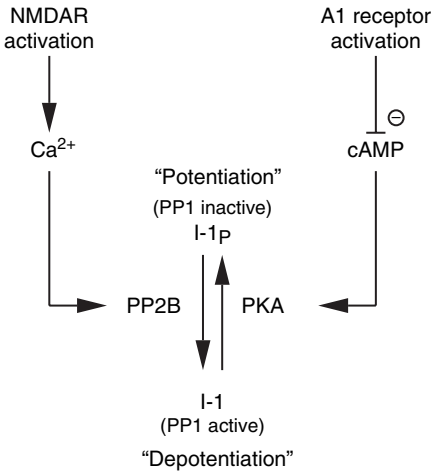


Figure 4 Converging pathways to depotentiation. Depotentiation occurs if synapses are given LFS in a narrow time window immediately following induction of LTP. Available evidence suggests that depotentiations results from dephosphorylation of postsynaptic proteins by protein phosphatase 1 (PP1). PP1 is activated when inhibitor 1 (I-1) is dephosphorylated by calcineurin (PP2B) at a PKA site. Stimuli that cause depotentiation include those that activate PP2B (e.g., NMDAR activation) and those that inhibit PKA (e.g., adenosine A1 receptor activation).

Upstream regulation of TS-DP can occur in different ways. One route for TS-DP induction appears to be the now-familiar pathway involving NMDAR stimulation (Fujii et al., 1991; O'Dell and Kandel, 1994; Barr et al., 1995; Xiao et al., 1996) followed by activation of PP2B and dephosphorylation of I-1 (O'Dell and Kandel, 1994; Zhuo et al., 1999). A second route to TS-DP induction, also leading to dephosphorylation of I-1, is the activation of A1 adenosine receptors (Larson et al., 1993; Staubli and Chun, 1996; Fujii et al., 1997; Huang et al., 1999a). A1 receptor activation inhibits adenylyl cyclase and, as a consequence, PKA (Dunwiddie and Fredholm, 1989). Injection of PKA activators into the postsynaptic neuron can prevent depotentiation caused by A1 receptor activation (Huang et al., 1999a).

Despite the apparent mechanistic similarities of TS-DP and NMDAR-LTD, the two phenomena differ. For example, TS-DP shows much less developmental regulation than LTD, and the patterns of activity that are optimal for induction are different (O'Dell and Kandel, 1994). In addition, TS-DP shows greater sensitivity to PP1 inhibitors and to deletion of the Aα isoform of PP2B than does LTD (O'Dell and Kandel, 1994; Zhuo et al., 1999). Interestingly, however, like LTD, TS-DP *in vivo* is dramatically facilitated by exposure of animals to a novel environment (Xu et al., 1998a).

A simple way to reconcile the findings is if we assume that LTP induction results in the transient exposure of postsynaptic phosphorylation sites to both protein kinases and phosphatases. Normally, the kinase activation that follows strong NMDAR stimulation leads to a net phosphorylation of these sites and LTP. However, the sites are also vulnerable to dephosphorylation if PP1 is activated. Because the phosphorylation sites are exposed, the threshold level of PP1 activation for TS-DP is much lower than that for NMDAR-LTD. Thus, while stimulation that induces LTD also can always produce TS-DP, the converse is not true. However, the common requirement for PP1 activation makes both forms of synaptic modification subject to very similar types of modulation.

4.17.8.2 Time-Insensitive Depotentiation

Under conditions in which *de novo* LTD is induced by LFS, the same stimulation can also reverse LTP that was induced hours before. The precise mechanism of LTD and LTP reversal may not be identical, however. For example, LTD *de novo* is associated with the dephosphorylation of GluR1 at a PKA site. However, the same induction protocol given 1 h after induction of LTP causes dephosphorylation of the CaMKII/PKC site instead (Lee et al., 2000; see **Figure 2(b)**). On the other hand, both LTD and LTP reversal *in vivo* are associated with a parallel decrease in AMPAR and NMDAR protein in the synaptoneurosomal biochemical fraction (Heynen et al., 2000).

4.17.9 LTD of the Cerebellar Parallel Fiber–Purkinje Cell Synapse

The second type of synapse that we want to use as an example to discuss LTD mechanisms is the parallel fiber–Purkinje cell synapse in the cerebellum. For comparison, we will also describe a more recently characterized form of LTD at the climbing fiber–Purkinje cell synapse. Why bother to study synaptic plasticity in an obscure and atypical part of the brain like the cerebellum? The answer is that it is one of only two locations where learning and memory can be understood at the level of circuits (the other being the amygdala; for comparison see Medina et al., 2002). In contrast, the hippocampus, for all of its experimental utility, receives information that is so

highly processed that its content cannot be easily characterized (what is the nature of the information conveyed by the perforant path?).

4.17.10 Cerebellar Anatomy and Some Useful Models

The cerebellum functions largely to integrate various forms of sensory information to smooth and fine-tune complex voluntary movements and reflexes (see Ito, 1984, for review). Therefore, cerebellar damage in humans is associated not with outright paralysis, but rather with dysmetric and ataxic syndromes, as well as impairments in motor learning. In addition, some recent work on human cerebellar lesions complemented by functional imaging studies has implicated the cerebellum in certain forms of non-motor procedural learning as well (see Schmahmann, 1997, for review).

The cerebellum comprises ~10% of the total weight of the human brain, but contains >50% of the total number of neurons, packed into the most infolded and convoluted structure in the brain. This degree of specialization suggests that, throughout evolution, fast, accurate, coordinated movements have been highly adaptive. To coordinate many joints and muscles, it is necessary that sensory and proprioceptive signals from any location in the body or sensory world be able to influence motor commands to any muscle in the body. Essentially, this requires a giant switchboard, which is implemented in the following way. The cerebellar circuitry is essentially composed of a relay station in the deep cerebellar nuclei (DCN) and a cortical 'side-loop' (see **Figure 5(a)**). The neurons of the DCN receive their main excitatory drive from glutamatergic mossy fibers which are the axons of a large number of precerebellar nuclei. The main outflow of information from this structure is carried by excitatory axons which originate from the large neurons of the DCN and project to premotor areas including the red nucleus and thalamus. In addition, there are small projection neurons in the DCN which are GABAergic (Kumoi et al., 1988; Batini et al., 1992) and send axons to the inferior olive (Fredette and Mugnaini, 1991).

The sole output of the cortical side-loop is the inhibitory, GABAergic (GABA: gamma-aminobutyric acid) projection from Purkinje cells to the neurons of the DCN (both large and small projection neurons are innervated; see De Zeeuw and Berrebi, 1995; Teune et al., 1998). Purkinje cells receive two major excitatory inputs, which are organized in very different ways. Each Purkinje cell is innervated by a single climbing fiber. This climbing fiber, which originates in the neurons of the inferior olive, will innervate ~10 Purkinje cells. This is potentially the most powerful synaptic contact in the brain, as each Purkinje cell receives ~1400 synapses from a single climbing fiber axon (Strata and Rossi, 1998). Climbing fibers also provide a very weak innervation of the DCN, consisting of a few synapses in the most distal dendrites, the function of which is poorly understood. In contrast, each Purkinje cell receives ~200 000 synapses from parallel fibers, which are the axons of granule cells. Because of the large number of granule cells (~50 billion) and the divergent output of their parallel fibers (each contacts ~1000 Purkinje cells), this synapse is the most abundant of any in the brain. Closing the loop, granule cells receive excitatory synapses from branches of the same mossy fibers which innervate the DCN directly. Because there are ~10 000-fold more granule cells than DCN cells, the innervation of granule cells by mossy fibers is highly convergent.

Putting this circuit together, it appears as if cerebellar output is driven by direct excitatory input from the mossy fibers and is modulated by the inhibitory input from the Purkinje cell axons, the latter of which will reflect computations and interactions in the Purkinje cell. These computations will be performed upon very subtle and informationally rich excitatory parallel fiber input and massive, synchronous excitation produced by the climbing fiber. This striking anatomical organization has inspired some notable models of motor learning. In particular, Marr (1969) proposed that the parallel fiber–Purkinje cell synapses could provide contextual information, that climbing fiber–Purkinje cell synapses could signal an 'error' in motor performance that required alteration of subsequent behavior, and that the conjunction of these two signals could strengthen the parallel fiber–Purkinje cell synapse to create a memory trace for motor learning. This model was modified by Albus (1971), who noted that a decrease in synaptic strength would be more appropriate, given the sign-reversing function of the Purkinje cell inhibitory output. Importantly, Albus also noted that this model is analogous to classical conditioning, with the parallel fibers conveying a conditioned stimulus (CS), the climbing fiber an unconditioned stimulus (US), and a depression of the parallel fiber–Purkinje cell synapse giving rise to a conditioned response (CR) via disinhibition of

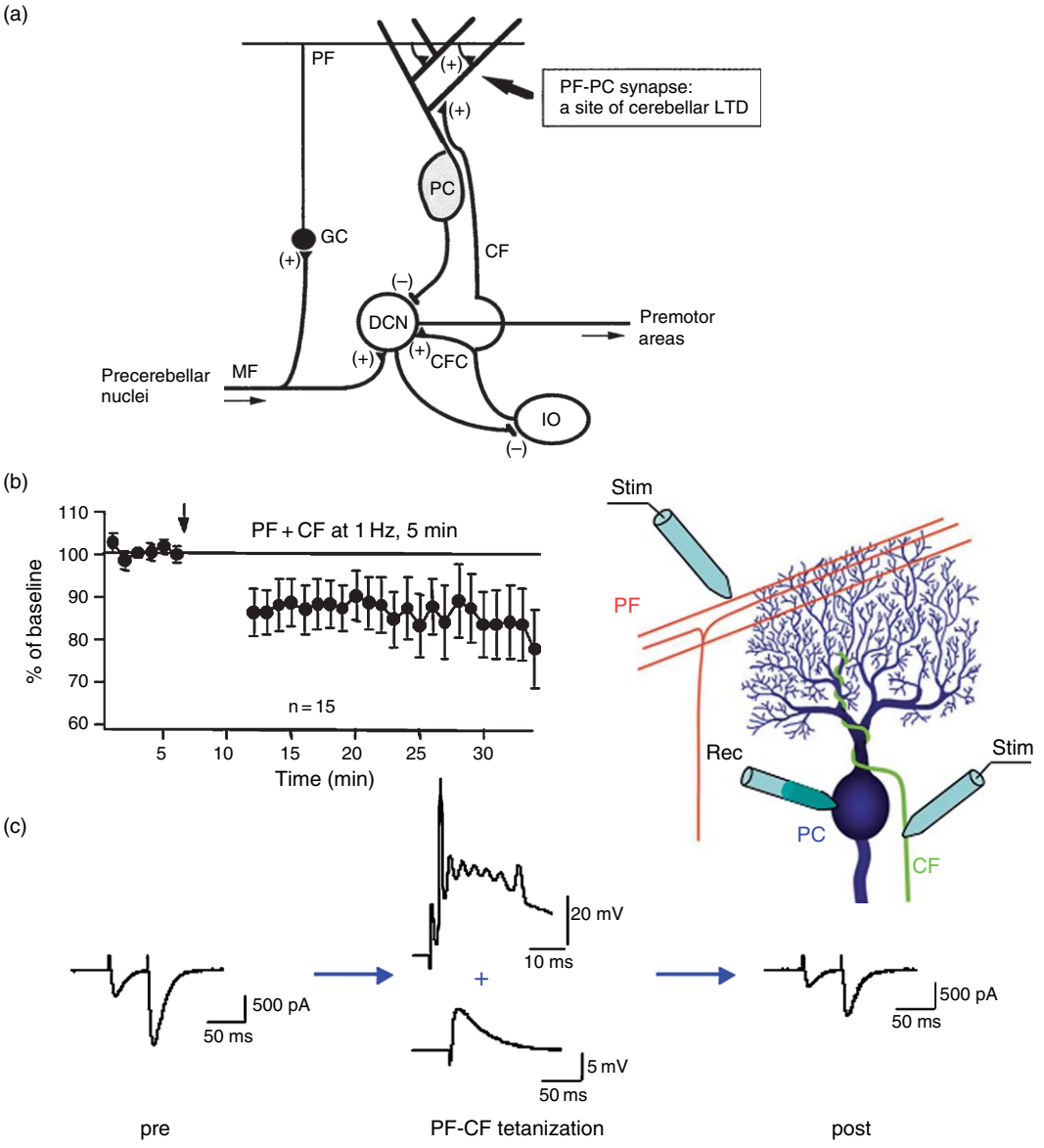


Figure 5 Basic cerebellar functional anatomy and LTD of the parallel fiber–Purkinje cell synapse. (a) A simplified diagram of cerebellar circuitry. Information flow through the main relay pathway consisting of precerebellar nuclei, their axons, the mossy fibers (MF), their targets in the deep cerebellar nuclei (DCN), and DCN excitatory axons projecting to premotor centers, is indicated with arrows. Excitatory synapses are denoted with a (+) and inhibitory synapses with a (−). IO, inferior olive; CF, climbing fiber; CFC, climbing fiber collateral; PC, Purkinje cell; GC, granule cell; PF, parallel fibers. (b) Left: parallel fiber LTD is obtained after paired parallel fiber and climbing fiber tetanization (1 Hz, 5 min; n = 15). Arrow indicates the time point of tetanization. Right: Diagram showing the electrode arrangement used for LTD induction *in vitro.* Whole-cell patch-clamp recording (rec) is used to monitor electrical responses to parallel fiber (PF) and/or climbing fiber (CF) stimulation. For extracellular stimulation, glass pipettes are used (stim) that are filled with ACSF. (c) Test responses to PF stimulation are recorded before and after tetanization in voltage-clamp mode. The paired-pulse facilitation ratio is monitored to screen for presynaptic changes. For tetanization, recordings are switched to current-clamp mode. CF activation results in a typical complex spike (top). The PF-EPSP is shown here in isolation (bottom), but in these recordings is masked by the complex spike. Following tetanization, the PF-EPSC amplitude is reduced.

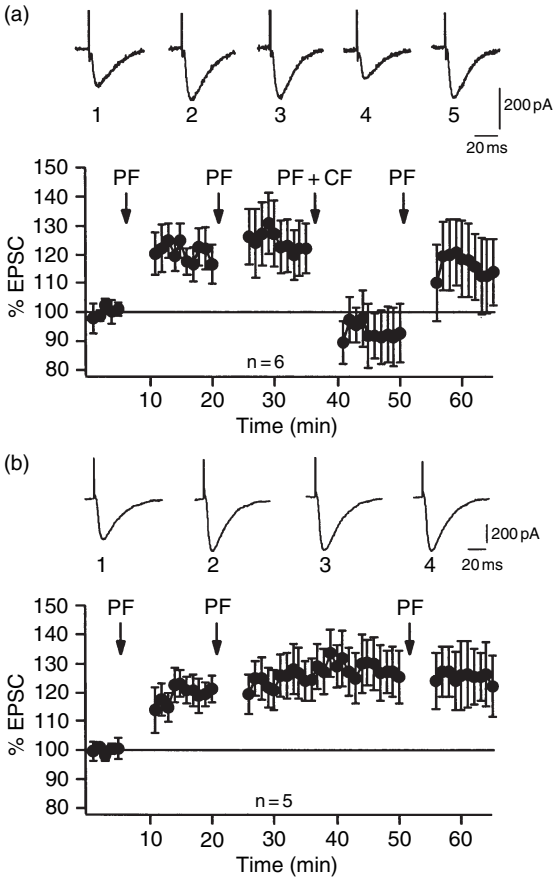


Figure 6 Reversal of parallel fiber LTP by LTD. (a) Saturated LTP is reversed by the application of the LTD protocol. Two LTP protocols were followed by an LTD protocol and, finally, by a third LTP protocol (n = 6). The LTD protocol consists of a paired parallel fiber and climbing fiber stimulation at 1 Hz for 5 min. The LTP protocol consists of the same parallel activation pattern in the absence of climbing fiber activation. Tetanization periods are indicated by the arrows. (b) Omission of the LTD protocol reveals LTP saturation after the application of two LTP protocols (n = 5). In (a) and (b), traces on top show EPSCs from the time points indicated. This figure is taken from Coesmans M, Weber JT, De Zeeuw CI, and Hansel C (2004) Bidirectional parallel fiber plasticity in the cerebellum under climbing fiber control. *Neuron* 44: 691–700, with permission from Elsevier.

the DCN. To place this model in a behavioral context, let us consider a well-characterized form of classical conditioning, associative eyeblink conditioning in the rabbit. Before training, an airpuff to the eye (US) gives rise to an immediate reflexive blink (the unconditioned response, UR). During training, a neutral stimulus such as a tone (CS) is paired with the airpuff stimulation so that the tone onset precedes the airpuff and the two stimuli coterminate. As the rabbit acquires the association, it performs a blink carefully timed to immediately precede the airpuff (CR). This associative learning can also be actively reversed. In well-trained animals which reliably perform CRs, this response can undergo rapid extinction if tone stimuli are repeatedly presented without airpuffs.

4.17.11 The Role of the Cerebellum in Associative Eyeblink Conditioning

There is extensive evidence to support the involvement of cerebellar circuits in associative eyeblink conditioning (see Kim and Thompson, 1997, for review). Similar evidence implicates the cerebellum in other forms of motor learning such as limb-load adjustment and adaptation of the vestibulo-ocular reflex (VOR; du Lac et al., 1995; De Zeeuw et al., 1998). Extracellular recording showed that populations of cells in the nucleus interpositus (a particular portion of the DCN) discharge during the UR before training and, in well-trained animals, begin to fire during the CS-US interval. This firing is predictive of and correlated with the performance of the CR, suggesting that the CR behavior is expressed in the firing rate and pattern of DCN neurons (McCormick and Thompson, 1984a,b; Berthier and Moore, 1986, 1990). This notion is further supported by the finding that microstimulation in the appropriate region of the nucleus interpositus elicited a strong eyelid response in either trained or untrained animals (McCormick and Thompson, 1984a). Moreover, during training, stimulation of mossy and climbing fibers can substitute for the CS and US, respectively (Mauk et al, 1986; Steinmetz et al., 1986, 1989).

The data obtained using lesions and reversible inactivation have been somewhat more complex (see Mauk, 1997, for review). A Marr/Albus model would predict that lesions of the cerebellar cortex would both delete the memory trace in previously trained animals and prevent further learning. Initially, it was observed that lesioning either the whole cerebellum (ipsilateral to the trained eye) or the anterior interpositus nucleus completely abolished the CR but *not* the UR (McCormick et al., 1982; McCormick and Thompson, 1984a,b; Yeo et al., 1985a; Steinmetz et al., 1992; but see Welsh and Harvey, 1989). These experiments suggested that cerebellar lesions abolished the memory trace for eyeblink conditioning, but their irreversibility made it difficult to dissociate this interpretation from a performance deficit. A more convincing case was made when experiments

showed that reversible inactivation of the DCN with muscimol (a $GABA_A$ receptor agonist) prevented the acquisition of the eyeblink CR, but not the performance of the UR (Krupa et al., 1993; Hardiman et al., 1996; Krupa and Thompson, 1997; but see Bracha et al., 1994). In contrast, inactivation of the superior cerebellar peduncle or red nucleus, sites through which excitatory DCN output is conveyed, prevented the expression of the CR during training, but not its acquisition, as evidenced by the fact that the CR was present after inactivation (Krupa et al., 1993; Krupa and Thompson, 1995). These studies suggest that the cerebellum and its associated projections are essential for acquisition and expression of the eyeblink CR. More specifically, the memory trace seems to be localized 'upstream' of the red nucleus, in the cerebellar cortex and/or the DCN.

While there is general agreement that lesions or inactivation of the DCN block the acquisition of the eyeblink CR, there has been considerable debate over the specific role of the cerebellar cortex in eyeblink conditioning. Reports using lesions and inactivation of the cerebellar cortex have ranged from those which have found a complete blockade of CR acquisition (Yeo et al., 1985b), to those which have slowed, but not prevented acquisition (Lavond and Steinmetz, 1989; Yeo and Hardiman, 1992), to those which have found no effect at all (McCormick and Thompson, 1984a,b). Some recent reports point to a potential resolution of this problem. Lesions which included the anterior cerebellar cortex (a region previously thought not to be important), or infusion of picrotoxin (a $GABA_A$ receptor antagonist, the opposite of muscimol) into the DCN to block Purkinje cell input, did not abolish the CR entirely but affected its timing (Perrett et al., 1993; Perrett and Mauk, 1995; Garcia and Mauk, 1998). Recently, a model has been proposed to explain these findings. In this model, the memory trace of the eyeblink CR is sequentially stored, initially as a depression of the parallel fiber–Purkinje cell synapse in the cerebellar cortex. This would result in an attenuation of Purkinje cell firing and hence Purkinje cell–DCN synaptic drive, thereby disinhibiting the DCN targets. This disinhibition, when coupled with activation of the mossy fiber–DCN synapse, could then potentiate the latter, resulting in storage of the CR at the mossy fiber–DCN synapse while the timing of the conditioned response is retained in the cerebellar cortex (Raymond et al., 1996; Mauk, 1997; Mauk and Donegan, 1997; Medina and Mauk, 1999).

4.17.12 Potential Cellular Substrates of Associative Eyeblink Conditioning

LTD of the parallel fiber–Purkinje cell synapse has been proposed as a cellular mechanism which could, at least in part, underlie the acquisition of associative eyeblink conditioning. This phenomenon, which was first described by Ito and colleagues (1982), results when the climbing fiber (corresponding to the US) and parallel fiber (corresponding to the CS) inputs are activated together at low frequencies (1–4 Hz; see **Figure 5(b)**). In addition, stimulation of parallel fibers alone can produce LTP of the parallel fiber–Purkinje cell synapse, thus providing a form of bidirectional control (Lev-Ram et al., 2002; Coesmans et al., 2004). LTD in the parallel fiber–Purkinje cell synapse requires association of parallel fiber (CS) and climbing fiber (US) activation and would result in decreased firing of the Purkinje cell, causing increased firing of DCN neurons and enhanced expression of the CR. Essentially, this is a cellular restatement of the Marr/Albus model. Conversely, repeated activation of the parallel fiber (CS) alone could, through enhanced inhibition resulting from parallel fiber LTP, decrease firing of the DCN and thereby reduce expression of the CR during extinction.

As indicated by the lesion and inactivation studies described above, it is likely that the parallel fiber–Purkinje cell synapse is not the only site of information storage during cerebellar motor learning. At a cellular level, extinction of the CR, as results from repeated application of a tone CS, could be mediated not only by LTP of the parallel fiber–Purkinje cell synapse, but also by LTD of the mossy fiber–DCN synapse. This idea is consistent with reports that both cortical lesions which include the anterior region (Perrett and Mauk, 1995) and reversible inactivation of the DCN with muscimol (Hardiman et al., 1996; Ramnani and Yeo, 1996) block CR extinction.

Recently, both LTD and LTP have been described at the mossy fiber–DCN synapse. Whereas LTD can be observed after high-frequency mossy fiber burst stimulation, either alone or paired with postsynaptic depolarization (Zhang and Linden, 2006), LTP can be elicited when high-frequency mossy fiber stimulation is paired with postsynaptic hyperpolarization followed by a rebound current (Pugh and Raman, 2006). The existence of mossy fiber LTP has been suggested by a model of Mauk and coworkers, in which disinhibition of the DCN

from reduced Purkinje cell input, when coupled with activation of the mossy fiber–DCN synapses, results in LTP of mossy fiber–DCN synapses, constituting a portion of the memory trace of the CR (Raymond et al., 1996; Mauk, 1997; Mauk and Donegan, 1997). A recent computational analysis has suggested that an LTP induction rule for the mossy fiber–DCN synapse that depends upon specific patterns of Purkinje cell input (plus ongoing mossy fiber activity) could constitute a memory trace that is unusually resistant to degradation by ongoing 'background' activity in the cerebellar circuit (Medina and Mauk, 1999). The dependence of mossy fiber LTP on paired hyperpolarization and subsequent rebound currents (Pugh and Raman, 2006), mimicking the response to Purkinje cell activity and the transient interruption of inhibition (e.g., related to a complex spike pause), fits this theoretical framework and underlines the importance of a specific timing of mossy fiber and Purkinje cell activity for LTP induction. A missing piece that remains in the puzzle, however, is whether previous parallel fiber–LTD induction (resulting in a reduction of the inhibitory tone imposed by Purkinje cells) not only leads to increased activity levels in DCN cells, but also facilitates the induction of mossy fiber LTP.

4.17.13 Parallel Fiber LTD Induction

4.17.13.1 Parametric Requirements

Cerebellar LTD was first described in the intact cerebellum (Ito et al., 1982) and, since that time, has been analyzed in acute slice preparations, primary cultures, acutely dissociated Purkinje cells, and macropatches of Purkinje cell dendrite. In slice or *in situ*, the standard induction protocol consists of stimulating the parallel and climbing fiber inputs together at low frequency (1–4 Hz) for a period of 2–6 min. This results in a selective attenuation of the parallel fiber–Purkinje cell synapse (typically a 20–50% reduction of baseline synaptic strength), which reaches its full extent in ~10 min and persists for the duration of the experiment, typically 1–2 h.

LTD is said to result from coactivation of parallel fibers and climbing fibers, but what are the precise timing constraints on this coactivation? This is an important point, because if parallel fiber LTD underlies associative eyeblink conditioning, then the temporal constraints on CS/US association should be reflected in the temporal constraints on LTD induction. One study, using intracellular recording in rabbit cerebellar slice, has indicated that LTD is optimally induced when climbing fiber stimulation precedes parallel fiber stimulation by 125–250 ms (Ekerot and Kano, 1989). Another study using a similar preparation has shown that LTD may be induced by climbing fiber–parallel fiber stimulation with an interval of 50 ms, but claims that LTD induced by climbing fiber–parallel fiber pairing will not occur unless disynaptic inhibition is blocked by addition of a $GABA_A$ antagonist (Schreurs and Alkon, 1993). Neither of these intervals (in which the US precedes CS) will support robust eyeblink conditioning. However, with slightly different stimulation protocols (small trains of parallel fiber stimulation instead of single pulses in one case) parallel fiber before climbing fiber pairing, at intervals which support eyeblink conditioning, may also be effective in inducing LTD in the absence of $GABA_A$ receptor blockade (Chen and Thompson, 1995; Schreurs et al., 1996). In a more recent study, which combined whole-cell patch-clamp recordings with two-photon Ca imaging, it was shown that LTD is optimally induced when parallel fiber activation precedes climbing fiber activation by 50–200 ms and that coincident parallel fiber and climbing fiber activation results in supralinear Ca signals (Wang et al., 2000). Thus, the timing requirements found in cerebellar motor learning paradigms can indeed be matched by timing requirements characterized in cerebellar slice preparations. Moreover, the optimal timing conditions for LTD induction also yielded the largest spine Ca signals, providing an explanation why this particular activation sequence was beneficial for LTD induction.

4.17.13.2 Climbing Fiber Signals

The climbing fiber contributes to LTD induction by causing sufficient postsynaptic depolarization (through activation of AMPARs) to strongly activate voltage-sensitive Ca channels in the dendrites, thereby causing a complex spike and a large Ca influx (see **Figure 7**; for review see Schmolesky et al., 2002). In fact, climbing fiber activation may be replaced in the LTD induction protocol by direct depolarization of the Purkinje cell (Crepel and Krupa, 1988; Hirano, 1990; Linden et al., 1991). Furthermore, LTD induction is blocked by postsynaptic application of a Ca chelator (Sakurai, 1990; Linden and Connor, 1991; Konnerth et al., 1992), electrical inhibition of Purkinje cells during parallel

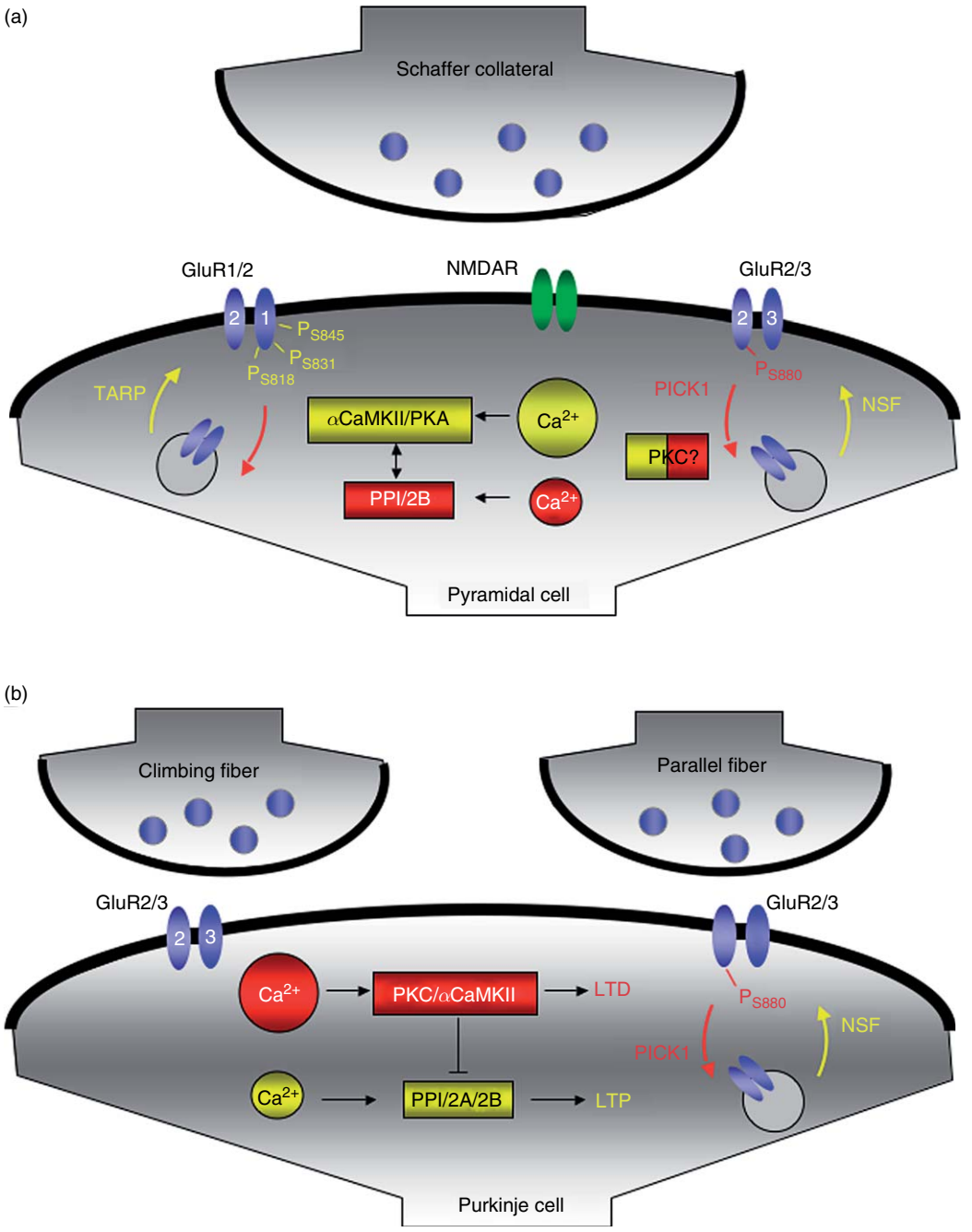


Figure 7 Comparison of LTP and LTD induction cascades at CA1 hippocampal (a) and cerebellar synapses (b). LTP induction cascades are shown in yellow, and LTD induction cascades in red. (a) LTP and LTD induction at Schaffer collateral synapses onto CA1 hippocampal pyramidal cells. Functional NMDARs are present in pyramidal cells, but not in Purkinje cells. Other calcium sources present in both types are neurons are not displayed. Note that PKA and protein phosphatase 1 (PP1) are not directly calcium activated. PKC has been implicated in hippocampal LTP, but also in the endocytosis of GluR2 subunits. Abbreviations are used as explained in the text. (b) LTP and LTD induction at cerebellar parallel fiber–Purkinje cell synapses. For simplicity, climbing fiber and parallel fiber terminals are shown to contact the same postsynaptic compartment; (b) is modified from Hansel C (2005) When the B-team runs plasticity: GluR2 receptor trafficking in cerebellar long-term potentiation. *Proc. Natl. Acad. Sci. USA* 102: 18245–18246, with permission from the National Academy of Sciences, USA.

fiber/climbing fiber conjunctive stimulation (Ekerot and Kano, 1985, 1989; Hirano, 1990; Crepel and Jaillard, 1991), or removal of external Ca (Linden and Connor, 1991). In addition, studies using optical indicators have shown large Ca accumulations in Purkinje cell dendrites following climbing fiber stimulation (Ross and Werman, 1987; Knöpfel et al., 1990; Konnerth et al., 1992). These Ca transients reach supralinear levels when the climbing fiber stimulation is paired with parallel fiber activation (see above; Wang et al., 2000). While these studies have suggested that Ca influx is the sole mediator of climbing fiber action, another view has come from studies which have examined a peptide released from climbing fiber terminals, corticotropin releasing factor. Miyata et al. (1999) have found that LTD induced by either parallel fiber/climbing fiber conjunction or parallel fiber/depolarization conjunction can be blocked by antagonists of the corticotropin releasing factor receptor in a slice preparation. Furthermore, parallel fiber/depolarization conjunction fails to induce LTD in slices prepared from rats in which climbing fibers were chemically prelesioned, but this may be restored with exogenous corticotropin releasing factor. These observations have led to the suggestion that corticotropin releasing factor plays a permissive role in LTD of parallel fiber synapses. A similar facilitatory role of corticotropin releasing factor can be found for LTD induction at climbing fiber–Purkinje cell synapses (see following; Schmolesky et al., 2007).

4.17.13.3 Parallel Fiber Signals

Parallel fiber activation results in glutamate release, which activates glutamate receptors in the Purkinje cell dendrite. While mature Purkinje cells do not express functional NMDARs, they are found on both cultured embryonic Purkinje cells and acutely dissociated Purkinje cells in early postnatal life (Linden and Connor, 1991; Rosenmund et al., 1992). Purkinje cells also express AMPARs of the GluR2-containing, Ca-impermeable variety (Linden et al., 1993; Tempia et al., 1996) as well as a particular metabotropic receptor, mGluR1, at high levels in the dendritic spines where parallel fiber synapses are received (Martin et al., 1992).

The first evidence indicating that activation of metabotropic receptors was required for parallel fiber LTD induction came from experiments using cerebellar cultures, which showed that agonists that activated both AMPA and metabotropic receptors (such as glutamate and quisqualate) could substitute for parallel fiber activation during LTD induction, but that agonists that failed to activate metabotropic receptors (such as AMPA or aspartate) could not (Kano and Kato, 1987; Linden et al, 1991). Complementary evidence was found in which metabotropic receptor antagonists blocked LTD induction (Linden et al., 1991; Hartell, 1994; Narasimhan and Linden, 1996; Lev-Ram et al., 1997a). These results, while they indicated that metabotropic receptor activation was required, did not specify which metabotropic receptor(s) were important for LTD induction. The first findings to address this issue were those of Shigemoto et al. (1994), who demonstrated that specific inactivating antibodies directed against mGluR1 could block LTD induction in cell culture. This result was confirmed and extended by two different groups using mGluR1 knockout mice (Aiba et al., 1994; Conquet et al., 1994).

Activation of mGluR1 results in the activation of phospholipase C and the consequent production of two initial products, inositol-1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol. The former binds to specific intracellular IP_3 receptors, resulting in the liberation of Ca from internal stores, while the latter results in activation of PKC. Are both of these products required for parallel fiber LTD induction? Purkinje cells express IP_3 receptors, particularly the type I isoform, at unusually high levels (Nakanishi et al., 1991), and it has been shown through photolysis of caged IP_3 that these receptors are functionally coupled to intracellular Ca release in situ (Khodakhah and Ogden, 1993; Wang and Augustine, 1995). Several lines of evidence have supported a role for IP_3 receptor activation in the induction of parallel fiber LTD. First, compounds which interfere with IP_3 receptor function have been shown to block LTD. Application of heparin, a nonspecific (Herbert and Maffrand, 1991; Bezprozvanny et al., 1993) inhibitor of the IP_3 receptor, blocked LTD induced by glutamate/depolarization conjunction in cultured Purkinje cells (Kasono and Hirano, 1995) or by parallel fiber/depolarization conjunction in Purkinje cells in a cerebellar slice (Khodakhah and Armstrong, 1997). A specific inactivating antibody directed against the IP_3 receptor was similarly effective (Inoue et al., 1998). Application of thapsigargin, a drug which depletes internal Ca stores through inhibition of the endoplasmic reticulum Ca-ATPase, also blocked LTD induction (Kohda et al., 1995). Thapsigargin would be expected to deplete Ca stores gated by both the IP_3 receptor and the ryanodine receptor, the latter of which mediates Ca-induced Ca

release. Second, photolysis of IP_3 in cultured Purkinje cells can induce LTD when combined with depolarization plus AMPAR activation (Kasano and Hirano, 1995). Similarly, IP_3 photolysis combined with depolarization can induce LTD in slices derived from either wild-type (Khodakhah and Armstrong, 1997) or mGluR1 knockout mice (Daniel et al., 1999). Finally, parallel fiber LTD is blocked in slices derived from a mutant mouse which lacks the type I IP_3 receptor (Inoue et al., 1998).

While these experiments would appear to provide a strong case for the involvement of IP_3 receptors in cerebellar LTD induction, it is worth noting that not all evidence has been consistent with this view. For example, thapsigargin application in slices was found to block LTD induced by bath application of the mGluR agonist trans-DL-1-amino-1,3-cyclopentanedicarboxylic acid (ACPD) together with depolarization, but not parallel fiber/depolarization conjunction (Hemart et al., 1995). Furthermore, Narasimhan et al. (1998) performed ratiometric imaging of free cytosolic Ca on both acutely dissociated and cultured Purkinje cells. It was determined that the threshold for glutamate pulses to contribute to LTD induction was below the threshold for producing a Ca transient. Furthermore, the Ca transients produced by depolarization alone and glutamate plus depolarization were not significantly different. In addition, the potent and selective IP_3 receptor channel blocker xestospongin C (an improvement over heparin) was not found to affect the induction of LTD in either acutely dissociated or cultured Purkinje cells at a concentration which was sufficient to block mGluR1-evoked Ca mobilization. Finally, replacement of mGluR1 activation by exogenous synthetic diacylglycerol in an LTD induction protocol was successful. At present it is not clear why an IP_3 signaling cascade is not required for induction of cerebellar LTD in these experiments using reduced preparations, while other experiments using both slice and culture preparations have suggested otherwise.

4.17.13.4 Second Messengers

Two major postsynaptic signals resulting from cerebellar LTD induction are 1,2-diacylglycerol and Ca. These signals are known to synergistically activate the enzyme PKC. The involvement of PKC in LTD induction was suggested by experiments in which PKC inhibitors blocked induction when applied during glutamate/depolarization conjunction in cultured Purkinje cells (Linden and Connor, 1991). Application of these compounds after LTD had been induced had no effect, suggesting that continued PKC activation is not required for LTD to persist. Blockade of LTD induction by PKC inhibitors has since been confirmed using several preparations including cerebellar slices (Hartell, 1994; Freeman et al., 1998), acutely dissociated Purkinje cells and Purkinje cell dendritic macropatches (Narasimhan and Linden, 1996), as well as cultured Purkinje cells derived from a transgenic mouse which expresses a PKC inhibitor peptide (De Zeeuw et al., 1998). These observations are complemented by the finding that bath application of PKC-activating phorbol esters induces an LTD-like attenuation of Purkinje cell responses to exogenous glutamate or AMPA (Crepel and Krupa, 1988; Linden and Connor, 1991) which occludes pairing-induced LTD. In a recent study using αCaMKII knockout mice, it was shown that αCaMKII is involved in cerebellar LTD induction as well (Hansel et al., 2006). Moreover, LTD was blocked when the CaMKII inhibitor KN-93 was bath applied, but not when its inactive analogue, KN-92, was used. These observations show that, similar to hippocampal LTP (but operating in an 'inverse' manner), multiple kinases are involved in the induction process.

In addition to PKC and αCaMKII activation, a number of studies have indicated that release of the gaseous second messenger, nitric oxide (NO) by the action of the Ca/calmodulin-sensitive enzyme NO synthase (NOS), is necessary for parallel fiber LTD induction. They have shown that an LTD-like phenomenon could be induced when climbing fiber stimulation was replaced by bath application of NO via donor molecules such as sodium nitroprusside (Crepel and Jaillard, 1990; Shibuki and Okada, 1991; Daniel et al., 1993; but see Glaum et al., 1992). Likewise, induction of LTD by more conventional means could be blocked by inhibitors of NOS (such as N^G-nitro-L-arginine), agents that bind NO in the extracellular fluid (such as hemoglobin), or genetic deletion of the neuronal isoform of NOS (Lev-Ram et al., 1997b). Application of NO donors, cGMP analogs, or cGMP phosphodiesterase inhibitors directly to the Purkinje cell (via a patch pipette) also resulted in depression of parallel fiber responses (Daniel et al., 1993; Hartell, 1994, 1996), while postsynaptic application of a NOS inhibitor did not block LTD induction (Daniel et al., 1993). In contrast, postsynaptic application of a specific guanylyl cyclase inhibitor was effective in blocking LTD induction (Boxall and Garthwaite, 1996;

Lev-Ram et al., 1997a). These findings suggested a model in which climbing fiber activation resulted in NO production, which then diffused to the Purkinje cell to activate soluble guanylyl cyclase. However, this model was complicated by the fact that both climbing fibers (Bredt et al., 1990; Vincent and Kimura, 1992; Ikeda et al., 1993) and Purkinje cells (Bredt et al., 1990; Vincent and Kimura, 1992; Crepel et al., 1994) lack NOS.

A proposal which addresses this complication has been that climbing fiber–evoked Ca influx into Purkinje cell dendrites causes K-efflux, which depolarizes adjacent parallel fiber terminals, resulting in Ca influx and the consequent activation of NOS in these compartments (Daniel et al., 1998). Another approach has been taken by Lev-Ram et al. (1995), who found that photolysis of caged NO loaded into Purkinje cells could substitute for parallel fiber activation in LTD induction. When NO photolysis was followed by direct Purkinje cell depolarization within a 50-ms window, LTD of parallel fiber EPSCs was produced. LTD induced in this manner could be blocked by a postsynaptic application of a Ca chelator or NO scavenger, but not external application of a NOS inhibitor or an NO scavenger. In contrast, LTD produced by parallel fiber/depolarization conjunction could be blocked by either an internally or externally applied NO scavenger, or an externally applied NOS inhibitor, but not an internally applied NOS inhibitor. This pattern of results suggests a model in which activation of parallel fibers causes an anterograde NO signal which acts inside the Purkinje cell. A subsequent investigation by this group showed that, when photolysis of caged Ca was used in place of Purkinje cell depolarization, the coincidence requirement for NO and Ca pairing was <10 ms, and the resultant LTD could be blocked by an inhibitor of soluble guanylyl cyclase (Lev-Ram et al., 1997a). However, when caged Ca and cGMP were used, the inhibition of guanylyl cyclase could be overcome, and the coincidence requirement was lengthened to ~200 ms.

The production of cGMP by this cascade is likely to be exerting its effect through activation of cGMP-dependent protein kinase (PKG). LTD induced by parallel fiber/depolarization conjunctive stimulation (Hartell, 1994) or photolytic NO/depolarization stimulation (Lev-Ram et al, 1997a) may be blocked with PKG inhibitors. While the mechanisms by which PKG might contribute to LTD induction are not known, one suggestion has been that phosphorylation of G-substrate by PKG could result in inhibition of protein phosphatases (Ito, 1990), which in turn promote LTP induction (Belmeguenai and Hansel, 2005).

In contrast to the extensive evidence indicating a requirement for a NO/cGMP/PKG cascade in slice preparations, LTD of glutamate currents produced without synaptic stimulation in cultured Purkinje cells is unaffected by reagents that stimulate (sodium nitroprusside) or inhibit (hemoglobin, N^G-nitro-L-arginine) NO signaling (Linden and Connor, 1992). Furthermore, in cerebellar cultures made from neuronal NOS knockout mice, LTD was indistinguishable from that in cultures from wild-type mice (Linden et al., 1995). In wild-type cultures, neither an activator of soluble guanylate cyclase, nor an inhibitor of type V cGMP-phosphodiesterase, nor inclusion of cGMP analogs in the patch pipette produced an LTD-like effect. Induction of LTD was not blocked by inclusion in the patch pipette of three different PKG inhibitors. These results suggest that a NO/cGMP/PKG cascade is not required for cerebellar LTD induction in culture.

A recent study suggests that in slices NO might indeed be required for LTD induction, but that NO is not released from parallel fiber terminals, but instead from interneurons (Shin and Linden, 2005). Using combined patch-clamp recordings and presynaptic confocal Ca imaging, the authors demonstrated that there are no NMDAR-mediated Ca transients in parallel fiber terminals, which had previously been suggested to promote NOS activation and subsequent NO production (Casado et al., 2002). Rather, functional NMDAR and NMDAR-mediated Ca signaling were found in the somata, dendrites, and presynaptic terminals of stellate cells (Shin and Linden, 2005). Thus, it is likely that under certain activation conditions NO diffuses from interneurons to Purkinje cells and facilitates LTD induction by promoting phosphatase inhibition.

4.17.14 Parallel Fiber LTD Expression

While considerable attention has been paid to the molecular mechanisms of cerebellar LTD induction, significant effort has only recently been focused upon its expression. A widely accepted notion has been that LTD is expressed, at least in part, as a down-regulation of the postsynaptic sensitivity to AMPA, as LTD may be detected using AMPA or glutamate test pulses in intact (Ito et al., 1982), slice (Crepel and Krupa, 1988), and culture (Linden et al., 1991)

preparations. These experiments have been extended with ultrareduced preparations that completely lack functional presynaptic terminals (Linden, 1994) including outside-out dendritic macropatches and acutely dissociated Purkinje cells (Narasimhan and Linden, 1996, Narasimhan et al., 1998) which provide definitive evidence for a postsynaptic locus of expression. This idea has received further support from a study showing that the coefficient of variation of parallel fiber EPSCs was altered by manipulations known to act presynaptically (such as transient synaptic attenuation produced by addition of adenosine), but was not altered by induction of LTD (Blond et al., 1997).

In CA1 pyramidal cells, synaptic plasticity is implemented by changes both in the properties of postsynaptic AMPA receptors and in the insertion/internalization balance of AMPA receptor subunits (see above). In contrast, parallel fiber LTD is not associated with changes in AMPA receptor properties, such as glutamate affinity, conductance, or kinetics (Linden, 2001; for review see Ito, 2001). Rather, synaptic plasticity at parallel fiber synapses seems to rely entirely on changes in AMPA receptor trafficking. In Purkinje cells, GluR1 expression is low (Baude et al., 1994), and GluR2/GluR3 heteromers constitute the majority of AMPA receptors. PF-LTD results from a clathrin-mediated endocytosis of GluR2 (Wang and Linden, 2000), which requires PKCα-dependent phosphorylation of GluR2 at serine-880 (Chung et al., 2003; Leitges et al., 2004). The internalization of GluR2 involves unbinding from GRIP (Dong et al., 1997) and binding to PICK1 (Xia et al., 1999, 2000; Chung et al., 2000; Steinberg et al., 2006), thus using the same molecular machinery as described for GluR2 endocytosis in CA1 pyramidal cells (**Figure 7**). The role of αCaMKII activation, which is needed for LTD induction (Hansel et al., 2006), in GluR2 subunit trafficking remains to be examined.

4.17.15 Another Type of Cerebellar LTD: Climbing Fiber LTD

In the classic Marr-Albus-Ito models of cerebellar function and learning, paired parallel fiber and climbing fiber stimulation leads to LTD at the parallel fiber synapses, but the climbing fiber input was considered invariant (Marr, 1969; Albus, 1971; Ito, 1984). This reputation is a result of early studies by Eccles and colleagues demonstrating a high degree of reliability of complex spike occurrence upon tetanization at frequencies exceeding 100 Hz (e.g., Eccles et al., 1966). However, a different perspective of the 'invariance' of the complex spike emerges when it comes to modifications of the complex spike waveform. The search for plasticity at climbing fiber synapses was actually started using recordings of climbing fiber–mediated EPSCs in voltage-clamp mode, enabling an electrophysiological isolation of synaptic events underlying the complex spike. Climbing fiber activation at 5 Hz for 30 s resulted in a lasting reduction of EPSC amplitudes (Hansel and Linden, 2000; Carta et al., 2006). This form of LTD at the climbing fiber input was not accompanied by changes in parallel fiber responses, but it turned out that climbing fiber LTD shares induction requirements with parallel fiber LTD, namely a dependence on postsynaptic Ca transients, mGluR1, and PKC activation (Hansel and Linden, 2000). Climbing fiber LTD is not accompanied by changes in the paired-pulse depression ratio (Hansel and Linden, 2000) and also does not alter the degree of AMPA receptor blockade by a low-affinity competitive antagonist, γ-D-glutamylglycine, which unbinds rapidly from AMPA receptors and can be used as a reporter for changes in glutamate release (Shen et al., 2002). These results suggest that climbing fiber LTD, just like parallel fiber LTD, is postsynaptically expressed. When discussing plasticity at the climbing fiber synapse, it has to be kept in mind that this is a very unusual type of synapse, as climbing fiber activity under physiological conditions results in an all-or-none complex spike. The waveform of complex spikes is composed of an initial Na action potential in the soma, followed by smaller spikelets on top of a plateau. These spikelets likely reflect calcium spike activity in the dendrite, although at the somatic level (where typically the recordings are performed) resurgent Na conductances might be involved as well (for review see Schmolesky et al., 2002). As complex spikes are the physiological responses to climbing fiber stimulation, climbing fiber plasticity was also characterized when complex spikes were recorded in current-clamp mode. Under these conditions, 5-Hz climbing fiber activation for 30 s resulted in a selective reduction of slow complex spike components (most reliably the first spikelet) (Hansel and Linden, 2000; see also Hansel et al., 2001). This effect could be mimicked by partial AMPA receptor blockade using NBQX (1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo-[f]quinoxaline-7-sulfonamide) (Weber et al., 2003), suggesting that

climbing fiber LTD is primarily caused by a reduction in AMPAR-mediated transmission, which subsequently affects dendritic Ca spike activity. This interpretation is supported by experiments in which whole-cell patch-clamp recordings and microfluorometric Ca measurements were simultaneously performed. These recordings showed that climbing fiber LTD is accompanied by a long-term depression of dendritic Ca transients (Weber et al., 2003). Finally, it was shown that climbing fiber LTD is associated with a reduction in the afterhyperpolarization (AHP) following complex spikes (Schmolesky et al., 2005), which might result from the depression of Ca transients. These observations show that LTD at the climbing fiber synapse affects all components of a climbing fiber response, namely the underlying synaptic transmission and subsequently the complex spike, the complex spike-evoked Ca transient, as well as the complex spike AHP. As will be discussed, these alterations (particularly the 'Ca-LTD') have a large effect on the function of Purkinje cells and on parallel fiber plasticity.

4.17.16 Interactions Between LTP and LTD at Parallel Fiber Synapses

Parallel fiber LTD is postsynaptically induced and expressed (for review see Hansel et al., 2001; Ito, 2001, 2002). A mechanism allowing for activity-dependent reversibility of LTD therefore needs to operate postsynaptically as well. Recently, a postsynaptic form of parallel fiber LTP has indeed been described (Lev-Ram et al., 2002; Coesmans et al., 2004). LTP had been reported in earlier studies under conditions when LTD was blocked (e.g., Sakurai, 1990; Shibuki and Okada, 1992), but in these studies the expression side of LTP was not specifically addressed. The first type of parallel fiber LTP that was actually examined in detail turned out to be a presynaptic phenomenon (Salin et al., 1996).

This presynaptic form of LTP is typically induced by brief (4–8 Hz) parallel fiber tetanization and requires presynaptic Ca influx (Salin et al., 1996; Linden, 1997) and activation of Ca-sensitive adenylyl cyclase I (Storm et al., 1998), an enzyme which is concentrated in granule cell presynaptic terminals. The resulting cyclic adenosine monophosphate elevation then activates PKA (Salin et al., 1996) in this same compartment (Linden and Ahn, 1999). A PKA substrate involved in the induction of presynaptic parallel fiber LTP is the active zone protein RIM1α (Lonart et al., 2003).

The postsynaptic form of parallel fiber LTP can be obtained with the same parallel fiber tetanization protocol used for the induction of parallel fiber LTD (1 Hz; 5 min), but in the absence of climbing fiber activity (Lev-Ram et al., 2002; Coesmans et al., 2004). Therefore, the polarity of synaptic gain changes at the parallel fiber input depends on the activity level of the heterosynaptic climbing fiber input. Climbing fiber activity can exert such function as a polarity switch, because LTD induction requires a larger Ca transient than LTP induction (Coesmans et al., 2004). This is a remarkable observation, as it indicates that bidirectional parallel fiber plasticity is governed by a Ca-threshold mechanism that operates inverse to its hippocampal counterpart (**Figure 7**). The additional Ca needed for parallel fiber LTD induction is contributed by climbing fiber–evoked complex spike activity (Konnerth et al., 1992; Wang et al., 2000). In fact, previous induction of climbing fiber LTD (and the associated reduction in dendritic Ca transients) reduces the probability for subsequent induction of parallel fiber LTD (Coesmans et al., 2004). Postsynaptic parallel fiber LTP depends on the activation of protein phosphatases 1, 2A, and 2B, but neither requires PKC activity (Belmeguenai and Hansel, 2005) or αCaMKII activity (Hansel et al., 2006). The postsynaptic expression side of this form of parallel fiber LTP suggests that it might provide a reversal mechanism for LTD. The postsynaptic expression of LTP was confirmed by the absence of changes in the paired-pulse facilitation ratio (Lev-Ram et al., 2002; Coesmans et al., 2004) and by the absence of changes in the degree of AMPA receptor blockade by the low-affinity competitive AMPA receptor antagonist γ-DGG (Coesmans et al., 2004). Postsynaptic LTD and LTP can indeed reverse each other: when LTP was saturated first by applying the LTP-inducing protocol twice, application of the LTD protocol resulted in a depotentiation (**Figure 6**). Subsequent application of the LTP protocol for the third time again potentiated the parallel fiber EPSCs, indicating that the previous LTD protocol had reversed LTP and that the LTP mechanism was not saturated any longer (Coesmans et al., 2004). The mutual reversibility of postsynaptic parallel fiber LTD and LTP is also suggested by the observation that LTP induction involves an NSF-dependent membrane delivery of GluR2 subunits (Kakegawa and Yuzaki, 2005), which are internalized during LTD (**Figure 7**).

4.17.17 Comparison of Bidirectional Plasticity at Hippocampal and Cerebellar Synapses

While this article largely focuses on the description of LTD mechanisms and the function of LTD, we have also discussed how at both hippocampal and cerebellar synapses LTD relates to LTP. This relation is crucial for an understanding of brain plasticity when it comes to the cellular machinery involved in synaptic memory storage, but also when it comes to the functions of LTP and LTD, respectively. A postsynaptic form of parallel fiber LTP was only recently discovered (Lev-Ram et al., 2002), but its arrival on the scene finally establishes bidirectional plasticity at parallel fiber synapses and allows us to directly compare the cellular mechanisms involved in LTP and LTD induction at hippocampal synapses to their cerebellar counterparts. To start with, at both types of synapses different 'tools' are available for plasticity: in contrast to pyramidal neurons, mature Purkinje cells lack functional NMDARs (Crepel et al., 1982) and only weakly express the AMPAR subunit GluR1 (Baude et al., 1994). Purkinje cells, in turn, express the orphan glutamate receptor $\delta 2$ (GluR$\delta 2$), which plays a not well-understood role in parallel fiber LTD (for review see Yuzaki, 2004), but is not expressed in pyramidal cells. Moreover, Purkinje cells lack back-propagating action potentials in the dendrites (Stuart and Häusser, 1994), but fire complex spikes in response to climbing fiber activation (for review see Schmolesky et al., 2002).

Our current understanding of plasticity mechanisms is not yet advanced enough to pinpoint, for example, what consequences the absence of functional NMDARs has for Purkinje cells, or the absence of GluR$\delta 2$ receptors for pyramidal cells. However, we can describe an emerging picture of remarkable differences between hippocampal and cerebellar plasticity, but also of astonishing similarities (see also Hansel, 2005). In several aspects, cerebellar plasticity provides a mirror image of hippocampal plasticity. As described in detail earlier, hippocampal LTP induction requires large Ca transients and the activation of protein kinases (e.g., CaMKII, PKA, PKC), whereas LTD relies on lower Ca transients and the activation of protein phosphatases. In cerebellar plasticity, the Ca and kinase/phosphatase dependencies are inverse to the hippocampal ones (**Figure 7**): LTD induction (by paired parallel fiber and climbing fiber activity) requires larger Ca transients than LTP induction (by parallel fiber activity alone) (Coesmans et al., 2004). Moreover, LTD is PKC dependent (Linden and Connor, 1991; De Zeeuw et al., 1998) and αCaMKII dependent (Hansel et al. 2006), whereas LTP induction requires the activation of protein phosphatases 1, 2A, and 2B (Belmeguenai and Hansel, 2005).

At the level of AMPAR trafficking, the mirror image–like arrangement of induction events is partially confirmed and partially breaks down. Kinase activity promotes the membrane insertion of GluR1 subunits, which dominate trafficking behavior in GluR1/GluR2 heteromers (Song and Huganir, 2002). Three GluR1 phosphorylation sites are discussed in the context of hippocampal LTP induction, namely serine-831 (CaMKII, but for conductance changes), serine-845 (PKA), and most recently, serine-818 (PKC). In GluR1 'phospho-free' mice, NMDAR-dependent LTD is blocked, which has been interpreted as further evidence for the hypothesis that a dephosphorylation at GluR1 promotes GluR1 endocytosis and LTD (Lee et al., 2003). This scenario would indeed provide a mirror image to the phosphorylation events involved in parallel fiber plasticity, which, however, is mediated by GluR2 subunit trafficking. PKCα-mediated phosphorylation at serine-880 causes GluR2 endocytosis and LTD (Leitges et al., 2004), while LTP is phosphatase dependent (PP1/2A/2B; Belmeguenai and Hansel, 2005) and is associated with a membrane insertion of GluR2 subunits (Kakegawa and Yuzaki, 2005).

However, the phosphorylation/AMPAR trafficking events involved in LTP and LTD, particularly at hippocampal synapses, are still not sufficiently well understood to reach such a comprehensive and simplifying view. At the moment it seems safe to say that the cellular events underlying LTP induction indeed differ significantly, simply because there is compelling evidence that hippocampal LTP is mediated in part by a membrane insertion of GluR1 subunits, which are only weakly expressed in Purkinje cells. However, GluR2 subunit trafficking, which clearly mediates parallel fiber plasticity, works similarly in pyramidal cells. GluR2 phosphorylation at serine-880 triggers a clathrin-mediated GluR2 endocytosis at both hippocampal (Man et al., 2000; Seidenman et al., 2003) and cerebellar synapses (Wang and Linden, 2000; Chung et al., 2003). On the other hand, NSF-GluR2 binding promotes GluR2 insertion at both types of synapses (Lüscher et al., 1999; Kakegawa and Yuzaki, 2005). Moreover, recent evidence

suggests that GluR2 rather than GluR1 endocytosis mediates LTD in CA1 pyramidal cells (for review see Malinow, 2003), which would suggest that both types of synapses use the same mechanism for LTD expression. How this scenario fits together with the obviously different Ca signaling requirements and kinase/phosphatase dependences of LTD induction at hippocampal and cerebellar synapses remains to be seen.

4.17.18 Is LTD of the Parallel Fiber–Purkinje Cell Synapse Involved in Motor Learning?

A fruitful approach to testing the hypothesized LTD/motor learning connection has been provided by the generation of mutant mice that lack proteins thought to be required for cerebellar LTD (see Chen and Tonegawa, 1997, for review). Several forms of knockout mice have been reported which have shown impairments in both parallel fiber LTD and motor learning. Mutant mice which lack mGluR1 have severely impaired cerebellar LTD (Aiba et al., 1994; Conquet et al., 1994; Ichise et al., 2000), consistent with previous studies using mGluR1 antagonists or inactivating antibodies. These mice had normal postsynaptic voltage-gated Ca currents and normal paired-pulse facilitation and depression of parallel and climbing fiber synapses, respectively. mGluR1 mutant mice were severely ataxic and showed impairments in several motor coordination tasks. When associative eyeblink conditioning was performed, these animals showed a partial deficit in CR acquisition (Aiba et al, 1994), which may not reflect an inability to produce a CR, but rather an inability to produce the optimal timing of the eyelid closure.

Other knockout mice also have LTD and motor learning deficits. Unfortunately, in some of these cases it is not understood how the missing protein functions in LTD induction. GluRδ2 is a protein which is expressed almost exclusively in Purkinje cell dendritic spines. While a point mutation in this receptor gives rise to a constitutive cation conductance and the *lurcher* phenotype (Zuo et al., 1997), the function of wild-type GluRδ2 is not clear. A GluRδ2 null mouse has impaired cerebellar LTD (Kashiwabuchi et al., 1995), consistent with reports that have used application of antisense oligonucleotides to suppress expression of this protein in culture preparations (Hirano et al., 1995; Jeromin et al., 1996). While the cerebellar cortex appears normal by light microscopy, electron microscopic analysis revealed an ~50% reduction in the number of parallel fiber–Purkinje cell synapses. This mouse is severely ataxic and is retarded in its ability to achieve a form of vestibular compensation: the righting response under a rotation load following unilateral middle ear destruction (Funabiki et al., 1995). These results are supported by more recent observations that injection of an anti-GluRδ2 antibody into the subarachnoidal supracerebellar space caused ataxic gait and poor rotorod test performance (Hirai et al., 2003).

A puzzling LTD defect is found in a mutant mouse which lacks glial fibrillary acidic protein (GFAP), which is, of course, not expressed in neurons. Since at least the early phase of cerebellar LTD may be expressed in reduced preparations that lack glia (Narasimhan and Linden, 1996; Narasimhan et al., 1998), it is likely that this knockout is exerting its effect indirectly. These mice show normal motor coordination, but are severely impaired in parallel fiber LTD and partially impaired in acquisition of associative eyeblink conditioning (Shibuki et al., 1996). Interestingly, these mice show clear improvement with training in a motor coordination task (the rotating rod), indicating that there are forms of motor learning that do not require cerebellar LTD.

There have been several major problems which have complicated the analysis of knockout mice. First, knockout mice have the gene of interest deleted from the earliest stages of development. As a result, these mice often have a complex developmental phenotype. For example, PKCγ, mGluR1 and GluRδ2 (but not GFAP) knockout mice all have cerebellar Purkinje cells that fail to undergo the normal developmental conversion from multiple to mono climbing fiber innervation in early postnatal life (Chen et al., 1995; Kano et al., 1995, 1997; Kurihara et al., 1997). Second, knockout of one gene sometimes produces compensatory upregulation in the expression of other related genes during development. In the CREBa-d (CREB: cAMP response element binding protein) knockout mouse, there is compensatory upregulation of the related transcription factor cAMP response element modulator (Hummler et al., 1994; Blendy et al., 1996). A third complicating factor is that knockout mice have the gene of interest deleted in every cell of the body, not just the cells of interest, making it more difficult to ascribe the knockout's behavioral effects to dysfunction in any one particular structure or cell type. This could be a potential problem for the analysis of behaviors such

as associative eyeblink conditioning and VOR adaptation, which are likely to require use-dependent plasticity at multiple sites, synapses received by both cerebellar Purkinje cells and their targets in the DCN or vestibular nuclei.

To address the latter two complications, an alternative approach has been used (De Zeeuw et al., 1998). Using the promoter of the Purkinje cell-specific gene *pcp-2* (also known as L7; Oberdick et al., 1990), transgenic mice have been created in which a selective inhibitor to a broad range of PKC isoforms (House and Kemp, 1987; Linden and Connor, 1991) is chronically overexpressed. This strategy ensures that, in L7-PKCI mice, PKC inhibition will be restricted to Purkinje cells, and that compensation via upregulation of different PKC isoforms will not succeed in blunting the biochemical effect of the transgene. This transgenic strategy resulted in nearly complete suppression of both cerebellar LTD as assessed in culture and adaptation of the VOR in the intact, behaving animal. In addition, L7-PKCI mice show impaired learning-dependent timing of conditioned eyeblink responses (Koekkoek et al., 2003). The phenotype of these animals was remarkably delimited. They showed normal motor coordination as measured by their ability to display the normal eye movement reflexes, optokinetic reflex, and VOR, as well as by several tests of gross motor coordination (rotorod, thin rod). Basal electrophysiological and morphological features of Purkinje cells were unaltered (with the exception that the Purkinje cells remained multiply innervated by climbing fibers). Similarly, conditional knockout mice lacking cGMP-dependent protein kinase type I (cGKI) selectively in Purkinje cells show impaired LTD and VOR gain adaptation (Feil et al., 2003).

While these mice with Purkinje cell–specific deficits represent a refinement in testing the relationship between parallel fiber LTD and motor learning, there are still caveats and complications which remain. First, the one basal physiological abnormality found in L7-PKCI mice is that about 50% of the Purkinje cells show a persistent multiple climbing fiber innervation, raising the possibility that this could be a cause of their failure to demonstrate VOR adaptation. However, this is unlikely because a PKCγ knockout mouse shows multiple climbing fiber innervation with normal cerebellar LTD and no motor learning deficit (Chen et al., 1995; Kano et al., 1995). Moreover, it has been shown more recently that the elimination of surplus climbing fibers is not entirely blocked in L7-PKCI mice, but only delayed, and that the elimination process is completed in 3- to 6-month-old mice (Goossens et al., 2001) A strategy to alleviate this kind of problem in the future will be to make the L7-PKCI transgene inducible in adult mice. Second, it has recently been reported that the climbing fiber–Purkinje cell synapse undergoes LTD, which, like parallel fiber LTD, requires activation of PKC (Hansel and Linden, 2000). Thus, even inhibition of PKC that is restricted to Purkinje cells cannot have its behavioral effects solely ascribed to parallel fiber LTD. Third, it is clear that PKC does not function in Purkinje cells only to produce LTD of various synapses. For example, it is known that voltage-gated K channels are modulated by PKC in Purkinje cells. Could this or some other function of PKC that is unrelated to LTD underlie the VOR adaptation deficit? While this is not an easy problem to address, future studies will benefit from *in vivo* recording during the appropriate behavioral tasks to distinguish among these possibilities.

While the mutant mouse studies cited here provide strong support for the widely accepted notion that LTD mediates forms of cerebellar motor learning (for review see De Zeeuw and Yeo, 2005), there are also examples of failures to establish such relationship: it has been shown that T-588, a drug that affects Ca release from internal stores, blocks parallel fiber LTD *in vitro* (Kimura et al., 2005), but does not impair associative eyelid conditioning (Welsh et al., 2005). Studies are on the way to examine the effects of disrupting PF-LTP on motor learning. These studies may provide additional insight into how synaptic plasticity contributes to learning and memory. In VOR gain conditioning, LTD is assumed to provide the cellular basis for increasing the VOR gain. When examining properties of reversibility, it has been found that complete reversal can be reached by gain-down training after gain-up training. In contrast, gain-up training after gain-down training led to an incomplete reversal (Boyden and Raymond, 2003; Boyden et al., 2004). It was suggested that this asymmetric reversibility was caused by an involvement of both pre- and postsynaptic LTP in the downregulation of the VOR gain, with only the postsynaptic form of LTP providing a true reversal mechanism. It will also be interesting to study the role of (postsynaptic) LTP in associative eyeblink conditioning. LTP can be observed after parallel fiber stimulation alone, which makes it an ideal candidate mechanism to mediate extinction of conditioned eyeblink responses, as extinction can be obtained by application of the conditioned stimulus alone (for review see Hansel et al., 2001).

4.17.19 Conclusion

In this article, we have provided a portrait of LTD by describing the phenomenon *per se* and by summarizing and explaining the cellular signaling cascades that are involved in LTD induction and expression. At the end of this article, we would like to address a question (once more) that appears particularly crucial for plasticity at large: if LTP provides a cellular correlate of information storage and learning (synaptic memory), why is there a need for LTD?

We would like to discuss this question using again the hippocampal area CA1 (and with it, glutamatergic synapses onto cortical pyramidal cells in general) and the cerebellum as examples.

We hasten to add, however, that the list of possible LTD mechanisms is not exhausted by the study of the two synapses we have highlighted in this article. Inhibitory synapses have also been shown to exhibit LTD (Marty and Llano, 1995; Aizenman et al., 1998). Other forms of LTD have been described in excitatory synapses at other locations, such as the striatum (Lovinger et al., 1993; Calabresi et al., 1999), amygdala (Li et al., 1998; Wang and Gean, 1999), neocortex (Artola et al., 1996; Egger et al., 1999), other parts of the hippocampal formation (Kobayashi et al., 1996; Manahan-Vaughan, 1998), and the olfactory bulb (Mutoh et al., 2005). Time will tell whether these forms of LTD use expression mechanisms which overlap with those we have described here.

By intuition, most researchers in the plasticity field would likely associate LTP with learning and LTD with forgetting. This might be due to the fact that a strengthening of synaptic transmission is easily perceived as information storage, and a weakening of synaptic strength as erasing previously stored information. Moreover, LTP and LTD, as characterized in the adult, have strong overlap with cellular mechanisms involved in strengthening (establishing) and weakening (disconnecting) synapses in the developing brain (e.g., Rittenhouse et al., 1999; for review see Singer, 1995), and synapse elimination certainly qualifies as a form of deleting previously present 'information.' Over the last years, experimental evidence from the hippocampus supports this view; agents that block signaling factors for LTP induction (e.g., NMDARs), or corresponding genetic manipulations, impair hippocampus-dependent learning, for example, in spatial learning tasks (for review see Silva, 2003). Along these lines, mutations that enhance LTP enhance learning. This effect was observed in transgenic mice overexpressing NR2B receptors (Tang et al., 1999), but also in transgenic mice expressing a constitutively active form of H-ras, which facilitates glutamate release through increased extracellular signal-regulated protein kinase (ERK)-dependent phosphorylation of synapsin I (Kushner et al., 2005). Similarly, the protein phosphatases PP1 and PP2B (calcineurin), which are involved in LTD induction, have been shown to constrain LTP and learning (Malleret et al., 2001; Genoux et al., 2002), suggesting that LTD itself constrains learning and promotes forgetting. On the other hand, a forebrain-specific calcineurin knockout resulted in a blockade of LTD, but selectively impaired hippocampus-dependent working and episodic-like memory tasks (Zeng et al., 2001), which suggests that LTD inhibition does not automatically facilitate learning. Moreover, LTD is involved in novelty acquisition (Manahan-Vaughan and Braunewell, 1999; Etkin et al., 2006). It might, therefore, be impossible to generally state that LTP equals learning and LTD equals forgetting. Rather, it will be necessary to look in detail at the type of synapse involved and the particular learning task. At cortical synapses, where the correlation to behavioral outputs is far more difficult to establish, the concept of LTD and LTP as extremes along an array of graded synaptic weights might be more useful. At this synaptic level, the need for reversal mechanisms (e.g., LTD for potentiated synapses) is more obvious, as reversibility prevents saturation and, consequently, the subsequent loss of plasticity. It has to be kept in mind, though, that LTP can act as much as a reversal mechanism for LTD as LTD can be a reversal mechanism for LTP.

In the cerebellum, it is easier to assign a role in motor learning to parallel fiber LTD, although even in the cerebellum this link is not established beyond any doubt (see earlier). A final question then is why the cerebellum uses LTD for learning. A possible answer can be found in the range of spontaneous action potential discharge rates observed in pyramidal cells and Purkinje cells. Recent *in vivo* whole-cell patch-clamp recordings in awake animals show that the spontaneous spike rates in cortical neurons are extremely low; e.g., in the somatosensory cortex they are well below 0.1 Hz (Margrie et al., 2002). In contrast, Purkinje cells have spontaneous discharge rates of 30 Hz, and simple spike rates can transiently exceed 200 Hz upon sensory activation (see Monsivais et al., 2005). These high discharge rates provide a strong, tonic inhibition to the target cells in the

DCN. Parallel fiber LTD leads to a disinhibition of DCN cells, facilitating transmission and/or LTP induction at mossy fiber–DCN synapses (Medina et al., 2002). Thus, it seems that Purkinje cells use different plasticity rules than pyramidal cells, because they are GABAergic projection neurons and because they belong to the few types of neurons with a high spontaneous discharge rate. These features assign different roles to Purkinje cells in the local cerebellar network than those assigned to pyramidal cells in their local networks and projection areas.

Ten years ago, the LTD club was very small, and LTD seemed like an obscure synaptic phenomenon. Indeed, it is probably safe to say that at the end of the 1980s there was little general interest in LTD, or even any strong conviction that homosynaptic depression existed at all outside the cerebellum. This situation has clearly changed. The major challenge for the next 10 years will be to see if the role of LTD in the brain lives up to its lofty theoretical promise.

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4.18 GABAergic Interneurons in Synaptic Plasticity and Information Storage

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4.18.1 Introduction

At the level of the hippocampal network, the net flow of information in the CA1 region is strongly modulated by the action of the nonpyramidal neurons, whose cell bodies are distributed throughout all layers of the hippocampus (for reviews, see Freund and Buzsaki, 1996; McBain and Fisahn, 2001). The vast majority of these cells are glutamic acid decarboxylase (GAD)–positive, inhibitory interneurons whose axons target different domains of the pyramidal cell dendritic tree. This selective targeting is important for the control of principal cell generation of somatic sodium spikes or dendritic calcium spikes (Miles et al., 1996). Moreover, fast γ-aminobutyric acid ($GABA_A$) receptor–mediated synaptic inhibition plays a critical role in determining the net excitability of the hippocampus. The frequency of tonic $GABA_A$-mediated inhibitory postsynaptic currents (IPSCs) received by pyramidal neurons is almost 10 times that of glutamatergic excitatory postsynaptic currents (EPSCs) (McBain and Dingledine, 1992; Soltesz and Mody, 1994). In addition to their control of cell excitability, interneurons also underlie the synchronization of pyramidal neuron firing (Cobb et al., 1995) and have been proposed to be involved in the generation of large-scale network oscillations (Buzsaki and Chrobak, 1995; Klausberger et al., 2003, 2004, 2005; Somogyi and Klausberger, 2005). Therefore, the occurrence of synaptic plasticity in nonpyramidal cells, in addition to that occurring in principal neurons, would greatly increase both the

power and the level of complexity of signal processing and of information storage in the hippocampus.

It is important to point out that interneurons are embedded in complex networks of highly interconnected cells. As such, they receive both excitatory and inhibitory inputs from a variety of sources as well as making inhibitory synaptic outputs onto many distinct structural targets within their particular circuit. Consequently, plasticity of the interneuron circuit may not only arise at the level of excitatory (or inhibitory) drive onto the interneuron but may also occur at the level of inhibitory input onto the cell's target. In this chapter, we consider, first, specific forms of synaptic plasticity of excitatory input onto interneuron populations, which is then followed by a review of the synaptic plasticity of GABAergic output generated by interneurons on their target cells.

4.18.2 Long-Lasting Plasticity of Excitatory Synaptic Input onto Interneurons

Despite rapid progress in elucidating the cellular mechanisms responsible for long-term plasticity at excitatory synapses between principal cells (Bliss and Collingridge, 1993; Nicoll, 2003; Collingridge et al., 2004), relatively little insight has been gained concerning mechanisms of plasticity of excitatory drive onto inhibitory interneurons. Moreover, excitatory transmission typically displays cell-target-specific regulation, indicating that the same rules governing plasticity at principal cell synapses cannot necessarily be applied to equivalent inputs onto interneurons (Maccaferri et al., 1998; Markram et al., 1998; Reyes et al., 1998; Scanziani et al., 1998; McBain et al., 1999; Toth et al., 2000; Toth and McBain, 2000). In an earlier review (McBain and Maccaferri, 1997), we described the technical complexities associated with trying to tease apart 'true' mechanisms of plasticity associated with excitatory synapses transmitted onto identified inhibitory interneurons from network effects from passively propagated forms of plasticity that had their origins at principal cell synapses. Although these issues are still highly relevant, since that time a number of careful studies have directly demonstrated a number of novel plastic mechanisms arising between excitatory synapses and local circuit inhibitory neurons. Interestingly, many of these studies show activity-dependent strengthening or weakening of excitatory synaptic transmission onto interneurons, which occurs via mechanisms distinct from those observed at principal cell synapses within the same subfield. In this section we highlight a number of these studies.

4.18.3 The MF-CA3 Network

In the hippocampus, mossy fiber (MF) axons arising from dentate gyrus granule cells provide excitatory glutamatergic input both to the dentate gyrus/hilar region and to area CA3. Within the CA3 subfield, mossy fiber axons synapse with both CA3 pyramidal cells (PYRs) and stratum lucidum inhibitory interneurons (SLINs). This synaptic arrangement generates a strong feedforward inhibitory circuit, as SLINs, which provide inhibitory inputs to CA3 PYRs, are more readily excited by MFs than are PYRs (Lawrence et al., 2004). Such feedforward inhibitory circuits provide exquisite control over network excitability through surround inhibition and by shaping temporal windows for input summation and spike generation in principal cell targets (Pouille and Scanziani, 2001; Lawrence and McBain, 2003; Mori et al., 2004). Thus, the plasticity of MF feedforward inhibition has the capacity to dramatically influence CA3 information processing.

Interestingly, MF axons innervate their divergent CA3 postsynaptic targets via anatomically distinct presynaptic elements: Inputs onto PYRs are formed by large, complex presynaptic boutons (mossy terminals), while SLINs are predominantly innervated by small en passant varicosities or filopodial extensions (Acsady et al., 1998) (**Figure 1**). Postsynaptically, α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid subtype glutamate receptors (AMPARs) at MF-SLIN synapses comprise a continuum ranging from GluR2-lacking, Ca^{2+}-permeable channels (CP-AMPARs) to GluR2-containing, Ca^{2+}-impermeable channels (CI-AMPARs), whereas mature MF-PYR synapses contain only CI-AMPARs (Toth and McBain, 1998; Lei and McBain, 2002).

4.18.3.1 MF–Stratum Lucidum Interneuron Synapses

Interneurons of the stratum lucidum are primarily targeted by the filopodial extensions and en passant MF synapses, but rarely by the larger MF boutons that typically contact principal cells (Acsady et al., 1998) (**Figure 1**). Given that the filopodial extensions originate from the large MF terminal, it might be assumed that any change in presynaptic release

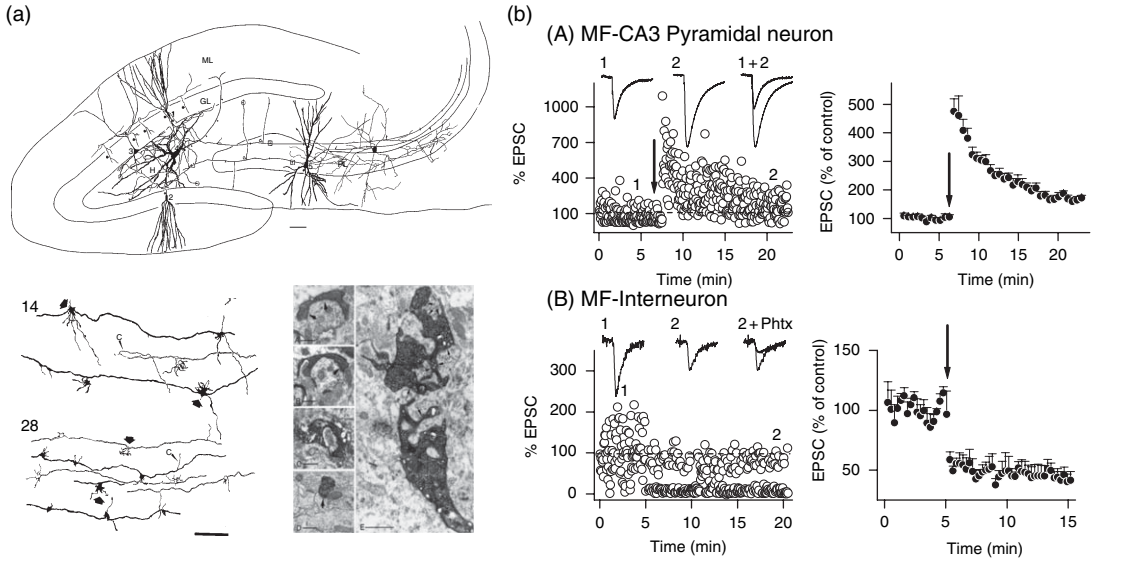


Figure 1 Basic anatomical elements of mossy fiber–CA3 circuitry. (a) The dentate gyrus and proximal portion of the hippocampus (mossy fibers [MFs]). Granule cells (labeled 1) give rise to extensive collateral plexuses, the MFs that are distributed throughout much of the hilus (H) and make synaptic contact with both dentate gyrus basket cells (2) and mossy cells (3). As the parent MF approaches the CA3 pyramidal cell layer (PL), the large presynaptic MF expansions begin to appear and are typically 140–200 μm apart for the entire length of the axon. These expansions form complex synapses on the proximal apical dendrites of pyramidal cells (4). The small filopodial or en passant terminals make synapses onto local circuit interneurons primarily located within the stratum lucidum (5). Scale bar, 100 μm. Left lower panel: Camera lucida drawings of Golgi-impregnated MFs illustrate the filopodial extensions emerging from the large complex MF terminal (large arrows). By postnatal day (P) 14, mature MF expansions are common. At P28 there is an increase in the number of MF expansions that have reached adult shape and size, and the lengths of the individual expansions have decreased to adult levels. Scale bar, 50 μm. Right lower panel: Electron micrographs of different terminal types along MFs in the CA3 region (A–C, E) and of a CA3 pyramidal cell terminal (D). All electron micrographs have the same magnification, for comparison of the relative size of the terminals. (A, B) A small en passant terminal establishes a single asymmetrical synapse on a dendritic shaft with long perforated postsynaptic density (arrows). (C) A filopodial extension of a mossy terminal forms a synapse (arrow) with a substance-P-receptor-immunoreactive interneuron. (D) The postsynaptic target of a pyramidal cell terminal is a simple spine of a CA1 pyramidal neuron. (E) A large, double-headed mossy terminal forms multiple contacts (arrows) with thorny excrescences of a CA3 pyramidal cell. The individual release sites are short. Scale bars, 0.5 μm (A–D) and 1 μm (E). (b) High-frequency stimulation (100 Hz for 1 s) of MF afferents onto CA3 pyramidal cells results in robust NMDAR-independent long-term potentiation, as evidenced by the increase in EPEC amplitude. Left panel represents an individual experiment with associated averaged EPSCs, as indicated by numbers above. Right panel represents pooled data. The identical induction paradigm results in a long-term depression at filopodial/en passant MF synapses onto CA3 stratum lucidum interneurons. These data highlight that, although expression of both forms of plasticity occur within the presynaptic terminal, an opposing change in synaptic strength occurs. Panel (a, top) reproduced from Claiborne et al. (1986) Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.; the stratum lucidum inhibitory interneuron (cell 5) was added to the original figure by JJ Lawrence and CJ McBain. Lower left panel in (a) reproduced from Amaral and Dent (1981) Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc. Lower right panel in (a) reproduced from Acsady et al. (1998) from the Society for Neuroscience. Panel (b) reproduced from Toth et al. (2000) from the Society for Neuroscience.

probability arising from long-term potentiation (LTP) in the large MF bouton would distribute evenly to all synapses, resulting in long-term changes at both MF–pyramidal cell and interneuron synapses. However, quite the opposite is the case. At MF–CA3 st. lucidum interneuron synapses, the same high-frequency nonassociative protocol that induces LTP at principal cell synapses induces two forms of long-term depression (LTD) at interneuron synapses (**Figure 1**; Lei and McBain, 2004; Pelkey et al., 2005). Postsynaptically, AMPARs at MF-SLIN synapses comprise a continuum ranging from GluR2-lacking, Ca^{2+}-permeable channels (CP-AMPARs) to GluR2-containing, Ca^{2+}-impermeable channels (CI-AMPARs) (Toth et al., 2000; Lei and McBain, 2002). Both CI-AMPAR- and CP-AMPAR-containing MF-SLIN synapses exhibit LTD in response to high-frequency stimulation (HFS) that arises from two distinct mechanisms (**Figure 2**; Maccaferri et al., 1998; Toth et al., 2000; Lei and McBain, 2002, 2004; Pelkey

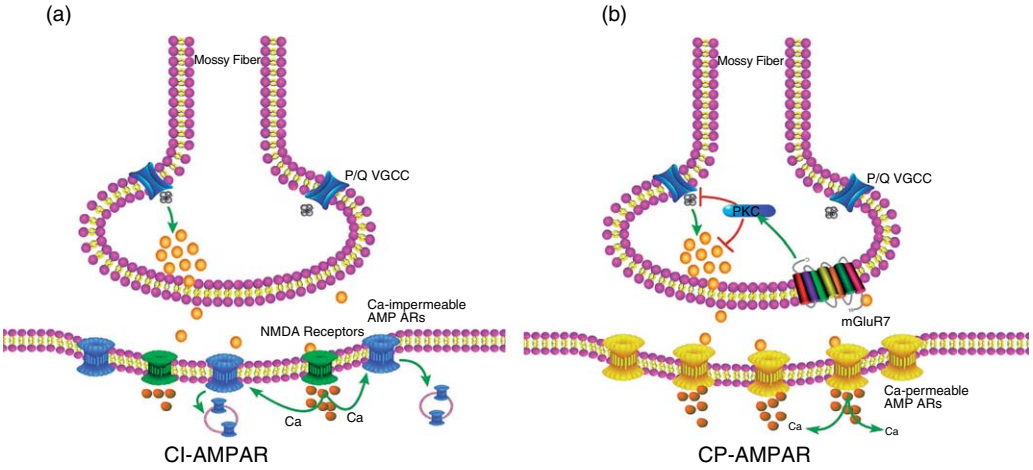


Figure 2 Two forms of mossy fiber-CA3 interneuron long-term depression (LTD). (a) Long-term depression at mossy fiber–interneuron synapses composed of Ca-impermeable α-amino-3-hydroxy-5-methyl isoxazole-4-propionic acid (AMPA) receptors has a postsynaptic locus of induction and expression, is *N*-methyl-D-aspartate (NMDA) receptor dependent, and involves an endocytosis of surface AMPA receptors reminiscent of NMDAR-dependent LTD observed at principal cell synapses. (b) LTD at mossy fiber–interneuron synapses composed of Ca-permeable AMPA receptors has a requirement for a postsynaptic increase in intracellular Ca but is expressed presynaptically. This form of LTD is NMDA receptor independent and requires activation of presynaptic mGluR7 and downstream PKC-dependent cascades to reduce transmitter release probability via a reduction of the voltage-gated Ca transient generated by P/Q Ca channels.

et al., 2005). Induction of each is blocked by inclusion of the Ca^{2+} chelator BAPTA in the postsynaptic compartment, indicating a postsynaptic induction locus for each (cf. MF CA3 pyramidal cell LTP described following). Activity-induced LTD of MF-SLIN transmission at CI-AMPAR synapses is blocked by inclusion of the *N*-methyl-D-aspartate receptor (NMDAR) antagonist, D-AP5, indicating that this form of plasticity is mediated in part by postsynaptic NMDAR activation. Expression of this form of LTD proceeds via subsequent AMPAR internalization by a mechanism similar, but not identical, to postsynaptically expressed LTD observed at excitatory synapses throughout the central nervous system (Lei and McBain, 2002, 2004). Consistent with a postsynaptic expression locus, this form of LTD is not accompanied by changes in the paired pulse ratio, the CV (coefficient of variation), or the synaptic failure rate – parameters typically used to monitor presynaptic mechanisms. Although none of these parameters on their own rigorously supports presynaptic changes (e.g., postsynaptic conversion of silent to active synapses will also change these parameters), an independent test of whether this NMDA receptor–dependent form of mossy fiber plasticity was postsynaptic made use of the low-affinity α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) receptor antagonist γ-D-glutamyl glycine (γ-DGG) (Lei and McBain, 2004). In these experiments, Lei and McBain (2004) demonstrated that the magnitude of block of the evoked synaptic event by γ-DGG was unaltered following induction of NMDA-dependent MF-interneuron long-term depression. These data argue that the transmitter-release concentration profile is unchanged following LTD expression. Taken together, these data support the conclusion that this form of plasticity has both its induction and expression locus within the postsynaptic compartment.

In contrast, LTD at CP-AMPAR containing MF-SLIN synapses is NMDAR independent and is expressed presynaptically as a reduction in transmitter release (Lei and McBain, 2004). Again, in contrast to postsynaptic NMDA receptor LTD at CI-AMPARs, this form of plasticity is accompanied by changes in failure rate, paired pulse ratio, and CV, arguing for a presynaptic expression locus. Consistent with this conclusion was the observation that the magnitude of block of the evoked EPSC by γ-DGG was greater following LTD expression. Together, these observations are consistent with NMDAR-independent LTD resulting from a reduction in presynaptic transmitter release through a mechanism that may arise as a reduction in transmitter release probability, by a reduction in multivesicular release, or a mechanism akin to reverse 'kiss and run' (Lei and McBain, 2004; Harata et al., 2006).

Of particular interest is that, whereas MF–CA3 pyramidal cell LTP involves PKA-dependent cascades, transmission and plasticity at either form of the MF-interneuron synapse is largely insensitive to the manipulations that elevate cyclic adenosine monophosphate (cAMP) levels (Maccaferri et al., 1998). In our original experiments, application of forskolin induced a robust potentiation of MF–pyramidal transmission, consistent with MF–principal cell plasticity arising via an adenylyl cyclase–cAMP mechanism involving rab3/Rim1a modulation of synaptic transmission (Nicoll and Schmitz, 2005). The same experiment applied to MF interneuron synapses revealed a complete lack of sensitivity to exogenously applied forskolin, indicating that perhaps cAMP-dependent cascades were either absent or rendered inactive at MF interneuron synapses. This compartmentalization of the biochemical machinery highlights one potential functional utility of the filopodial versus large terminal arrangement of the MF presynaptic complex. How the cAMP-dependent cascade is segregated across the interterminal compartments is presently not understood.

LTD at MF–stratum lucidum Ca^{2+}-permeable AMPA receptor synapses is blocked by antagonists protein kinase C (PKC) (Pelkey et al., 2005) (PKC pathways are also implicated in MF–basket cell synapse LTP; see following for discussion). Furthermore, activation of mGluR7, which specifically localizes to MF terminals opposing stratum lucidum interneurons and not PYRs (Shigemoto et al., 1996, 1997), is an important step in the induction of presynaptic MF-SLIN LTD (**Figure 2(b)**; Pelkey et al., 2005). A brief application of the Group III agonist, L-AP4, at a concentration capable of activating mGluR7 induces a long-lasting depression of excitatory transmission between MFs and CP-AMPAR synapses. This synaptic depression far outlasts the drug application time and is not reversed by subsequent application of a broad-spectrum Group III mGluR antagonist, indicating that L-AP4 induces a chemical form of LTD at these synapses. This chemical form of LTD shares all of the hallmark features of activity-dependent LTD at these synapses, an increase in the paired pulse ratio, increased CV, and failure rate consistent with a presynaptic locus of expression. Interestingly, application of L-AP4 in the absence of continued synaptic stimulation failed to convert a short-term depression into LTD. Furthermore, introduction of the calcium chelator 1,2-bis(*o*-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid (BAPTA) into the postsynaptic compartment blocked chemical LTD induction. Taken together, these data suggest that, like HFS-induced LTD at these synapses, there exists a requirement for postsynaptic activity for chemical LTD induction. Finally, of particular interest, blockade of mGluR7 activation during HFS not only prevented LTD induction but also revealed a previously unobserved posttetanic potentiation.

As it turns out, mGluR7 not only functions as a conventional autoreceptor at these synapses but also acts as a metaplastic switch at MF-SLIN synapses. Importantly, mGluR7 activation and cell surface expression governs the direction of plasticity. In naïve slices, mGluR7 activation during HFS generates MF-SLIN LTD, depressing presynaptic release through a PKC-dependent mechanism. Following agonist exposure, mGluR7 undergoes internalization, unmasking the ability of MF-SLIN synapses to undergo presynaptic potentiation or de-depression in response to the same HFS that induced LTD in naïve slices. Thus, the selective mGluR7 accumulation at MF terminals contacting SLINs and not PYRs provides cell-target-specific plasticity and bidirectional control of feedforward inhibition (Pelkey et al., 2005).

Recently one of the downstream targets of mGluR7 activation at these synapses was elucidated. Pelkey et al. (2006) observed that transmission at MF filopodia is supported primarily by P/Q-type voltage-gated calcium channel activity, with only a minor role for N-type channels. This presynaptic calcium channel arrangement is identical to that observed at the large MF bouton inputs into pyramidal cells and ruled out a role for differential voltage-gated calcium channel distribution as a mechanism to explain the functional divergence of the two presynaptic release sites. Of particular importance, they showed that LTD induced by either exogenous application of L-AP4 or HFS resulted in a persistent depression of the presynaptic voltage-gated calcium channel signal monitored by two-photon microscopy. Importantly, this depression of the calcium transient arose through a preferential depression of P/Q calcium channels: Blockade of P/Q channels prevented both the depression of synaptic events and calcium transients by either L-AP4 or HFS. These data indicate that synaptic depression at MF–CP-AMPAR interneuron synapses arises by an mGluR7-dependent persistent reduction in P/Q calcium channel function.

Bidirectional synaptic plasticity of excitatory synaptic transmission onto interneurons has been reported at three other synapses: dentate gyrus granule

cell–basket cell synapses, CA3 pyramidal cell–stratum radiatum interneuron synapses, and CA1 pyramidal cell–stratum oriens–alveus interneuron synapses. In the next section each of these synapses is discussed in turn.

4.18.3.2 MF–Dentate Gyrus Basket Cell Synapses

In experiments employing paired recordings between granule cells and basket cells of the dentate gyrus, or low-frequency stimulation of afferent fibers, Jonas and colleagues (Alle et al., 2001) demonstrated an associative form of MF posttetanic potentiation (PTP) and LTP. Analysis of failures, coefficient of variation, and paired pulse modulation indicated that both PTP and LTP are expressed in the presynaptic compartment. The mechanism was reminiscent, but not identical, to MF–CA3 pyramidal cell LTP. One major discrepancy between MF–pyramidal cell synapse LTP and MF–basket cell LTP was the presynaptic second messenger systems engaged. While MF–CA3 pyramidal cell LTP involves PKA-dependent cascades, MF–basket cell LTP primarily involved a PKC-dependent cascade. In addition, induction of associative LTP was blocked by postsynaptic infusion of the Ca^{2+} chelator BAPTA, indicating a postsynaptic locus of MF–basket cell LTP induction. The induction locus for MF–CA3 pyramidal cell LTP remains somewhat controversial (Nicoll and Schmitz, 2005). Interestingly, by using a nonassociative protocol, in which the postsynaptic cell was held under voltage clamp and incapable of firing action potentials, the same authors demonstrated a long-term depression at MF–basket cell synapses. This observation is consistent with the LTD observed at MF synapses onto stratum lucidum interneurons in the CA3 hippocampus (Maccaferri et al., 1998; Toth et al., 2000) (see earlier for further details). In conclusion, these data again highlight that, although both forms of LTP (MF–CA3 pyramidal cell and MF–basket cell) are expressed presynaptically, there are major differences in both the induction and expression mechanisms that are critically dependent on the identity of the postsynaptic target (Lawrence and McBain, 2003).

4.18.3.3 CA3 Collateral–Stratum Radiatum Interneuron Synapses

McMahon and Kauer (1997) made the important observation that excitatory synapses onto CA1 stratum radiatum interneurons weaken (LTD) in response to a high-frequency stimulation paradigm that typically strengthens (LTP) transmission at the same synapses made onto CA1 pyramidal cell synapses. This was the first demonstration of HFS–induced long-term depression at an excitatory synapse onto a local circuit inhibitory interneuron. Moreover, this LTD was not synapse specific, with depression being triggered at multiple excitatory synapses onto the same interneuron despite HFS of only a single set of synapses. This form of heterosynaptic depression was observed in numerous morphological cell types that were considered to participate in feedforward inhibitory input and suggested that LTD in response to HFS may be a common principal among interneurons.

In a related expansion of this initial observation, Dingledine and colleagues demonstrated that excitatory synaptic transmission between CA3 collateral–stratum radiatum interneuron synapses composed of CP-AMPARs also depressed in response to a HFS induction paradigm (Laezza et al., 1999). This form of LTD was originally reported to arise via a presynaptic mGluR7-dependent form of depression, similar to that observed at MF–stratum lucidum interneuron synapses (Pelkey et al., 2005). However, a subsequent study from the same group then demonstrated a form of bidirectional plasticity at these synapses that depended on postsynaptic membrane potential and NMDAR activation (Laezza and Dingledine, 2004). At synapses expressing CP-AMPARs, HFS of CA3 pyramidal cell collaterals, while voltage clamping the cell at depolarized potentials to relieve the Mg block of the NMDA receptor, resulted in LTP. LTP was prevented by hyperpolarizing the cell to −70 mV, strongly depolarizing the cell to 0 mV (presumably to reduce Ca influx through the NMDAR), or when BAPTA was introduced into the postsynaptic cell. Together these data argued for an entirely postsynaptic induction and expression locus for LTP. This study suggests that stratum radiatum interneurons have an exquisite sensitivity to postsynaptic Ca levels and that only small changes in the Ca driving force are required to convert LTP (induced at −30 mV) into LTD (LTD was observed at synapses clamped at both 0 mV and −70 mV), perhaps reflecting differential Ca entry via both NMDA receptors and CP-AMPARs. Unfortunately, no role was described or considered for the presynaptically expressed mGluR7 in LTP induction or expression, which they had previously implicated as a requirement for LTD at the same synapses. However, the data would

suggest that activation of postsynaptic NMDA receptors is capable of overriding the HFS-induced, mGluR7-dependent changes in presynaptic transmitter release properties associated with LTD.

It is worthwhile pointing out that this novel bidirectional plasticity is fundamentally different from the mGluR7-dependent plasticity described at MF–CP AMPAR stratum lucidum interneuron synapses. When NMDAR signaling is left intact and postsynaptic cells are held close to resting membrane potential, CP-AMPAR containing MF-SLIN synapses exhibit only LTD following HFS (Lei and McBain, 2002), while CP-AMPAR containing collateral–radiatum interneuron synapses give nonuniform responses to HFS (Laezza and Dingledine, 2004). Moreover, rather than NMDAR activation or membrane depolarization, potentiation of MF-SLIN synapses occurs in response to HFS only following the *removal* of mGluR7 from presynaptic terminals.

4.18.3.4 CA1 Pyramidal Cell–Stratum Oriens–Alveus Interneuron Synapses

Excitatory synapses made onto a variety of stratum oriens–alveus interneuron subpopulations express an mGluR1-dependent form of long-term potentiation in response to postsynaptic depolarization and theta burst stimulation of excitatory afferents (**Figure 3**). This form of LTP is NMDAR independent but requires postsynaptic activation of mGluR1a receptors and a consequent postsynaptic Ca elevation (Perez et al., 2001; Lapointe et al., 2004). Confirmation that this form of plasticity is critically dependent of metabotropic glutamate receptor function came from the important observation that LTP is absent in the mGluR1-knockout mouse. Interestingly, like a number of other forms of interneuron plasticity, it occurs only at CP-AMPAR synapses but surprisingly does not appear to require Ca flux through the CP-AMPAR for induction or expression (Topolnik et al., 2005). Although induction required activation of postsynaptic mGluR1a, expression was accompanied by changes in both the paired pulse ratio and coefficient of variation, indicating a role for presynaptic expression. Another interesting feature of this form of LTP was its tight correlation with the presence of presynaptic group II mGluRs but not group III mGluRs (Lapointe et al., 2004). Recently, this same group identified an important piece of the signaling cascade puzzle required for mGluR1a-dependent LTP (Topolnik et al., 2005). Using two-photon imaging of dendritic Ca signals in oriens-alveus interneurons, they convincingly demonstrated that mGluR1a activation induces a Ca elevation that arises in part from transient receptor potential (TRP) channel activation, with a minor component arising from Ca release from ryanodine-sensitive intracellular stores. The mGluR1a/TRP Ca signal depends on both Src tyrosine kinase and

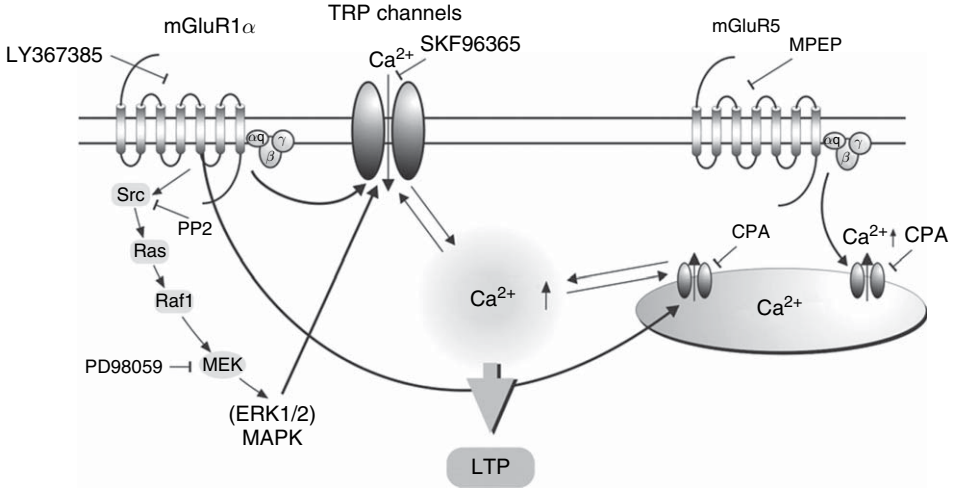


Figure 3 mGluR1-dependent interneuron plasticity. Schematic representation of distinct mGluR1 and mGluR5 Ca^{2+} signaling pathways and their role in long-term potentiation (LTP) induction in O/A interneurons mGluR1 activation is linked to two parallel Ca^{2+} signaling pathways: one leading to Ca^{2+} entry via tryptophan (TRP) channels and a second producing Ca^{2+} release from intracellular stores. Src/ERK-dependent Ca^{2+} entry via TRP channels and intracellular release are necessary for LTP induction. mGluR5 activation causes intracellular Ca^{2+} release that does not require Src/ERK and TRP channels and does not appear to be involved in LTP induction. (Topolnik L, Azzi M, Morin F, Kougioumoutzakis A, and Lacaillc JC (2006) mGluR1/5 subtype-specific calcium signalling and induction of long-term potentiation in rat hippocampal oriens/alveus interneurones. *J. Physiol.* 575: 115–131.)

extracellular signal-regulated protein kinase (ERK) activation (**Figure 3**). This raises an important issue that suggests that ERK signaling is necessary for receptor-activated Ca signaling in interneurons, whereas in principal cells it may produce long-term changes involving gene expression and protein synthesis (Kelleher et al., 2004a, b; Topolnik et al., 2006). One important feature of this plasticity is that the ERK inhibitor PD98059 and the SERCA pump inhibitor CPA (cyclopiazonic acid) not only prevent LTP induction but also uncover a novel form of long-term depression (Topolnik et al., 2006). Similar effects were also observed using SKF96365, a TRP channel inhibitor.

4.18.3.5 NMDAR-Dependent LTP at Schaffer Collateral Stratum Radiatum Interneuron Synapses

Early attempts to identify long-term plastic changes in excitatory synaptic transmission onto interneurons focused on detecting potential NMDAR-dependent mechanisms, since this is the most common form of plasticity observed at hippocampal principal cell excitatory synapses. At principal cell synapses, NMDAR-dependent LTP relies on a transient Ca elevation accompanying NMDA receptor activation, requires activation of Ca-dependent CaMKII, and is thought to arise via a mechanism of 'unsilencing' NMDAR-containing synapses involving the trafficking of AMPA receptor subunits (for reviews, see Nicoll, 2003; Collingridge et al., 2004). Successful attempts to observe a similar NMDAR-dependent form of LTP (or LTD) on interneurons of the CA1 hippocampus were few (Ouardouz and Lacaille, 1995; Cowan et al., 1998; Laezza and Dingledine, 2004). Maccaferri and McBain (1996) performed an extensive search for NMDAR-dependent LTP, using the perforated patch recording configuration to prevent perturbation of the intracellular environment, whose preservation appears critical for LTP induction at principal cell synapses. This approach yielded no evidence for NMDAR-dependent LTP on a number of subpopulations of CA1 interneuron. Given that NMDAR-dependent LTP in principal cells requires activation of CaMKII and that interneurons are generally considered to lack CaMKII, it was perhaps not surprising that this form of LTP was absent from inhibitory interneuron synapses.

In the intervening years, although a number of other forms of LTP and LTD have been described at excitatory synapses on interneurons, evidence for a hebbian form of interneuron LTP requiring NMDA receptors was elusive. However, in 2005, Lamsa et al. (2005) described a hebbian form of NMDAR-dependent LTP at excitatory synapses made onto feedforward inhibitory neurons of the CA1 stratum radiatum. Again, using the perforated patch clamp recording configuration, they demonstrated that, like NMDAR-dependent LTP onto principal cells, pairing-induced plasticity on interneurons was blocked by inclusion of an NMDAR antagonist, was not accompanied by changes in the paired pulse ratio, and was absent in recordings made with whole-cell pipettes. This form of LTP occurred in about 40% of cells tested and, importantly, occurred on cells subsequently identified as spine free, indicating that NMDAR-dependent plasticity is not limited to spines on pyramidal cells. This observation is consistent with the observation that some interneuron types can form spatially restricted Ca domains within their aspiny dendrites (Goldberg et al., 2003). Another important conclusion of this study was the observation that strengthening of excitatory transmission onto interneurons could also influence the degree of feedforward inhibitory drive onto pyramidal cells. This, the authors argued, was a feature important in preventing a potential 'degradation' of the feedforward pathway during NMDAR-dependent LTP of principal cell synapses, and they emphasized that this may be a key feature in ensuring the "fidelity of information processing" (Lamsa et al., 2005).

Several issues here are worthy of further discussion. Although on the surface this form of LTP shares many phenomenological properties with NMDAR-dependent LTP at principal cell synapses, the actual downstream mechanisms of NMDAR activation are completely unexplored. However, given the apparent lack of CaMKII in interneurons (Sik et al., 1998) and the observation that interneurons typically lack silent synapses (Nusser et al., 1998b; although see Wang and Kelly, 2001), it is unlikely that it shares a mechanism identical to NMDAR-dependent LTP at Schaffer collateral synapses onto principal cells. Interestingly, this feature is also true for the NMDAR-dependent long-term depression observed at MF–CI AMPAR interneuron synapses, since interneurons also lack calcineurin (Sik et al., 1998), a key signaling cascade required for NMDAR-dependent LTD (see earlier discussion). Taken together, it would appear that, although interneurons are capable of generating NMDAR-dependent forms of plasticity, the key downstream signaling cascades

responsible for plasticity expression are likely to differ from their principal cell counterparts.

Second, a consideration of the network connectivity of inhibitory interneurons raises an important issue *against* the author's claim that NMDAR LTP on feedforward interneurons is important for the preservation of the "fidelity of information processing." The axons of inhibitory interneurons target hundreds, if not thousands, of principal neuron targets. Furthermore, Schaffer collateral–mediated excitatory transmission onto feedforward interneurons is robust, and in the case of parv albumin (PV)-positive, CB1 receptor–negative interneurons, which provide the bulk of feedforward inhibitory input, the excitatory synaptic event in the naïve slice is on average almost one order of magnitude greater than the concomitant event recorded onto principal cells (Glickfeld and Scanziani, 2006). The authors argue that any mechanism that strengthens Schaffer collateral inputs onto principal cells without a similar strengthening of the inhibitory circuit would lead to a potential imbalance of excitation within the system. However, consideration of the circuit would suggest that any potentiation of excitatory transmission onto feedforward interneurons would result in a massive recruitment of divergent inhibition onto large numbers (potentially thousands) of principal cells, potentially eroding the CA1 excitatory circuit and compromising the temporal fidelity of network signaling (Somogyi and Klausberger, 2005). Interestingly, the original description of plasticity at stratum radiatum interneurons by McMahon and Kauer (1997) demonstrated that stratum radiatum interneuron synapses *weakened* in response to a paradigm that strengthened synaptic transmission onto CA1 pyramidal cells. Moreover, a similar scenario was observed within the MF–CA3 network, where the same paradigm that strengthened MF–CA3 pyramidal cell excitatory synapses *weakened* transmission between MF and interneuron synapses. Clearly, much is left to be uncovered regarding both the mechanism(s) and network consequences of plasticity at excitatory synapses onto interneurons.

4.18.4 Long-Lasting Plasticity of Interneuron-Generated GABAergic Output

As detailed in the introduction to this chapter, GABAergic interneurons are essential elements for shaping cortical circuit dynamics and play critical roles in the regulation of cellular excitability, spike timing, network oscillations, and threshold for the induction of synaptic plasticity. To achieve these functions, GABA is released onto specific postsynaptic domains of target cells by different classes of interneurons (Gulyas et al., 1993; Miles et al., 1996). Therefore, the study of long-term plasticity of interneuronal output, specifically occurring at the level of the GABAergic synapse, is an important element for our understanding of network function dynamics. The integration of synaptic plasticity of excitatory input and GABAergic output will exert profound effects on the overall network function. As a result, although a direct demonstration is still lacking, long-term plasticity of interneuronal output is predicted to have an important impact on brain function and behavior. Furthermore, any long-term decrease in inhibition could also lower the threshold for epileptiform activity *in vitro* (Miles and Wong, 1987; Steltzer et al., 1987) and potentially underlie a number of pathological processes.

Analogous to what we have discussed above for excitatory input, we now consider the evidence for LTP and LTD of GABAergic synaptic transmission in mammalian cortical networks of the developing and mature brain. We discuss their molecular mechanisms and consequences for network processing, and later we briefly examine other forms of GABAergic plasticity relevant for network signaling. An important distinction that needs to be remembered here is that while the previous section considered excitatory input onto interneurons, all of the present discussion reviews recordings of GABAergic input onto pyramidal neurons. Finally, long-term plasticity of GABAergic input to specific types of interneurons is a virtually unexplored field and is not addressed here.

4.18.4.1 Activity-Dependent Long-Lasting Plasticity of GABAergic Input in the Hippocampus and Neocortex: General Considerations

Long-term potentiation and depression of GABAergic synaptic input have been studied in both the mammalian neocortex and hippocampus *in vitro,* with paradigms reminiscent of those used to study conventional forms of plasticity of excitatory input. However, in comparison to classical long-term plasticity of excitatory synaptic transmission, this field is relatively new, and most of the relevant mechanisms are incompletely understood. Furthermore,

the comparison of results from different laboratories is often problematic, since there is a general lack of standard protocols, which complicates the emergence of clear, unifying principles.

Before considering examples of plasticity at specific synapses, a few general points are worth mentioning to avoid confusion. Induction of long-term plasticity at excitatory inputs typically requires activity at the various types of synaptic ionotropic and/or metabotropic glutamate receptors and typically does not require activation of receptors for neurotransmitters other than glutamate. In contrast, induction of synaptic plasticity at GABAergic synapses often requires cross-talk *between* receptors for different neurotransmitters and neuromodulators, further complicating their study. For example, the pharmacological isolation of evoked GABAergic responses that is required for a careful comparison before and after any induction protocol, by necessity, often prevents the activation of glutamate receptors that may be critical for the induction of plasticity itself. Therefore, in some studies, pharmacological isolation of GABAergic test responses is temporarily relieved during the induction protocol, whereas other studies may maintain the blockade throughout the experiment (e.g., compare McLean et al., 1996, with Shew et al., 2000). Such an experimental design may introduce an uncontrollable source of inconsistency, preventing a true comparison of results from different laboratories. Nevertheless, several factors have been recognized as essential in determining the large experimental variability of the results. First, the stage of brain development appears to be especially important. $GABA_A$ receptor–mediated signaling in early neonatal life is excitatory and generates depolarizing IPSPs (dIPSPs, Owens et al., 1996). $GABA_A$ receptors are mostly permeable to chloride (Borman et al., 1989), and chloride homeostasis in neurons is developmentally regulated by the early expression of the Na-K-Cl cotransporter NKCC1 that accumulates chloride intracellularly (Plotkin et al., 1997), in contrast to the later expression of the neuron-specific K-Cl cotransporter KCC2, which results in chloride extrusion and a negative shift in the $GABA_A$ equilibrium potential (Rivera et al., 1999). GABA released onto neonatal pyramidal neurons can depolarize the postsynaptic membrane and hence can directly activate voltage-dependent calcium channels. Furthermore, GABA-mediated dIPSPs can remove the voltage-dependent blockade of NMDARs by Mg^{2+}, a role that is well established as critical for 'classical' NMDAR-dependent LTP of glutamatergic input (Bliss and Collingridge, 1993) and that is mediated by AMPA receptors in mature neurons. Therefore, early in development, GABAergic postsynaptic potentials can directly lead to Ca^{2+} influx or provide the associative signal required for Ca^{2+} entry via NMDARs, in case of simultaneous glutamatergic activity (Ben-Ari et al., 1997). Second, the pattern of stimulation used in the induction protocol appears to be more critical than what is typically observed for glutamatergic synaptic plasticity. For example, both high-frequency tetanization and theta-patterned stimulation are effective induction protocols to trigger LTP at glutamatergic synapses, whereas differential effects have been described with either paradigm at GABAergic inputs. Third, regional brain specificity needs to be taken into careful consideration, and it cannot be assumed that identical molecular mechanisms operating in the hippocampal network are valid for every other cortical region. For example, specific differences have been described in GABAergic plasticity in the hippocampus compared to visual cortex. Fourth, and finally, GABAergic plasticity is often studied by evoking GABAergic input following the stimulation of many fibers originating from undetermined interneuronal types. Yet it is critically important to remember that the cellular diversity of the interneurons generating the GABA release is staggering, and therefore the dynamic properties of averaged events across a number of interneuron subpopulations may blur the results of these types of experiments. Ideally, the specific plastic properties of unitary events originating from a well-identified interneuron should be studied. However, this has been achieved in only a few cases.

4.18.4.2 Activity-Dependent Long-Lasting Plasticity of GABAergic Input in the Neonatal Hippocampus

Bidirectional synaptic plasticity of GABAergic input has been repeatedly described in the neonatal hippocampus. Homosynaptic LTP and LTD of $GABA_A$ receptor–mediated IPSP/Cs have been recorded under a variety of conditions (Gaiarsa et al., 2002). Their induction is generally postsynaptic and is triggered by postsynaptic Ca^{2+} entry. However, the specific sources of Ca^{2+} entry appear critical in determining the direction of plasticity (see **Figure 4**). Ca^{2+} influx via voltage-dependent calcium channels results in LTP, whereas entry via NMDARs or an elevation resulting from Ca^{2+}-induced Ca^{2+} release results in

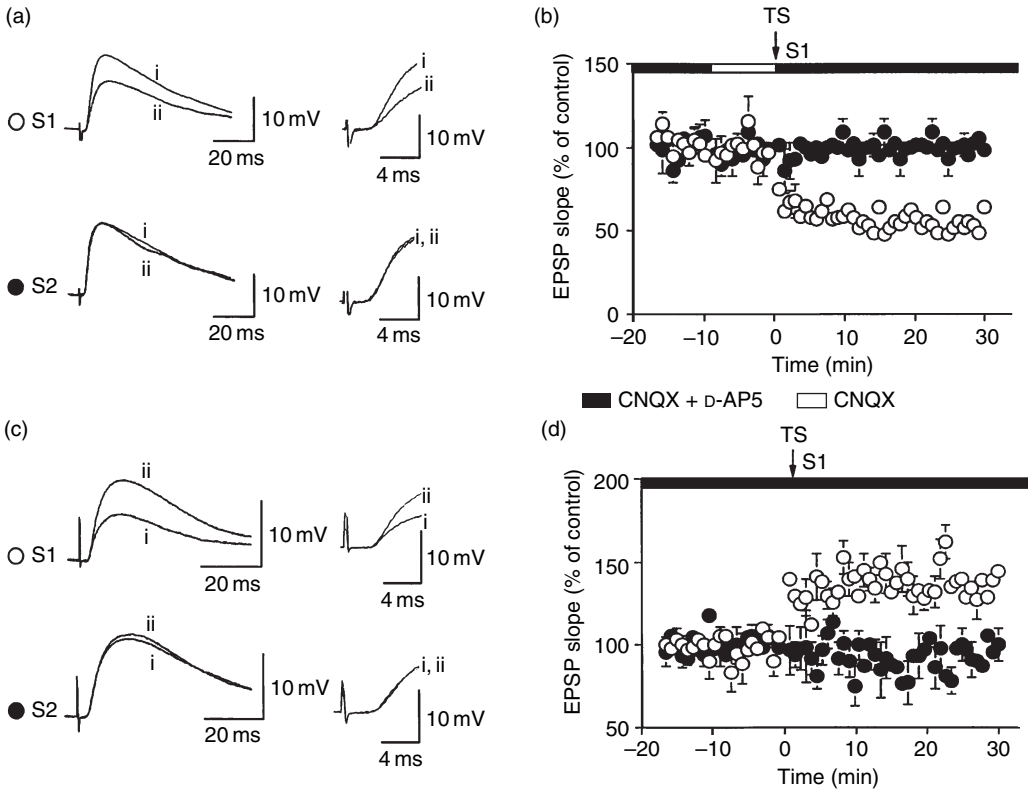


Figure 4 Bidirectional synaptic plasticity of γ-aminobutyricacid (GABA)-ergic inhibitory postsynaptic potentials (IPSPs) in the CA3 neonatal hippocampus. Evoked dIPSPs were recorded in the continuous presence of CNQX and D-AP5 in order to isolated GABAergic input. Notice, however, that the presence/absence of *N*-methyl-D-aspartate receptor (NMDAR) activity during the conditioning protocol determines the direction of the plasticity. Insets show averaged responses from the conditioned (S1) and control (S2) pathway. (a, b) Homosynaptic long-term depression (LTD) is induced by tetanic stimulation (TS) in the absence of the NMDAR blocker D-AP5. Insets in (a) show evoked responses before (i) and after the induction of LTD (ii). (c, d) Homosynaptic long-term potentiation (LTP) is triggered by TS after pharmacological blockade of NMDARs. Reprinted from McLean HA, Caillard O, Ben-Ari Y, and Gaiarsa JL (1996) Bidirectional plasticity expressed by GABAergic synapses in the neonatal rat hippocampus. *J. Physiol. (Lond.)* 496: 471–477, Blackwell.

LTD (McLean et al., 1996; Caillard et al., 2000). The locus of expression in both cases is likely to be presynaptic. LTP in neonatal and juvenile slices was associated with an increase of spontaneous and miniature IPSC frequency, but not amplitude (Caillard et al., 1999a; Shew et al., 2000). Similarly, LTD was associated with an increase in the coefficient of variation of evoked $GABA_A$ receptor–mediated synaptic currents and a decrease in the frequency, but not amplitude, of Sr^{2+}-induced asynchronous $GABA_A$ quantal events (Caillard et al., 1999b). If the release properties of the presynaptic terminals are involved in long-term plasticity, then a retrograde signal is most likely involved. In the case of LTP, neurotrophins acting via TrkB receptor–mediated phosphorylation have been proposed as candidates (Gubellini et al., 2005).

Neonatal long-term plasticity of GABAergic input has been proposed to play a critical role in the refinement of initially coarse patterns of synaptic connections in cortical circuits. Several observations make this hypothesis particularly attractive. First, neonatal plasticity is constrained by a limited period of development, which correlates well with the period of functional maturation of the network examined (Komatsu, 1994; McLean et al., 1996; Gubellini et al., 2001). Second, conditioning protocols that induce long-term plasticity are also effective in transforming silent into functional synapses. For example, the same protocols that are effective in inducing plasticity can turn on synapses in cells that previously displayed a complete absence of activity (Gubellini et al., 2001). Third and last, conditioning protocols used to trigger synaptic plasticity can be

very similar to the types of activity that neurons experience *in vivo*. For example, LTP can be induced by repeated bursts at low frequency that are reminiscent of the firing patterns recorded *in vivo* (Leinekugel et al., 2002).

4.18.4.3 Activity-Dependent Long-Lasting Plasticity of GABAergic Input in the Juvenile/Adult Hippocampus

In the juvenile-adult CA1 hippocampus, LTP of fast IPSPs and IPSCs has been reported following theta-patterned stimulation (Perez et al., 1999; Patenaude et al., 2003), whereas no change, LTD, or LTP (Stelzer et al., 1987; Wang and Stelzer, 1996; Lu et al., 2000; Shew et al., 2000; Chevaleyre and Castillo, 2003; Patenaude et al., 2003) have been reported following tetanic HFS. The induction of both types of plasticity is Ca^{2+} dependent and is prevented by perfusion of Ca^{2+} chelators into the postsynaptic cell (Patenaude et al., 2003). However, theta-pattern stimulation of unitary IPSCs was reported as ineffective in interneuron → pyramidal cell paired recordings, suggesting that a multifiber cooperative action may be required for induction. This is consistent with the observation that theta-patterned induced LTP can be prevented by blockers of either $GABA_B$ receptors or group I/II mGlu receptors (Patenaude et al., 2003), in a manner similar to that previously described in the visual cortex (Komatsu, 1996). The molecular pathway leading to tetanus-induced LTD of IPSPs critically depends on the activation of NMDARs and on the phosphatase calcineurin (Lu et al., 2000). Calcineurin dephosphorylates $GABA_A$Rs through the direct binding of the calcineurin catalytic domain to the second intracellular loop of the $\gamma 2$ subunit (Wang et al., 2003). In contrast to LTD, the molecular pathway leading to LTP remains unclear in these *in vitro* models. However, studies of plastic changes of GABAergic inhibition in the dentate gyrus of control versus kindled rats have provided functional and ultrastructural evidence for modification both of the number and of specific $GABA_A$ receptor subunits expressed at the synapse (Otis et al., 1994; Buhl et al., 1996; Nusser et al., 1998a). Thus, similar mechanisms might potentially be involved in LTP induced *in vitro* by HFS patterns.

In addition to mechanisms of plasticity that are reminiscent of classical LTP and LTD of excitatory synapses onto principal cells, the functional heterogeneity within the hippocampal interneuron population has also revealed novel, cell type–specific forms of plasticity. One specific form of long-term plasticity is expressed at GABAergic connections originating from a subset of interneurons whose presynaptic terminals express type 1 cannabinoid receptors (for a review, see Freund et al., 2003). Endocannabionid-mediated retrograde signaling activates these receptors (Alger, 2002) and reduces neurotransmitter release (Hajos et al., 2000; Katona et al., 1999). Tetanic and theta-burst stimulation of stratum radiatum afferents in the absence of ionotropic glutamatergic synaptic transmission lead to activation of mGluRs located within the pyramidal cell dendritic tree that trigger the retrograde release of endocannabinoids and a consequent selective LTD of transmission from CB1-expressing GABAergic terminals (Chevaleyre and Castillo, 2003). This form of LTD appears to be localized to a restricted subcellular area and affects only a limited subset of GABAergic terminals on pyramidal cell dendrites (Chevaleyre and Castillo, 2004). The complete molecular cascade and molecular mechanisms leading to the expression and maintenance of this form of LTD are presently unknown. This cannabinoid-dependent form of LTD has been suggested to play a critical role in priming LTP of excitatory connections (Carlson et al., 2002; Chevaleyre and Castillo, 2004).

4.18.4.4 Activity-Dependent Long-Lasting Plasticity of GABAergic Input in the Visual Cortex

LTP of fast evoked inhibitory synaptic transmission in the rat visual cortex has also been studied in some depth. Consequently, a detailed picture of the molecular cascades required for its induction is beginning to emerge. In general, visual cortex $GABA_A$ LTP in rats is induced by brief repetitive stimulation at high frequencies in the presence of antagonists of ionotropic glutamate receptors (Komatsu, 1994). Interestingly, when NMDARs were left unblocked during the conditioning protocol, $GABA_A$ LTD was observed (Komatsu and Iwakiri, 1993), indicating the potential for bidirectional plasticity. This type of plasticity is homosynaptic and, therefore, restricted to the conditioned pathway. Furthermore, it is also associative, such that weak conditioning stimulation to one pathway, which alone failed to induce LTP, induced LTP when strong conditioning stimulation was simultaneously applied to another independent pathway

(Komatsu, 1994). LTP can be observed both in neonatal and adult slices, although its threshold appears lower in younger animals (Komatsu, 1994). Importantly, induction is critically dependent on postsynaptic Ca^{2+} loading, mediated by IP3-induced Ca^{2+} release. Although the test responses to single stimuli in these studies were mediated exclusively by $GABA_A$ receptors, HFS used during induction protocols also activated $GABA_B$ and mGlu receptors (Komatsu, 1996). Activation of $GABA_B$ receptors during the induction protocol appeared essential for LTP, whereas mGluR activity was not required. At present, the role played by $GABA_B$ receptors remains unclear. One possibility is that they could be involved in raising postsynaptic calcium concentrations via IP3-induced Ca^{2+} release, but no direct demonstration has been provided yet. Another interesting observation in visual cortex $GABA_A$ LTP is that the activity of adrenalin and serotonin receptors appeared to be required during the conditioning stimuli. Indeed, application of alpha1-adrenoreceptor or 5-HT_2 receptor antagonists reduced the probability of inducing plasticity after a conditioning stimulus (Komatsu, 1996). This raised the possibility that postsynaptic $GABA_B$ receptor activity during the induction protocol may act by facilitating IP3-induced Ca^{2+} release triggered by adrenaline or serotonin (Komatsu, 1996). Maintenance of LTP depends on the activity of presynaptic GABAergic terminals and is mediated by presynaptic Ca^{2+} entry through multiple types of Ca^{2+} channel (Komatsu and Yoshimura, 2000). Furthermore, Yamada et al. (2005) showed that noradrenergic receptors may also play a role in the modulation of the maintenance of HFS-induced LTP. The authors showed that application of noradrenaline after tetanic stimulation in a reduced external calcium concentration that normally does not allow LTP resulted in potentiation of the conditioned, but not the test, pathway. Pharmacological analysis of this result indicated that the effect was mediated by alpha2 and beta adrenergic receptors (Yamada et al., 2005).

Analogous to our previous discussion, a lower threshold for induction of this plasticity exists during a critical developmental period, suggesting a particular role in development. Finally, during development, GABAergic synapses may play a role in the selective responses of visual cortical cells to features of visual stimuli such as orientation, length, or direction of a slit (Hubel and Wiesel, 1962), as suggested by the reduction of selectiveness following application of the $GABA_A$ receptor antagonist bicuculline (Sillito, 1975, but see Nelson et al., 1994). For example, optimal visual stimuli that produce strong spiking activity may result in activation of NMDARs and hence LTD of inhibition, consequently enhancing the optimal visual response. In contrast, nonoptimal stimuli producing only moderate spiking activity may selectively potentiate inhibition and result in an overall depression of the responses triggered by the stimuli.

4.18.4.5 Spike Timing–Dependent Plasticity of GABAergic Input in the Neocortex

In analogy with spike timing–dependent synaptic plasticity of glutamatergic inputs (Markram et al., 1997; for a review see Dan and Poo, 2004), work on reciprocally connected pairs of inhibitory and excitatory neurons in neocortical slices has shown that bidirectional modulation of unitary GABAergic input depends on the relative timing of pre- and postsynaptic activity (Holmgren and Zilberter, 2001). This type of GABAergic spike timing–dependent plasticity is also Ca^{2+} dependent, but the molecular cascade of events following Ca^{2+} elevations has not been elucidated yet. The mechanism proposed by the authors is that Ca^{2+}-dependent protein kinases may directly modulate the activity of postsynaptic $GABA_A$ receptors (Holmgren and Zilberter, 2001). Further work will be required to test this hypothesis. The significance of this form of plasticity is strengthened by the similarity of the induction protocol used *in vitro* to the activity observed *in vivo*. Indeed, bursting patterns of pre- and postsynaptic activity like the ones used in Holmgren and Zilberter (2001) are likely to occur during learning episodes and therefore could trigger long-term plasticity at GABAergic synaptic connections (Buzsaki et al., 1996; Thomas et al., 1998; King et al., 1999; Paulsen and Sejnowski, 2000).

4.18.4.6 Plasticity of $GABA_A$ Receptor Reversal Potential

Activity-dependent long-term plasticity can regulate synaptic transmission in several distinct ways. For example, the transmitter release properties of presynaptic terminals can be modified, or the number and/or the functional properties of postsynaptic receptors can be changed. Above, we have highlighted several examples of plasticity, which are mediated by these two general mechanisms.

However, responses mediated by $GABA_A$ receptors at inhibitory synapses depend also on the transmembrane chloride gradient (Bormann et al., 1987), which determines the polarity of the postsynaptic response, that is, whether the response is depolarizing (excitatory) or hyperpolarizing (inhibitory). Woodin et al. (2003) and Fiumelli et al. (2005) have reported that postsynaptic spiking of principal cells can induce local or global changes in the chloride reversal potential and hence depress the size of postsynaptic currents mediated by $GABA_A$ receptors in hippocampal cell cultures and slices from juvenile animals (**Figure 5**). These effects were mediated by Ca^{2+} entry via L-type Ca^{2+} channels and subsequent PKC activation (Woodin et al., 2003; Fiumelli et al., 2005). This type of plasticity required expression of KCC2, the chloride cotransporter, and significantly, was absent when KCC2 was blocked pharmacologically, suggesting that PKC depresses the activity of the transporter either by direct phosphorylation or by an indirect pathway. A similar modulation of EC1, but opposite in sign, was reported in neonatal slices by Ouardouz et al. (2006).

4.18.4.7 Plasticity of $GABA_B$ Receptor–Mediated Input in the Hippocampus

Synaptic inhibition in the hippocampus is mainly mediated by fast $GABA_A$ and slow $GABA_B$ receptor–mediated postsynaptic potentials (Dutar and Nicoll, 1988). However, in comparison to fast inhibition, studies focusing on the activity-dependent synaptic plasticity of $GABA_B$ receptor–mediated synaptic input are in their infancy. Intriguingly, $GABA_B$ receptors and G protein–activated inwardly rectifying K+ (GIRK) channels, which are responsible for the slow postsynaptic potential (Luscher et al., 1997), have been localized in spines (Drake et al.,

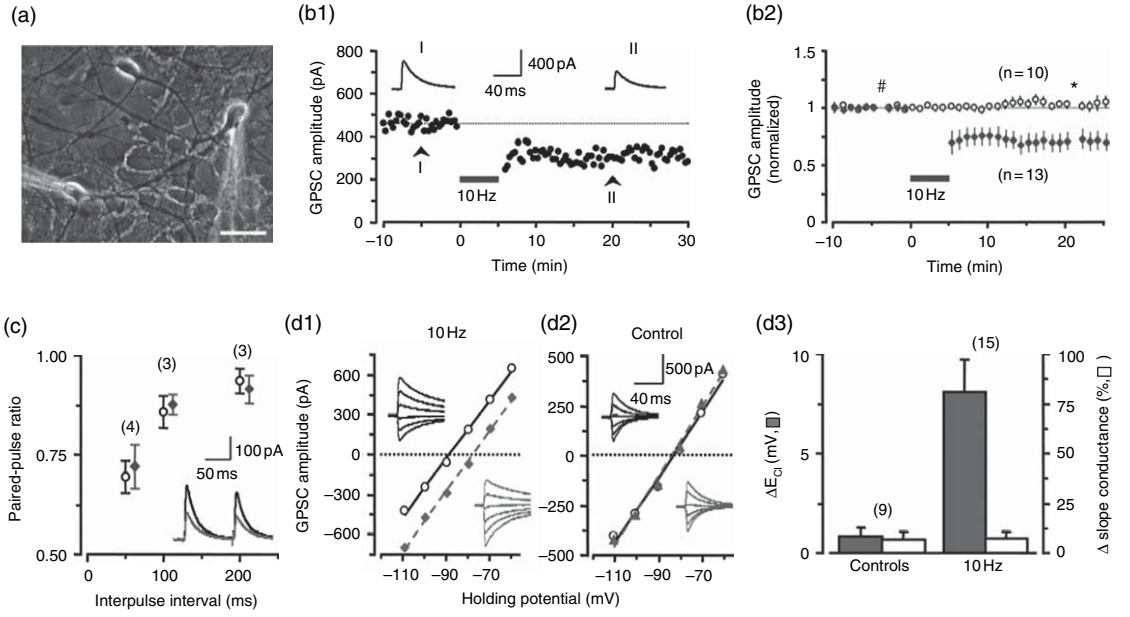


Figure 5 Long-term depression of GABAergic unitary responses recorded in pairs of cultured hippocampal neurons resulting from postsynaptic spiking–induced plasticity of E_{GABAA}. An image of a pair of connected neurons; scale bar, 20 μm. (b1) Single experiment showing that postsynaptic spiking at 10 Hz for 5 min induces long-term depression (LTD) of unitary GABAergic postsynaptic currents (GPSCs). Insets show average traces before (i) and after (ii) the conditioning protocol. (b2) Summary plot from two sets of experiments in which postsynaptic spiking was (filled symbols) or was not (empty symbols) applied. # and ∗ indicate the time selected to measure I/V relationships of the unitary event and estimate their reversal potentials (see panels d1 and d2). (c) Postsynaptic spiking does not alter the mean paired pulse ratio at various interevent intervals, suggesting the lack of presynaptic effects. (d1) I/V relationships of the GABAergic postsynaptic currents before (solid line) and after (dotted line) the conditioning protocol. Notice the shift in the reversal potential. In contrast, d2 shows the stability of I/V relationships and estimated reversal potentials in experiments where no conditioning protocols were used (panel b2, empty symbols). (d3) Summary graph indicating the changes in reversal potential and conductance of unitary GABAergic currents in control experiments (no conditioning protocol) and experiments in which postsynaptic spiking was used to trigger LTD (10 Hz). Reprinted from Fiumelli W, Cancedda L, and Poo MM (2005) Modulation of GABAergic transmission by activity via postsynaptic Ca^{2+}-dependent regulation of KCC2 function. *Neuron* 48: 773–786, © 2005, with permission from Elsevier.

1997; Kulik et al., 2003), suggesting the possibility that the same mechanisms operating in spines and leading to LTP of excitatory synaptic transmission might trigger LTP of slow inhibition. Huang and colleagues (2005) directly tested this hypothesis in the hippocampus and induced $GABA_B$ LTP following conventional stimulation patterns that are effective in the induction of glutamatergic LTP. Furthermore, when the cascade of events involved in LTP of $GABA_B$ receptor–mediated input was examined, it was found to depend on steps identical to the ones described for excitatory LTP. $GABA_B$ LTP was NMDAR and CaMKII dependent (Huang et al., 2005). However, the same authors also showed that $GABA_B$, but not glutamatergic LTP, was absent in mice lacking the RNA binding protein Nova-2 (Ule et al., 2003, 2005), thus indicating that specific control of $GABA_B$ LTP does exist. In the context of network signaling, selective $GABA_B$ LTP could sharpen the coincidence detection of excitatory synaptic input because of the late hyperpolarization, which would reduce the efficacy of late-arriving excitatory synaptic input (Huang et al., 2005).

4.18.4.8 Plasticity of Interneuronal Resting Membrane Potential

Last, we discuss a nonsynaptic form of long-term plasticity, which might nevertheless have important implications for GABAergic interneuron output. Ross and Soltesz (2001) described a long-lasting depolarization of resting membrane potential in hippocampal dentate gyrus interneurons following high-frequency tetanic stimulation. Because the conditioned interneurons now rest at more depolarized membrane potentials, they are more likely to respond to excitatory input by producing action potentials and hence release GABA onto their downstream targets. The induction mechanisms involved in this form of plasticity require activation of Ca^{2+}-permeable AMPA receptors and the consequent rise of intracellular Ca^{2+}, but not NMDAR, activity. Maintenance of this new persistently depolarized membrane potential was suggested to depend on modulation of the rate of Na^+/K^+ ATPase pump function (Ross and Soltesz, 2001).

In conclusion, although the field has received much less attention when compared to excitatory input, activity-dependent long-term plasticity of GABAergic input is an important mechanism that is likely to boost the flexibility of neuronal circuits in the brain. However, the heterogeneity of interneuronal populations and the complexity of the mechanisms involved suggest that considerably more work is required before a clear picture can emerge.

4.18.5 Toward the Future: Plasticity of GABAergic Networks?

Although for many years the focus on synaptic transmission in mammalian cortical system has been almost exclusively concentrated on chemical synapses, ongoing work in the last decade has also clearly shown that interneurons of cortical circuits are functionally organized through electrical coupling in GABAergic networks. In particular, dual recordings made from various types of interneurons have shown that electrical coupling is particularly strong among cells of the same class (reviewed by Hestrin and Galarreta, 2005; but see also Simon et al., 2005; Zsiros and Maccaferri, 2005). Electrical coupling of GABAergic networks appears especially important for the promotion of action potential synchronization between connected cells. Although the direct demonstration of a link *in vivo* is still lacking, this may help explain the stereotyped temporal pattern of activity that specific subsets of interneurons show in the hippocampus *in vivo* during different brain states (Klausberger et al., 2003, 2004, 2005). Long-term plasticity of electrical coupling has been shown in nonmammalian systems (Yang et al., 1990; for a review, see Pereda et al., 2004), and recent work has suggested that mechanisms similar to conventional LTP and LTD might operate at electrical synapses of the mammalian brain (Landisman and Connors, 2005). This possibility is virtually unexplored at present in mammalian circuits of the neocortex and hippocampus, but it is highly likely to become a strong focus of interest in the coming years.

4.18.6 GABAergic Plasticity and Implication for Network Functions

What are the consequences of synaptic plasticity in interneurons for network function? Although many hypotheses exist, few experiments have directly tackled this question. Network activity in a given cortical system is typically mediated by the integration of excitatory input with feedforward and feedback inhibitory loops. Steltzer et al. (1994) tackled this question by recording excitatory and inhibitory postsynaptic responses in hippocampal

pyramidal neurons and interneurons after tetanic stimulation of stratum radiatum. Their results highlighted that LTP of excitatory responses in pyramidal neurons was accompanied by a compartment-specific depression of dendritic, but not somatic, GABAergic input. This is especially intriguing in the light of the fact that pyramidal neurons have multiple action potential initiation sites (Golding and Spruston, 1998; Larkum et al., 2001), and that specific roles for dendritic- and somatic-targeting interneurons have been shown by Miles et al. (1996). Thus, the integration of plasticity in interneurons and principal cells could be related to the switch between two hypothetical modes of operation of pyramidal neurons, which have been proposed to underlie encoding and retrieval of memories (Paulsen and Moser, 1998). Other studies have highlighted LTD of fast inhibition as a critical component of E-S potentiation (Lu et al., 2000; Staff and Spruston, 2003; but see Marder and Buonomano, 2004). E-S potentiation (Bliss and Linch, 1988) is a phenomenon associated with classical LTP, which is described by a change in the input–output curve of a neuron. In other words, E-S spike potentiation associates the same sized excitatory postsynaptic potential (EPSP) with a higher probability of initiating a spike. The establishment of plasticity in feed-forward and feed-back inhibitory circuits may therefore change the EPSP-spike coupling properties of neurons and their precision of firing (Fricker and Miles, 2000, 2001).

4.18.7 General Conclusions

In this albeit brief overview of the state of inhibitory interneuron plasticity, we have tried to emphasize the current state of the field and highlight the complexities associated with plasticity of incoming excitatory afferents as well as discuss the nascent field of plasticity of the inhibitory output from a variety of well-defined interneuron circuits. If there is a single 'take-home' message from the above discussion, it is that no single general principal accounts for either the plasticity of excitatory synaptic transmission onto interneurons or the plasticity of their GABAergic inhibitory output. Moreover, often the same protocols that induce plasticity at principal cell targets either fail to induce plasticity or result in a long-lasting plastic change of opposite polarity at interneuron inputs and outputs. This, in hindsight, is not particularly surprising, given the emergence of the myriad types of cortical interneuron subpopulations, all of which are tuned to a specific function within the cortical network in which they are embedded. It is also important to appreciate that interneurons receive input, often from a wide array of afferent fibers, as well as making their output onto a wide array of diverse cellular targets. We would predict that each and every one of these inputs and outputs will have their own particular set of rules regarding both short- and long-term plasticity. A complete understanding of these plasticity rules will only be obtained by the careful and often arduous task of systemically studying each and every one. However, we hope that this review will help fuel such an endeavor over the coming years, and we predict that we are only on the cusp of truly understanding the role plasticity plays in tuning the role of interneurons in the cortical network.

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4.19 Neurofibromatosis Type I Learning Disabilities

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4.19.1 Learning Disabilities

Learning disabilities are characterized by specific impairments restricted to certain domains of mental function. This is in contrast to the broad impairment seen in mental retardation disorders, which cause generalized impairments in intellectual and social development (Kronenberger and Dunn, 2003; Kelly, 2004). The simplest definition of a learning disorder is a discrepancy between tests of intellectual capability and actual achievement (Kronenberger and Dunn, 2003). Intellectual capability is typically quantified with IQ tests such as the Wechsler scales (WISC-IV, WAIS-III) and the Stanford Binet (SB-IV). These tests provide reliable measures that are standardized across specific age groups (Palumbo and Lynch, 2006). Achievement is also determined using standardized tests that assess performance level in multiple academic areas (e.g., Woodcock-Johnson (WJ-III) (Palumbo and Lynch, 2006). Learning disability is diagnosed when performance in tests of achievement is significantly below predictions based on IQ score.

There are several ways to categorize learning disabilities. The DSM-IV places learning disabilities into three major categories: reading disorder, mathematical disorder, and disorders of written expression (Kronenberger and Dunn, 2003; Kelly, 2004). In addition to these categories, the DSM-IV also recognizes nonverbal learning disorders and learning disorders not otherwise specified. These latter categories represent disorders which lead to academic underachievement but do not fall neatly into one of the first three DSM-IV categories of learning disability (Tramontana, 2006). The DSM-IV categories are also considered distinct from other developmental disorders such as motor skills disorder and communication disorder. Finally, other diagnostic systems, such as those used in neurology, are based on more specific descriptions of learning disabilities such as dyslexia (reading disorder) (Kelly, 2004).

The criteria for diagnosis of learning disabilities are quite complex. Further, they describe patterns of learning problems but may not capture the entire clinical picture or reflect fundamental differences in neuronal mechanisms. For diagnosis and treatment to

be successful, it may be useful to consider a more comprehensive picture that reflects underlying causes and affected cognitive systems. The role of neuroscience is to provide insights into neurological systems and circuits as well as cellular, molecular, and genetic mechanisms that are required for normal function. In this chapter, we focus on investigations of a specific disorder, neurofibromatosis. The integration of basic and clinical neurosciences has led to significant strides in our understanding of learning disabilities associated with NF1. For example, our lab and others have identified specific changes in neuronal function and isolated therapeutic targets that are currently being used to develop novel treatment options for patients with NF1. The integrated approach that led to these advances could lead to similar advances in other cognitive disorders.

4.19.2 Neurofibromatosis Affects Several Brain Systems and Impairs Multiple Cognitive Functions

Neurofibromatosis type I (NF1), also known as von Recklinghausen neurofibromatosis, is an autosomal dominant neurological disorder. Patients with NF1 have widespread clinical symptoms in multiple organ systems (Lynch and Gutmann, 2002). Classic disease characteristics include the presence of neurofibromas, the benign peripheral nerve tumors composed of proliferating Schwann cells, and fibroblasts that give the syndrome its name. Other classic diagnostic features of NF1 are pigmented lesions of the skin called café au lait spots, hamartomas of the iris termed Lisch nodules, and pseudoarthrosis of the tibia. Additional symptoms can range from pain, bone dysplasia, and multiple types of tumors including optic gliomas (Sagar, 2004). Importantly, NF1 also causes high rates of learning disabilities and cognitive symptoms. Cognitive symptoms of NF1 pose one of the most significant sources of lifetime morbidity for patients (North et al., 1997).

Cognitive symptoms in NF1 occur in a specific, domain-restricted pattern, rather than in a globally generalized manner. This is evidenced by the lack of performance differences between NF1 patients and unaffected siblings on many neuropsychological measures considered sensitive to overall cognitive impairment (Kayl and Moore, 2000). For example, patients with NF1 have IQs in the normal range, with only a slight downward shift in distribution. However, their academic and social gains can be limited by learning disabilities and cognitive impairments in four main areas: Reading/vocabulary, attention, executive function and planning, as well as visuospatial function (Hyman et al., 2005).

Learning disability is a common symptom of NF1; it is present in 30%–65% of this population. This wide range is due in part to studies using differing definitions of a learning disorder, as well as the use of small groups without appropriate controls (North et al., 1997; North, 2000). NF1 patients show some characteristics of both nonverbal- and verbal-type learning disability. Components of nonverbal learning disability seen in NF1 include consistently poor performance in tests of visuospatial functioning and spatial learning (North, 2000; Kayl and Moore, 2000), notable impairments in the ability to perceive social cues, poor organizational skills, and increased impulsiveness (North et al., 1997; North, 2000). NF1 patients also show aspects of verbal learning disorder. Specifically, patients with NF1 have deficits in expressive and receptive language, vocabulary, visual naming, and phonologic awareness. In fact, reading and spelling are repeatedly found to be impaired more severely than predicted by IQ in NF1 patients (Hyman et al., 2005). Consistent with these impairments in language-based learning, NF1 patients show poorer academic achievement in reading and writing, compared to their unaffected siblings. Importantly, when IQ is controlled for, children with NF1 do not show a higher incidence of learning problems in mathematics, a core feature of nonverbal learning disability (Kayl and Moore, 2000).

The pattern of learning disabilities seen in NF1 does not fall cleanly into one of the DSM-IV defined categories. Instead, the occurrence of multiple types of learning problems suggests that a more fundamental learning process is impaired in NF1 patients, rather than a deficit in a specialized area such as language or mathematics. Therefore, it is useful to examine the symptoms of NF1 in terms of basic cognitive functions. Learning deficits in NF1 patients suggest dysfunction in the hippocampal memory system. NF1 patients perform poorly on spatial learning and memory tasks that have been shown to depend on the hippocampus and adjacent medial temporal cortex. Damage to these areas is known to produce profound amnesia for spatial information and difficulties navigating through space (Burgess et al., 2002; Squire et al., 2004). Reading comprehension and vocabulary learning impairments seen in NF1 may also be related to hippocampus dysfunction. Learning and remembering new facts and general information

about the world requires the hippocampus and surrounding medial temporal cortex (Schmolck et al., 2002; Bayley and Squire, 2005). For example, patients with damage to this area often have considerable difficulty learning and retrieving new vocabulary words (Verfaellie et al., 2000). A similar impairment could contribute to the learning problems that NF1 patients exhibit in school.

It is important to recognize that in addition to possible hippocampal-dependent learning disabilities, deficits in other behavioral and cognitive domains may play an important role in academic and social achievement problems seen in NF1. This has been suggested by studies showing that up to 40% of children with NF1 who were identified as academic underachievers were also normal on neuropsychological tests for learning disabilities (Kayl and Moore, 2000). In such cases, underachievement is likely due to deficits in attention, planning, and organization skills (Dilts et al., 1996; North, 2000; Kayl and Moore, 2000; Koth et al., 2000; Hyman et al., 2005).

Patients with NF1 consistently show planning impairments that are more severe than would be predicted by IQ (Hofman et al., 1994; Hyman et al., 2005). Further, there is a high comorbidity, ranging from 30% to 50%, between NF1 and attention deficit disorder (Hofman et al., 1994; Mautner et al., 2002; Hyman et al., 2005; Pavol et al., 2006). Comorbid attention deficit disorder affects not only academic but also social development for children with NF1 (Barton and North, 2004). Children with NF1 tend to have social problems and appear socially awkward and withdrawn, in comparison to their siblings and to children with other chronic, life-threatening illnesses (Kayl and Moore, 2000). Some evidence suggests that social deficits are related to poor interpersonal skills, which occur as a result of decreased attention to social cues.

Interestingly, severity of symptoms in planning/organization seems to be independent of attention deficit symptoms. This suggests that attention deficit does not cause symptoms of poor planning and organization in NF1 but, rather, NF1 affects each of these domains of cognitive function (Hyman et al., 2005). Symptoms in these domains are functionally and anatomically related. Attention, planning, and behavioral organization are elements of executive function. Executive functions are a set of higher-order cognitive functions that allow adaptive, goal-directed behavior. Domains of executive function which are impaired in NF1 are thought to critically depend on frontal/subcortical circuits (Ferner et al., 1996; Stuss and Levine, 2002). Specifically, the prefrontal cortex is thought to play a key role in processes such as working memory, control of attention, planning, and rule following (Ferner et al., 1996; Stuss and Levine, 2002; Otani, 2003). Limitations in planning, organization, and control of attention seen in NF1 are also commonly present in syndromes involving impaired prefrontal executive function (Ferner et al., 1996; Stuss and Levine, 2002; Powell and Voeller, 2004). Such evidence implicates prefrontal cortex pathology in NF1. Indeed, functional imaging studies show hypoactivation of prefrontal cortex in NF1 patients during a rhyming task (Billingsley et al., 2003). Improved understanding of how NF1 affects prefrontal cortex function will be essential to understanding the complex cluster of seemingly prefrontal deficits associated with the disorder.

The third major domain of cognitive impairment seen in NF1 is visuospatial. Visuospatial problems are one of the most consistent symptoms seen in NF1. Robust deficits in tests of visuospatial function, such as the Judgment of Line Orientation (JLO) task, occur in over half of patients with NF1 (Hyman et al., 2005). Function in the visuospatial domain is affected more consistently than other neuropsychological domains, and also more severely than predicted by general IQ (Hyman et al., 2003). In fact, combined performance in multiple tests of visuospatial function can discriminate children with NF1 from unaffected children (Schrimsher et al., 2003). Visuospatial processing and spatial working memory required for tasks such as the JLO are thought to be mediated by a network involving the prefrontal, parietal, and visual cortices (Ng et al., 2000; Lanca et al., 2003). Prefrontal cortex dysfunction may contribute to visuospatial impairments in NF1. However, these impairments occur independently of prefrontal cortex-related symptoms such as attention impairments (Schrimsher et al., 2003). Therefore, it is thought that visuospatial processing deficits in NF1 are primarily related to parietal cortex dysfunction.

Finally, motor performance is impaired in NF1 patients. Fine motor coordination and motor speed are decreased in NF1. Visuomotor integration is also impaired so that patients with NF1 have poor hand–eye coordination and balance. As a result, over half of the children with NF1 have problems with tasks such as handwriting that require visuomotor integration and fine motor skills (Hyman et al., 2003). The profile of motor impairments seen in NF1 involves functions classically assigned to the cerebellum (Timmann and

Diener, 2003). Indeed, the principal cells of the cerebellum, the Purkinje cells, express high levels of neurofibromin, the protein encoded by the NF1 gene (Gutmann et al., 1995). Further characterization of cerebellar pathology in NF1 will be important to understand the mechanisms underlying motor impairments associated with the disease.

Large, detailed sibling-controlled studies of patients with NF1 have revealed the extent and high frequency of cognitive symptoms associated with the disease. These studies have demonstrated specific cognitive domains in which symptoms cluster. These cognitive domains are thought to depend on specific brain systems such as hippocampus, prefrontal cortex, and parietal cortex, suggesting that dysfunction in these areas may underlie NF1 symptoms. Examining the genetic causes of NF1 can provide further insight into the type of dysfunction that occurs on a molecular and neuronal level within these brain areas.

4.19.3 Genetic Mechanisms Responsible for Neurofibromatosis

NF1 is caused by mutations to the *Nf1* gene (Wallace et al., 1990; Viskochil et al., 1990), many resulting in loss of function of its protein product, neurofibromin. Affected individuals are heterozygotes, as homozygous mutations are lethal (Friedman, 1999). The *Nf1* gene is one of the largest in the genome, encompassing 60 exons (Marchuk et al., 1991; Li et al., 1995). Interestingly, up to 50% of patients with NF1 have spontaneous mutations of the gene, with no genetic or phenotypic evidence of NF1 in their parents (Huson et al., 1989; Friedman, 1999). The remaining 50% of patients with NF1 inherit it from their parents. Inheritance of NF1 shows an interesting pattern of complete penetrance but variable expressivity (Ward and Gutmann, 2005). Every person with an inactivating mutation in one allele of the *Nf1* gene shows some symptoms of NF1. However, NF1 shows highly variable expressivity. Clinical presentation of NF1 ranges from minimal symptom severity to extremely high symptom load. The reason for this variable expressivity is currently not clear. Explanations for the variable expressivity include variable inheritance of genetic modifiers and differing effects of mutation type and location within the *Nf1* gene. Inheritance of genetic modifiers is thought to account for the majority of variability in NF1 expression. Genetic studies have examined the degree to which inheritance correlates with expression of symptoms, including neurofibromas and referral for remedial education. Monozygotic twins affected by NF1 have a high correlation in symptom severity. The strong genetic component in symptom expression in NF1 is consistent with modulation of disease symptoms by inherited genetic modifiers (Easton et al., 1993). For example, proteins encoded by genetic modifiers may interact with the neurofibromin signaling pathway to exacerbate effects of *Nf1* mutations or confer protection by compensating for the loss of neurofibromin function. Identification of these modifiers could provide additional prognostic information for patients and potentially act as new therapeutic targets.

The type of *Nf1* gene mutation could contribute to the variable expressivity of symptoms in this disorder. Different types of mutations, including point and frame shift mutations, occur throughout the *Nf1* gene. No clear relationship has been identified between the location of the mutation in the gene and the range of symptoms seen (Viskochil, 2002). However, patients who carry large deletions of the *Nf1* gene present with a more severe type of NF1, called the microdeletion syndrome, which involves more extreme symptoms such as mental retardation. This is significant because the occurrence of the microdeletion syndrome may account for the slightly elevated rate of mental retardation seen in the NF1 patient population (4%–5%) compared to the general population (2%–3%) (Venturin et al., 2004).

In addition to the variability in severity of individual symptoms, it is becoming clear that different mechanisms account for the evolution of different symptoms. Generalized symptoms seen in NF1, such as cognitive symptoms, are caused by the heterozygous mutation of one allele of the *Nf1* gene. More focal symptoms, such as formation of neurofibromas, require loss of both alleles of NF1. Reduction to homozygosity (loss of the remaining NF1 wild-type allele in heterozygous tissue) can occur throughout life, due to somatic mutation. This creates localized clusters of cells that show homozygous inactivation of the *Nf1* gene on a background of tissue with heterozygous loss of *Nf1* function. Reduction to homozygosity is thought to underlie a number of serious NF1 symptoms, including peripheral neurofibromas and CNS gliomas (Zhu et al., 2002; Bajenaru et al., 2003; Ward and Gutmann, 2005).

Investigation of the genetics of NF1 has led to improvements in the diagnosis of the disease and extended our understanding of its symptoms. As

NF1 is caused by a single gene with 100% penetrance, diagnosis of the disease can be confirmed by genetic analysis. This is in contrast to diagnosis in other diseases without a known genetic origin, which is purely dependent on symptom expression. For the NF1 patient population, genetic diagnosis improved characterization of the cognitive profile and evaluation of the prevalence of each symptom. For example, prior studies had indicated that mental retardation was a prominent feature of patients with NF1, due to ascertainment biases caused by underdiagnosis of patients without central nervous system (CNS) lesions (North, 2000). However, more comprehensive cognitive assessments in the entire NF1 patient population demonstrated instead specific patterns of cognitive strengths and weaknesses and low normal IQ. Additionally, identification of the *Nf1* gene has allowed the derivation of genetic animal models of NF1. The study of these models has led to significant insights into the molecular, cellular, and system mechanisms affected by mutations in the *Nf1* gene.

4.19.4 Animal Models of Neurofibromatosis

Animal models of genetic disorders have a variety of purposes. Some are made to mimic the human disease as closely as possible, so that disease mechanisms can be studied and potential treatments tested. Other animal models are derived to address specific questions within a complex cluster of biological phenotypes. The study of NF1 has involved a wealth of animal models derived primarily in mouse and *Drosophila*.

Mice with a heterozygous null mutation of the *Nf1* gene ($Nf1^{+/-}$) have compelling genetic and behavioral parallels with human NF1, making them an excellent system in which to study the mechanisms of cognitive deficits associated with NF1. The sequence, transcriptional regulation, and downstream targets of *Nf1* are conserved across species, including mouse and human (Bernards et al., 1993; Hajra et al., 1994). The majority of mutations (70%) to the *Nf1* gene in patients lead to synthesis of a truncated, nonfunctional version of the encoded neurofibromin protein (Thomson et al., 2002; Shen et al., 1996). The $Nf1^{+/-}$ mouse model was made by deleting exon 30 of the *Nf1* gene, which leads to an unstable, quickly degraded transcript (Jacks et al., 1994). Like patients with NF1, the $Nf1^{+/-}$ mouse model is heterozygous for this loss of function mutation.

Interestingly, behavioral analysis of the $Nf1^{+/-}$ mice revealed that they exhibit features of the learning deficits and executive function impairments seen in NF1 patients. Specifically, the $Nf1^{+/-}$ mice show spatial learning impairments and attention deficits. Learning deficits in the $Nf1^{+/-}$ mice were first revealed in a classic test of spatial learning, the hidden platform version of the Morris water maze. In this task, mice learn to navigate to a platform hidden just beneath the water in a round pool. The mice are thought to learn the position of the fixed hidden platform in reference to cues surrounding the pool. Impairments of the $Nf1^{+/-}$ mice in this test are specific to spatial learning and memory, as the $Nf1^{+/-}$ mice display normal visual learning in the visible version of the water maze (Silva et al., 1997; Costa et al., 2002). In this nonspatial control task, the mice learn to navigate to a visible platform. This phenotype is not unlike the profile of learning deficits seen in NF1 patients, where spatial learning is severely affected but visual learning is spared (Hyman et al., 2005).

In addition to spatial learning deficits, NF1 commonly involves attention deficits. Like patients, the NF1 mouse model also displays behavioral attention deficits (Li et al., 2005). Attention is tested in mice using a lateralized reaction time task. This task uses an apparatus originally described for a five-choice serial reaction time task (Robbins, 2002), a standard operant chamber with a curved side wall containing five nose-poke apertures. Mice start a trial with a sustained nose poke at a center aperture. At this point, the far-left or far-right aperture is illuminated for a fixed period (2, 1, or 0.5 s). The illuminated aperture acts as a target, which mice must notice and nose poke for a food reward. Performance of this task requires sustained attention on both spatially separated potential targets (Jentsch, 2003). When the target aperture is illuminated for short intervals of 0.5 s, $Nf1^{+/-}$ mice show a significantly increased rate of omissions, which indicates an inability to sustain visuospatial attention (Li et al., 2005). This task demonstrates behavioral attention deficits in $Nf1^{+/-}$ mice that are similar to attention deficits seen in patients.

Since the $Nf1^{+/-}$ mice model both learning and attention deficits seen in NF1 patients, mechanisms underlying these deficits can be studied using these mice. Spatial learning in both mice and humans is thought to depend on hippocampus while attention is thought to critically involve prefrontal cortex (Dalley et al., 2004). Changes in the biochemistry

and physiology of these brain areas can be examined in the *Nf1*$^{+/-}$ mice in order to identify dysfunction and to study the contribution of each brain area to the overall pattern of cognitive symptoms seen in NF1. This approach provides insights into the neuronal changes that underlie cognitive symptoms of NF1. Other approaches to the study of NF1 use different animal models which have been developed to address more specific questions about NF1 pathogenesis.

The *Nf1* gene encodes several functional elements, including a ras GTPase–activating domain (Ballester et al., 1990; Xu et al., 1990) and an adenylyl cyclase–activating domain (Tong et al., 2002). To evaluate the behavioral importance of each of these functions, animal models were developed with mutations affecting individual domains. For example, exons 20–27a of the *Nf1* gene encode a ras GTPase–activating domain (GAP domain) (Upadhyaya et al., 1997). A mouse model was made carrying a deletion of exon 23a in the GAP domain of *Nf1*. This deletion modifies the affinity and GAP activity of the mutant neurofibromin. Behavioral analysis of this mutant mouse demonstrates the importance of the GAP domain to cognitive function, including spatial learning (Costa et al., 2001). Importantly, previous studies in humans had identified cognitive deficits in a patient with a point mutation in the GAP domain of the *Nf1* gene (Upadhyaya et al., 1997; Klose et al., 1998; Weiss et al., 1999). These studies suggest that the ras-GAP function of neurofibromin is critical for normal cognition, and they confirm that mice can be used to model the cognitive deficits associated with NF1.

Additionally, studies with *Drosophila* models of NF1 revealed a behavioral role for the interaction between *Nf1* and adenylyl cyclase. In *Drosophila*, homozygous deletion of *Nf1* (*Nf1*$^{-/-}$) is not lethal, so *Drosophila* lines that are null for *Nf1* can be used. These lines can be engineered to carry partial homozygous deletions of *Nf1*, resulting in flies with truncated neurofibromin proteins. Behavioral studies in such fly lines demonstrate that restoring adenylyl cyclase activation in *Nf1* can rescue biochemical and behavioral deficits of the *Nf1*$^{-/-}$ flies (Guo et al., 2000; Hannan et al., 2006). Further, *Drosophila* genetics can utilize mutagenesis-based screens to identify novel genes which interact with *Nf1* (i.e., modifiers). *Drosophila* models greatly extend our ability to address the intricate biochemical functions of *Nf1*, examine the behavioral role of each of its biochemical functions, and identify effectors and modifiers of *Nf1*.

Nf1 mutations can affect various signaling pathways, and animal models, such as the ones described above, are being used to identify the contribution of each of these pathways to the phenotype of NF1 mice. By disrupting specific functional domains of the *Nf1* gene, these animal models isolate the contribution of each domain to behavioral and physiologic dysfunction. Like *Nf1*, many other disease-related genes encode multifunctional proteins which participate in complex biochemical cascades. Animal models provide a unique tool to integrate these biochemical properties with cellular and behavioral phenotypes.

Identification of genetic and molecular mechanisms of diseases, such as NF1, leads to another important question: Since the genetic mutation is inherited from birth, are the symptoms of the disease caused by the impact of the disease allele on CNS development, or are the symptoms caused by the effects of the gene mutation on function of the mature brain? Mice or *Drosophila* carrying conditional gene deletions can help to address this question. Conditional mutations can be created in mice using the cre-loxP system, a powerful tool widely used for restricting gene deletions to specific time frames, cell types, or areas. The system utilizes Cre recombinase, a bacteriophage enzyme which mediates site-specific recombination between DNA sequences (loxP sites) flanking the gene of interest (floxed gene). The floxed gene is expressed normally until deleted by Cre recombinase (Yu and Bradley, 2001; Morozov et al., 2003; Ahmed et al., 2004). For NF1, a mouse line was engineered with loxP sites flanking exons 31–32 of the *Nf1* gene (*Nf1*$^{flox/flox}$). The floxed *Nf1* gene acts like a wild-type allele prior to delivery of Cre recombinase. Mice carrying one floxed *Nf1* allele and one deleted *Nf1* allele (*Nf1*$^{flox/-}$) show the same phenotypes as *Nf1*$^{+/-}$ mice; for example, they have the same survival profile. Once the deletion of the floxed *Nf1* allele occurs, following delivery of Cre recombinase, the *Nf1*$^{flox/-}$;cre+ mice become homozygous knockouts (*Nf1*$^{-/-}$) (Zhu et al., 2001). Breeding the floxed *Nf1* allele mice to mice with a Cre recombinase gene driven by a specific promoter can result in *Nf1* mice with deletions of the gene in specific tissues and at specific times. For example, promoters can be chosen that are expressed later in development so that the resulting mutants exhibit adult deletion of the *Nf1* gene (Zhu et al., 2001; Morozov et al., 2003). Additionally, viral vectors can be used to deliver Cre recombinase to the *Nf1*$^{flox/+}$ mouse at specific times and to specific systems (Morozov et al., 2003; Ahmed et al., 2004).

Disease-related genes often encode multifunctional proteins that participate in a range of complex biochemical pathways within cells. This makes it difficult to isolate specific pathways leading to dysfunction. The encoded proteins are also expressed in multiple cell types such as glia, interneurons, and primary neurons that could interact to mediate complex network properties (Gutmann et al., 1995). Therefore, it is often difficult to determine exactly which systems contribute to dysfunction and how. Animal models can be used to identify causal relationships between the biochemical, physiologic, and behavioral effects of disease-causing mutations. Thus, they are an effective tool in understanding which mechanisms underlie specific phenotypes of the disease. In NF1, several types of animal models have been used to understand the cognitive phenotypes of the disease. As stated above, the $Nf1^{+/-}$ mice expresses a nonfunctional allele of the *Nf1* gene, and their phenotype mimics specific behavioral aspects of the human disease (Jacks et al., 1994; Costa et al., 2002). Other mouse and *Drosophila* NF1 models carry mutations targeted to specific functional domains of the *Nf1* gene, allowing the role of these domains to be examined (Costa et al., 2001; Hannan et al., 2006). Finally, animal models with conditional mutations can be used to examine temporal, cell type, or brain system–specific effects of *Nf1* deletion. For example, conditional mutations can be induced in a temporally controlled manner to identify particular times when *Nf1* is critical for function. In combination with clinical data, animal models, such as the ones used to study NF1, are powerful tools in the study of the pathogenesis of the disease.

4.19.5 Biochemical and Physiological Functions of Neurofibromin

The neurofibromin protein has multiple biochemical roles. It acts as a ras-GAP (GTPase activating protein) to negatively regulate ras signaling, and it can also serve as an activator of adenylyl-cyclase. Both pathways play a role in mediating signaling events from the membrane to downstream targets within the neuron. Loss of neurofibromin results in constitutive increases in ras intracellular signaling. Evidence for a role of neurofibromin in the modulation of ras signaling has been obtained in patient and mouse mutant studies. In NF1, ras signaling can be increased to the point that it becomes independent of the constraints of extracellular triggers such as growth factor activation. For example, $Nf1^{-/-}$ sensory neurons no longer require the extracellular growth factor brain-derived neurotrophic factor (BDNF) to survive and mature, presumably due to constitutive level of activity of the ras pathway when it is no longer subject to negative regulation by neurofibromin (Vogel et al., 1995).

The ras protein is part of a large superfamily of GTPases that mediate signaling from membrane receptors to intracellular cascades of kinases. Ras activation requires this protein to be tethered to cell membrane. At the membrane, ras can interact with downstream effectors such as raf kinase, which then activate signaling cascades such as the MEK/MAPK pathway (Boguski and McCormick, 1993; Weiss et al., 1999). These kinases then induce a number of downstream processes, including transcription of immediate early genes leading to short- and long-term changes in neuronal function. The ras protein cycles between the inactive GDP-bound state and the active GTP-bound state. The activity state of ras is determined by a balance of activating proteins (GEFs, guanine nucleotide exchange factors, which allow bound GDP to be released so that GTP can bind) and inactivating proteins (GAPs, GTPase activating proteins, which increase the endogenous GTP hydrolyzing activity of ras) (Bernards and Settleman, 2004). Neurofibromin , as a ras-GAP, is an important negative regulator of ras signaling (Weiss et al., 1999).

Experimental evidence demonstrates that increased ras activity in NF1 plays a critical role in causing impaired learning and cognition. For example, mice in which the ras-GAP activity of *Nf1* was modified by deleting exon 23a of the *Nf1* gene show impairments in behavioral tests of learning and memory similar to those found in the null allele (Costa et al., 2001). Additionally, genetic or pharmacologic normalization of ras activity in $Nf1^{+/-}$ mice rescued behavioral learning and memory deficits. Ras activity was shown to be abnormally increased in the brain of $Nf1^{+/-}$ mice (Li et al., 2005). Genetic rescue was done by breeding these $Nf1^{+/-}$ mice to mice with a heterozygous deletion of *K-ras*. In the resultant double mutants, the decreased total levels of *K-ras* compensate for the increased activation state of the ras pathway caused by the *Nf1* deletion, leading to normalized levels of Ras activity. In contrast to the $Nf1^{+/-}$ mice, these double mutants showed normal performance in the Morris water maze, a test of spatial learning and memory (Costa et al., 2002). Similar results were also obtained by crossing the $Nf1^{+/-}$ mice to mice with heterozygous deletion of N-ras, a different isoform of ras.

Farnesyl-transferase inhibitors (Costa et al., 2002) and lovastatin (Li et al., 2005) can also rescue the learning and memory deficits of the *Nf1*$^{+/-}$ mice. Both of these pharmacologic interventions were shown to decrease ras activity. Therefore, these interventions rescue the biochemical deficits thought to be at the heart of behavioral deficits in *Nf1*$^{+/-}$ mice. For example, lovastatin, a cholesterol synthesis inhibitor that can be safely prescribed to patients, was shown to lower levels of active ras-GTP and its downstream effector, p-MAPK, in the cortex and hippocampus of *Nf1*$^{+/-}$ mice (Li et al., 2005). Both farnesyl-transferase inhibitors (Weiss et al., 1999) and lovastatin (Mendola and Backer, 1990; Xu et al., 1996) are thought to interfere with important aspects of the isoprenylation and activation of ras. Importantly, besides rescuing learning and memory deficits, lovastatin was shown to also reverse the attention impairments of the *Nf1*$^{+/-}$ mice (Li et al., 2005). The ability of ras manipulations to successfully rescue behavioral deficits in the *Nf1*$^{+/-}$ mice demonstrates that the ras-GAP function of neurofibromin is critical for cognitive function.

One of the key functions of Ras is its role as an intracellular mediator of the receptor tyrosine kinases (Bernards and Settleman, 2005). This family of transmembrane receptors is characterized by phosphorylation of intracellular tyrosines upon ligand binding. Tyrosine phosphorylation then leads either to direct binding and inactivation of ras-GAPs such as neurofibromin, or to indirect activation of ras-GEFs (Weiss et al., 1999; Patapoutian and Reichardt, 2001). The growth factor receptors make up a major class of receptor tyrosine kinases, which are likely to be important to NF1 pathology. The formation of peripheral tumors in NF1 is thought to be related to increased growth factor sensitivity. This leads to increased cellular growth, survival, and abnormal function (DeClue et al., 2000; Li et al., 2002; Gottfried et al., 2006). Changes in growth factor signaling may also contribute to the changes in neuronal physiology and resulting cognitive symptoms seen in NF1. BDNF normally signals in neurons through transmembrane receptors such as the TrkB receptors, which activate the ras pathway (Patapoutian and Reichardt, 2001). In neurons with deletion of *Nf1*, constitutive ras activation results in neurons that can survive and extend neurites independently of BDNF (Vogel et al., 1995). Normally, BDNF-dependent signaling protects inactive inhibitory synapses from being pruned (Swanwick et al., 2006). If these pathways are constitutively activated, then activity-dependent shaping of inhibitory synapses and networks may not occur and inappropriately networked inhibitory terminals may persist in the NF1 mice. Additionally, BDNF modulates increases in vesicular release probability at inhibitory terminals through synaptic vesicle–associated proteins such as synapsin 1 (Li et al., 2002). Finally, BDNF enhances mRNA and protein expression of GAD, the rate-limiting enzyme in GABA synthesis (Li et al., 2002; Ohba et al., 2005). It is possible that these functions of BDNF are abnormal in NF1 neurons, thus resulting in changes in GABAergic activity. While the role of neuronal BDNF signaling in NF1 remains speculative, increased GABAergic tone has been suggested in *Nf1*$^{+/-}$ mice. This increased GABAergic release seems to cause deficits in long-term potentiation, a form of synaptic plasticity thought to underlie learning and memory (Costa et al., 2002).

In addition to its role as a ras-GAP, neurofibromin acts as an activator of adenylyl cyclase (Tong et al., 2002). Mutation of the *Nf1* locus in *Drosophila* leads to decreased activity of the cAMP/PKA pathway and to learning and memory deficits. The adenylyl cyclase pathway has been shown to be important for regulation of neuronal development and learning in *Drosophila*. Learning defects in the *Nf1* null flies can be rescued by expression of a constitutively active form of PKA (Guo et al., 2000). This suggests that associative learning impairments in *Nf1* null flies are due to decreased activation of adenylyl cyclase. Interestingly, *Nf1* can increase adenylyl cyclase activity in a ras-dependent manner in *Drosophila*. This form of signaling occurs in response to growth factors such as EGF. *Nf1* can also directly increase adenylyl cyclase activity in a ras-independent manner, through its C-terminal domain. This function of *Nf1* is required for stimulation of adenylyl cyclase by neurotransmitters such as serotonin or histamine (Hannan et al., 2006). *Nf1* loss in *Drosophila* causes some phenotypes, such as small body size, through direct decrease of adenylyl cyclase activity (Tong et al., 2002). Other behavioral functions of *Nf1*, such as circadian rhythm modulation in *Drosophila*, are MAPK dependent and require the GAP domain instead (Williams et al., 2001). It is currently unclear whether these biochemical effects of homozygous *Nf1* deletion are relevant to the behavioral and cognitive deficits associated with NF1, since these deficits are thought to be caused by heterozygous mutations and the effects on adenylyl cyclase are only observed in homozygous mice or flies. In both mouse and *Drosophila* models, heterozygous deletion of *Nf1* did not grossly affect regulation of

the adenylyl cyclase pathway (Tong et al., 2002). Nevertheless, it is conceivable that *Nf1* plays a role in maintaining the relative balance between ras- and cAMP-dependent signaling (Weeber and Sweatt, 2002).

Drosophila models also highlight the behavioral importance of the GAP domain of *Nf1*. Further, the *Drosophila* model demonstrates the complex interplay between the effects of *Nf1* mutation in multiple biochemical pathways, each of which acts in different signaling contexts. Perhaps this biochemical complexity contributes to the highly variable nature of the symptoms seen in NF1.

4.19.6 Interactions Between Different Cell Types in Neurofibromatosis

Symptoms of NF1 are caused not only by disruption of molecular signaling within each cell but also by the effect of *Nf1* mutation on the interaction between different cell types. Although all cells in patients carry the mutant *Nf1* locus, it is possible that the key symptoms in NF1 are caused by the effects of mutation in a subset of these cells. Heterogeneity in the effects of *Nf1* mutation in different cell types may be due to cell-type specific differences in either the expression of *Nf1* or the signaling molecules that interact with *Nf1*. In addition, somatic reduction to homozygosity can create clusters of homozygously deleted cells of one type among tissue that is heterozygous for *Nf1* deletion. As a result of this heterogeneity, symptoms can occur in one cell type as a result of dysfunction in another cell type. For example, some evidence suggests that glial dysfunction in NF1 is not cell autonomous. Glial dysfunction in NF1 patients results in astrocytic gliosis with upregulated glial fibrillary acidic protein (GFAP) expression (North, 2000) and glial-based tumors (Sagar, 2004; Ward and Gutmann, 2005) in the CNS. Data from mouse models suggest that glial dysfunction is caused by an abnormal interaction of glia with neurons expressing mutant *Nf1*. An elegant set of experiments using neuronal specific deletion of *Nf1* have demonstrated that neuronal loss of *Nf1* is sufficient to induce glial changes such as astrogliosis. Mice carrying a floxed *Nf1* gene were crossed to mice where Cre expression was limited to mature neurons by being driven by the synapsin promoter. Offspring from this cross carried a neuronal-specific, homozygous deletion of *Nf1*. *Nf1* deletion from neurons resulted in an increased number of reactive, GFAP-positive astrocytes (Zhu et al., 2001). Glial-derived tumor formation also requires *Nf1* loss in nonglial cell types. Loss of both alleles of *Nf1* in glial cells must occur on an $Nf1^{+/-}$ background in order for tumor formation to occur (Zhu et al., 2002), suggesting that the interaction between $Nf1^{+/-}$ neurons and $Nf1^{-/-}$ glia is permissive for glioma formation.

This example is presented to highlight the importance of identifying primary disease mechanisms. In this case, the changes that occur in astroglia in NF1 CNS may occur as a secondary consequence of *Nf1* loss in neurons. Patients with NF1 commonly demonstrate astrocytic gliosis as well as unidentified bright objects (UBOs). UBOs are areas of T2 magnetic resonance imaging hyperintensity related to edema within the myelin and localized abnormal glial proliferation (North et al., 1997). However, no consistent correlation, and certainly no predictive relationship, has been found between the number of UBOs and scores on cognitive tests. This result is consistent with the idea that neuronal changes, such as increased ras signaling, cause the cognitive deficits associated with NF1.

4.19.7 Increased GABAergic Inhibition in the Neurofibromatosis Mouse Impairs Learning and Memory

Studies in the NF1 mouse suggest that ras-dependent deregulation of inhibitory GABAergic networks is a central mechanism underlying behavioral impairment. Deletion of *Nf1* in the mouse leads to a ras-dependent increase in inhibitory GABAergic currents in the hippocampus. Increased GABAergic tone creates a loss of balance between inhibitory and excitatory processes within hippocampal networks and impairs plasticity. The resulting hippocampal dysfunction is thought to underlie the learning deficits seen in the $NF1^{+/-}$ mice.

In initial studies, learning was assessed in $NF1^{+/-}$ mice using the Morris water maze. This task was chosen because it is extremely sensitive to hippocampus dysfunction (Morris et al., 1982; Morris et al., 1990; Cho et al., 1999). During training, mice were placed in an opaque pool of water and learned to find a submerged platform using spatial cues (Morris, 1984). $NF1^{+/-}$ mice learned this task significantly slower than wild-type animals. For example, in a test after 7 days of training, most wild-type mice had already learned the spatial location of the platform, while most $NF1^{+/-}$ mice still had not. This finding is consistent with hippocampus dysfunction in these animals. A closer

examination of synaptic plasticity in a hippocampal region critical for spatial learning (CA1) found decreased levels of long-term potentiation (LTP). LTP is an increase in synaptic transmission that is observed following high-frequency stimulation (Bliss and Lomo, 1973; Bliss and Collingridge, 1993; Malenka and Nicoll, 1999). In the hippocampus, there is strong evidence that LTP is required for learning of spatial cues and contextual information (Richter-Levin et al., 1995; Martin et al., 2000; Martin and Morris, 2002). The hippocampal LTP impairment in the $NF1^{+/-}$ mice appeared to be caused by increased local inhibitory tone. Input–output curves demonstrated hyperactivity of local inhibitory circuits in CA1, as evidenced by abnormally large evoked inhibitory currents. Further, the $NF1^{+/-}$ LTP deficit can be rescued by lowering hippocampal inhibition using picrotoxin, a GABA(A) antagonist (**Figure 1**).

Interestingly, the LTP impairments seen in the $NF1^{+/-}$ mice are specific to certain strengths and patterns of stimulation. Hippocampal LTP deficits occur in response to theta burst stimulation (TBS), a form of

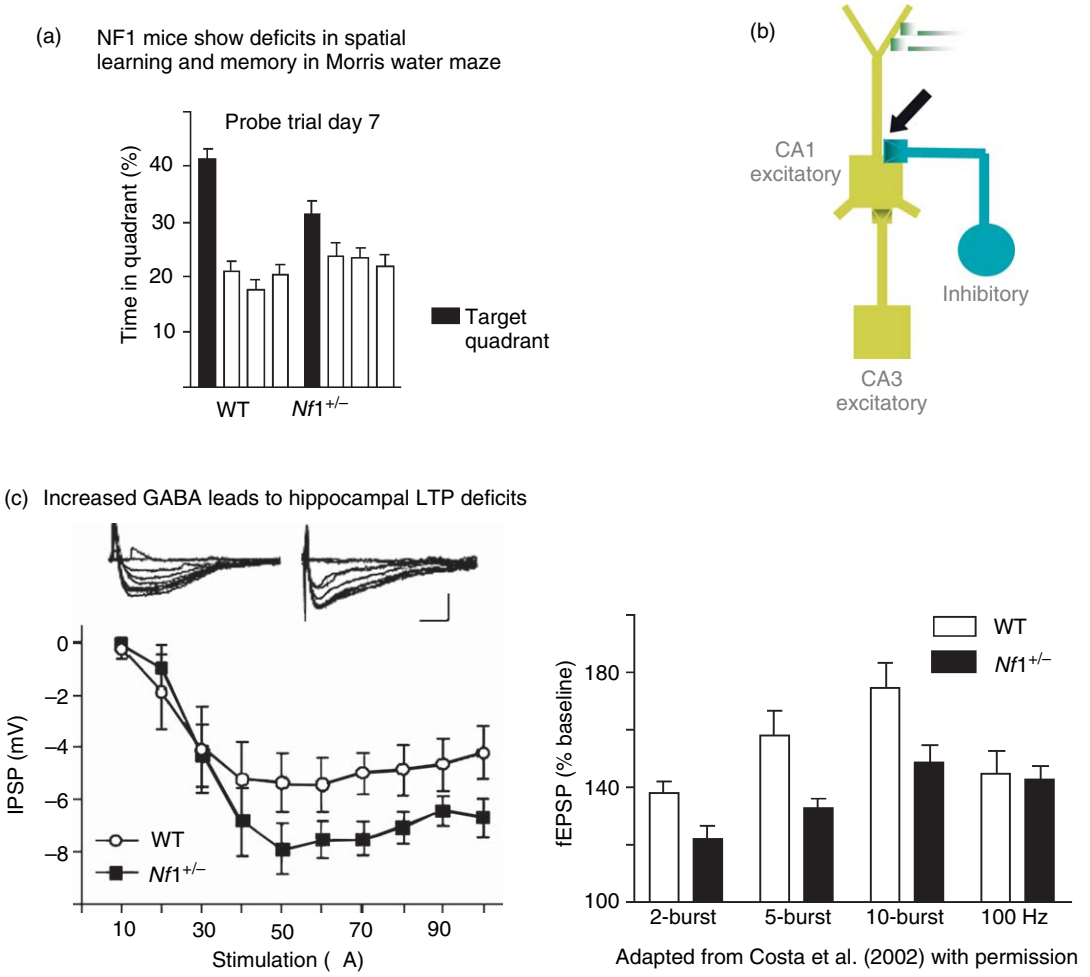


Figure 1 Learning and memory deficits in the NF1 mouse model are caused by increased local inhibition in hippocampal networks. (a) *Nf1*$^{+/-}$ mice show impaired spatial learning, as seen in this day 7 probe trial of the Morris water maze. The percentage of time spent in the target quadrant (*y*-axis), where the hidden platform had been, is significantly less in *Nf1*$^{+/-}$ mice compared to wild-type (WT) littermates. (b) schematic of hippocampal CA3–CA1 circuit, with local inhibitory interneurons. Inhibitory synapses onto CA1 pyramidal neurons (arrow) have abnormally high GABA release in *Nf1*$^{+/-}$ mice. (c) (left) Larger inhibitory potentials (IPSP) are evoked in *Nf1*$^{+/-}$ mice compared to WT littermates. Input–output curves were recorded by whole-cell patch in CA1 pyramidal neurons. Inhibitory potentials were evoked with local field stimulation of increasing amplitude (*x*-axis). (right) long-term potentiation (LTP) deficit in *Nf1*$^{+/-}$ mice at the CA3 to CA1 pathway. LTP deficit is seen when induced by theta burst (2-, 5-, 10-burst), but not 100-Hz stimulation. LTP is measured as the increase in the slope of evoked potentials (EPSPs) 40 min following induction, and is represented as a percentage increase over the baseline EPSP seen prior to induction (*y*-axis).

stimulation that mimics endogenous firing patterns in the hippocampus (Otto et al., 1991; O'Keefe and Recce, 1993; Skaggs et al., 1996). TBS recruits and potentiates synapses from both inhibitory interneurons and excitatory neurons (Chapman et al., 1998; Perez et al., 1999). Since interneurons seem to be overactive in the $NF1^{+/-}$ mice, the increased inhibition prevents normal levels of LTP from being induced. In contrast, LTP deficits in $NF1^{+/-}$ mice do not occur when nonphysiological intense stimulation parameters (e.g., 100 Hz) are used for LTP induction. These intense stimulation parameters strongly potentiate excitatory pathways, which can perhaps overcome the increased inhibition. This type of stimulation is also known to be less effective than theta burst stimulation at recruiting inhibitory activity (Chapman et al., 1998; Perez et al., 1999).

On a behavioral level, the learning deficit exhibited by $NF1^{+/-}$ mice can also be overcome with additional training. Overtraining allows $NF1^{+/-}$ mice to perform spatial learning tasks as well as their wild-type littermates (Silva et al., 1997). Under these conditions, it may be the case that excitatory pathways in $NF1^{+/-}$ mice eventually potentiate enough to compensate for increased inhibition. Physiologic impairments in $NF1^{+/-}$ are limited to specific stimulation paradigms and conditions. This may lead to the specific pattern of behavioral impairments seen in $NF1^{+/-}$ mice. In contrast to these mice, which model a learning disability disorder, plasticity impairments are seen even after 100-Hz stimulation in a rodent model of mental retardation (e.g., the tuberous sclerosis rat) (von der Brelie et al., 2006). It is likely that the severity of the behavioral impairment is related to the severity of the neurophysiological abnormalities.

The NF1 mouse model has clearly demonstrated that neurofibromin is an important regulator of inhibitory tone in neuronal networks. This regulation is functionally important, as its disruption significantly impairs behaviors, such as the acquisition of spatial information. These findings raise the possibility that increased activity in inhibitory networks underlies the learning deficits observed in NF1 patients.

4.19.8 Increased GABAergic Activity and Learning Disabilities in Neurofibromatosis Patients

We found that deletion of NF1 in mice increases hippocampal inhibition, reduces plasticity, and impairs spatial learning (Costa et al., 2002). Spatial learning has been shown to depend on the hippocampus in both humans and rodents (Burgess et al., 2002; Squire et al., 2004). Therefore, learning deficits observed in NF1 patients may also be caused by reduced plasticity and enhanced inhibition in hippocampus. Several aspects of learning deficits in NF1 patients suggest dysfunction in the hippocampal memory system. For example, NF1 patients perform poorly on spatial learning and memory tasks. Lesion and imaging studies in the general population have demonstrated that performance in these tasks depends on the hippocampus and adjacent medial temporal cortex. Damage to these areas produces profound amnesia for spatial information and difficulty in navigating through space (Bohbot et al., 2000; Burgess et al., 2002; Squire et al., 2004). Imaging studies (PET, fMRI) have also found activation of the hippocampus and surrounding cortex when subjects navigate through virtual mazes or when they recall routes they have learned in their natural environment. The most significant effects are often seen on the right side of the brain, although the left hippocampus and surrounding cortex are also thought to contribute (Maguire, 1997; Maguire et al., 1999). It should be noted that the hippocampus is only part of a larger network that supports navigation including the parietal cortex, posterior cingulate cortex, and prefrontal cortex. Its specific function is thought to be the formation and storage of topographical maps of the external environment (O'Keefe, 1978). When the hippocampus is damaged or dysfunctional, these maps cannot be formed or recalled, and spatial navigation is impaired. In NF1 patients, spatial learning and memory deficits suggest partial hippocampal dysfunction. Further, the NF1 animal models predict that hippocampal dysfunction should be caused by increased local inhibition. In this case, we can predict that activation of the hippocampus during spatial memory tasks would be reduced in NF1 patients relative to normal controls. Functional imaging studies can be used to evaluate these predictions.

Hippocampus dysfunction may also underlie the vocabulary and reading problems seen in NF1 patients. Damage to the hippocampus in normal individuals produces multiple memory problems, including an inability to acquire new facts and general information about the world (Schmolck et al., 2002; Bayley and Squire, 2005). For example, amnesic patients have considerable difficulty learning and retrieving new vocabulary words (Verfaellie et al., 2000). Although NF1 patients are clearly not amnesic, a milder hippocampal deficit akin to the one described in $NF1^{+/-}$ mice could contribute to the vocabulary and reading problems associated with

NF1. Imaging studies could be used to explore the possible relationship between deficits in hippocampal activation and vocabulary and reading problems. For example, learning of novel lexicon has been studied in the general population and was found to activate both prefrontal and hippocampal networks. With extended training, the hippocampus shows reduced activity, while the prefrontal cortex shows increased activation. Subjects that have smaller decreases in hippocampal activity with repeated training were found to have better recall of word lists (Breitenstein et al., 2005). Therefore, hippocampal engagement throughout the task appears to maximize learning of word definition associations. Training-dependent changes like these could also be examined in NF1 patients to see how activation in regions such as the hippocampus and prefrontal cortex differs from normal individuals during learning and retrieval of new information. The effects of drug treatment (i.e., lovastatin) on activity in specific brain regions could also be evaluated and correlated with performance in these patients.

4.19.9 Balanced Inhibition and Normal Prefrontal Cortex Function

Previous studies in the $NF1^{+/-}$ mice found high levels of ras activity not only in the hippocampus but also in prefrontal areas (Li et al., 2005). It is likely that ras-dependent increased inhibition is also seen in prefrontal cortex, as in hippocampus. Further, cognitive symptoms seen in NF1 patients are suggestive of prefrontal cortex dysfunction. To better understand the contribution of prefrontal cortex dysfunction to behavioral symptoms of NF1, it is important to consider the known function and physiology of this area. The prefrontal cortex in both mouse and humans plays an important role in mediating executive functions. Executive functions are those required for flexible, adaptive, goal-directed behavior and extrapolation to future goals (Miller, 2000; Stuss and Levine, 2002; Uylings et al., 2003; Dalley et al., 2004; Powell and Voeller, 2004). These include attention, organization, and planning, domains in which significant impairments are seen in NF1 patients (Dilts et al., 1996; Koth et al., 2000; North, 2000; Hyman et al., 2005). In mediating these behaviors, prefrontal cortex encodes representations of task-related information, incorporating cue relevancy, response outcomes, and rewards. This information is available to prefrontal cortex through a broad range of inputs from multimodal sensory areas, memory systems such as hippocampus, and striatal and midbrain systems which encode reward and expectation. Many of these connections are reciprocal, allowing for feedback loops to be established. Additional outputs from prefrontal cortex to motor cortex and striatum allow prefrontal activity to modulate behavior (Miller and Cohen, 2001; Uylings et al., 2003). Activity of prefrontal inhibitory networks is thought to be critical for normal prefrontal function (Goldman-Rakic, 1995; Lewis et al., 2005). In particular, balanced levels of inhibition and excitation in prefrontal cortex are thought to be important for tuning of activity during attention, working memory (Rao et al., 1999; Rao et al., 2000), and behavioral planning (Constantinidis et al., 2002).

Increased prefrontal inhibition may prevent normal control and allocation of attention. Behavioral attention involves cognitive control skills such as inhibition of irrelevant stimuli and inappropriate responses. Tasks that require such cognitive control normally recruit the prefrontal cortex as part of a cortical-striatal network (Ridderinkhof et al., 2004; Chudasama and Robbins, 2006; Arnsten, 2006). Hypoactivation of this network is associated with impairments in attention. For example, impaired performance in cognitive control tasks is seen in attention-deficit/hyperactivity disorder (ADHD). When performing these tasks, subjects with ADHD show decreased activation of prefrontal cortex and striatal structures (Vaidya et al., 2005; Smith et al., 2006). This prefrontal hypoactivity, seen in adolescents and adults independently of previous medication status (Smith et al., 2006), appears to be an intrinsic feature of ADHD thought to underlie behavioral deficits. In particular, the inattentive subtype of ADHD is thought to be caused primarily by prefrontal cortex dysfunction. In NF1, attention deficit, especially of the inattentive subtype, is a common cognitive symptom (Diamond, 2005). It is interesting to speculate that prefrontal hypoactivation may also occur in NF1 patients, leading to the symptoms of ADHD associated with this disorder. Prefrontal hypoactivation might be expected in NF1, as prefrontal increases in inhibition could increase the threshold for activation of local excitatory networks. This could prevent normal engagement of prefrontal cortex. Future studies addressing this hypothesis should examine activation of prefrontal cortex in patients with NF1 during cognitive tests of attention and response inhibition. Such studies

will be important to understand a possible connection between a putative prefrontal hypoactivation and the attention deficits described in NF1 patients.

In addition to attention, prefrontal activity contributes to other executive functions such as working memory, behavioral planning, and response organization (Goldman-Rakic, 1995; Miller, 2000; Miller and Cohen, 2001; Stuss and Levine, 2002; Dalley et al., 2004; Powell and Voeller, 2004). Working memory involves online maintenance and manipulation of information (Goldman-Rakic, 1995). Planning a series of events is a complex task which taxes working memory. Planning requires encoding multiple actions in a manner that bridges the delay between response initiation and eventual attainment of a future goal (Owen, 1997). Performance of working memory and planning tasks requires tuned, persistent activation of prefrontal cortex (Funahashi et al., 1991; Goldman-Rakic, 1995; Mushiake et al., 2006). Some evidence suggests that during planning tasks, ensembles of neurons in prefrontal cortex encode each of the actions being planned. Such evidence, obtained through single-cell recordings in the prefrontal cortex of monkeys, demonstrates that activity of these ensembles appears to be persistent and temporally orchestrated. Thus, neuronal ensembles in prefrontal cortex encode both the direction and order of actions planned through their spatial and temporal tuning (Mushiake et al., 2006). This observation is consistent with a role for this region in encoding an organized sequence of actions to reach a goal.

Both spatial and temporal tuning in prefrontal cortex is thought to be modulated by local inhibitory networks (Rao et al., 1999, 2000; Constantinidis et al., 2002). To get a sharp tuning curve, it is thought that activity of single neurons must be increased over that of their neighbors by local inhibition. This model is supported by data demonstrating that spatial tuning is disrupted quite significantly by modifying inhibitory tone in prefrontal cortex. Increasing local GABA levels leads to ensembles that respond nonspecifically and encode a broad range of movement directions. Blocking local inhibition in prefrontal cortex similarly leads to decreased spatial tuning in some prefrontal neurons. Interestingly, in other prefrontal neurons, disinhibition unmasks spatial tuning, which may reflect previously learned tasks or contexts (Rao et al., 2000). Therefore, prefrontal inhibition may play a role in determining relevancy of a cue to a task as well as sharpening spatial tuning. This is further demonstrated in behavioral data. Pharmacologic disruption of prefrontal inhibition impairs accuracy and increases omissions and inappropriately timed responding in working memory tasks (Sawaguchi et al., 1989). The increased incidence of early responding also demonstrates the importance of inhibition for proper temporal tuning. Prefrontal ensembles representing temporally distinct aspects of working memory tasks are activated in sequence during behavior (Funahashi et al., 1991). Cross-inhibition is seen among prefrontal ensembles during these tasks, often between ensembles that are active at different times (Constantinidis et al., 2002). This potentially allows temporal organization of behavior. For example, at the beginning of a task, cross-inhibition may allow ensembles representing early actions to inhibit those encoding later actions.

Therefore, the precise balance of prefrontal cortex inhibition is crucial to the orchestration of activity required for behavioral organization and planning. In NF1, where increased inhibition is seen in the mouse model, task-relevant tuning of prefrontal cortex neurons may be disrupted. This could cause behavioral impairments in planning and behavioral organization, which are common in NF1 patients. Future studies should utilize the NF1 mouse model to examine the capacity of prefrontal neuronal networks to sustain persistent and properly tuned activity. The mouse model can also be used to determine whether any prefrontal dysfunction seen is caused by increased inhibition. Experiments examining prefrontal cortex in the NF1 mouse offer powerful opportunities to identify the pathogenesis and potential therapeutic avenues for planning and attention deficits seen in NF1.

4.19.10 Parietal Cortex Dysfunction and Visuospatial Deficits in Neurofibromatosis

In addition to attention deficit and planning impairments, patients with NF1 show visuospatial deficits. In fact, one of the most robust impairments seen in NF1 is a visuospatial processing deficit commonly demonstrated in the JLO task. Deficits in complex visuospatial tasks are classically referred to parietal cortex dysfunction (Goldstein and Silverman, 2005). Indeed, fMRI studies show that the JLO task recruits parietal cortex (Ng et al., 2000). Like other cortical areas, function of parietal cortex requires balanced inhibition and excitation. As enhanced inhibition causes functional and behavioral deficits in a mouse

model of NF1, it is important to consider whether abnormal inhibition in parietal cortex could lead to visuospatial impairments such as deficits in JLO.

Parietal cortex is a multimodal area of the brain that creates integrated spatial representations of the external world. Parietal representations combine multiple sensory aspects of the environment and goal-directed motor outputs (Culham and Valyear, 2006). Transformation between different sensory modalities, such as touch and vision, and modulation of information from one sensory modality by another also involves the parietal cortex. Therefore, this area plays a critical role in putting together all the information that is being processed about a surrounding to create an integrated, whole representation of that environment (Grefkes and Fink, 2005). These representations are then available for other brain areas to use for manipulations in working memory such as rotations or integration with motor responses such as eye movement or grasping. This interaction with other brain areas means that in addition to pure sensory processing, parietal cortex function is important for online monitoring of the interaction of the individual with the environment (Andersen and Buneo, 2002; Culham and Valyear, 2006). Parietal activity is thought to maintain a stable and updated representation of space while the individual moves through and manipulates it. Individuals with NF1 show generalized visuospatial and visuomotor impairments, which may be caused by broad parietal cortex dysfunction.

Like other cortical areas, information is arranged in the parietal cortex so that subregions of this area are concerned with specific functions. Visuospatial deficits seen in the JLO are consistent with dysfunction of a specific compartment of the parietal cortex, the caudal intraparietal cortex. The caudal intraparietal cortex (CIP) receives extensive projections from visual cortex and processes three-dimensional representations of objects, in particular encoding orientations of object surfaces (Shikata et al., 2001; Grefkes and Fink, 2005) The anterior intraparietal cortex also responds to orientation of objects but is thought to be downstream of CIP processing. In the CIP, neurons are tuned to the orientation of the longitudinal axis of objects, with a topographic organization of neurons which preferentially fire to specific orientations. Further, these neurons show activity during delay periods, which may be a short-term memory trace of surface orientation (Grefkes and Fink, 2005). Activity of a subset of these neurons therefore encodes the presence of a set of object features. In this way, combined activity of ensembles of CIP neurons allows a visual reconstruction of an object in the environment. The JLO test asks individuals to distinguish between lines of different orientations and to make a response following a delay. Therefore, this task may depend on CIP orientation encoding.

Orientation coding in the CIP may be disrupted by increased activity of local inhibitory networks. In other cortical areas, parts of the cortex that are maximally activated by and therefore represent a stimulus feature also maximally recruit lateral inhibition, which represses activity of neighboring areas of cortex. In this way, the borders of neuronal ensembles or columns become more sharply defined. In visual cortex, manipulations that increased inhibition were shown to broaden orientation columns (Hensch and Stryker, 2004). Increased inhibition in parietal cortex, such as that seen in hippocampal areas of the $Nf1^{+/-}$ mouse, may also broaden ensembles. These larger ensembles could disrupt the specificity of object feature encoding in parietal cortex. Redundancy in encoding could lead to impairments such as those seen in the JLO task, where the specific orientation of a line must be distinguished from test lines of similar orientations. Such hypotheses regarding dysfunction of parietal cortex in NF1 can be explored using animal models of NF1.

While impaired parietal function in NF1 is thought to underlie JLO and general visuospatial processing deficits, it is important to consider that this is based on imaging studies in normal subjects and brain damage effects observed in otherwise normal patients. In NF1, however, other brain areas which interact intimately with the parietal cortex may also be dysfunctional. *Nf1* is expressed throughout cortex, and so inhibition is potentially increased throughout the brain, leading to deficits across multiple cortical areas which participate in visuospatial processing. For example, in areas such as visual cortex, encoding of orientation information also depends on tuning of orientation columns by balanced inhibition (Hirsch and Martinez, 2006). Therefore, in NF1 it is possible that poorly tuned orientation columns in visual cortex could also induce or exacerbate deficits in classically parietal cortex dependent tasks such as the JLO. Further, some of the multimodal associations mediated by parietal cortex also involve prefrontal cortex and hippocampus, areas which are suspected to function abnormally in NF1.

When an individual navigates through space toward a goal, parietal cortex interacts with the

hippocampus to manage external and self-motion cues. Parietal cortex encodes a series of representations of body-centered movements as well as associated visual changes as these movements are executed. This information is then converted to a representation of a single, fixed route relative to environmental cues. This integrated representation of a route through space can then be encoded and learned by the hippocampus. Since the hippocampus interacts closely with parietal cortex, seemingly parietal visuospatial and visuomotor integration deficits seen in NF1 can also be caused by changes in hippocampal activity (Save and Poucet, 2000). This possibility is particularly relevant since the mouse model of NF1 is known to have impaired hippocampal function. To fully understand which brain systems drive visuospatial and visuomotor deficits associated with NF1, it will be important to supplement classic neuropsychological testing. Neuropsychological testing batteries are not designed to isolate the individual contributions of brain structures that are recruited together during a task such as the JLO. Therefore, it may be productive to additionally use tools that systematically probe multiple aspects of parietal function to determine whether this structure is impaired in NF1 patients. Functional imaging studies provide another important tool to examine parietal cortex dysfunction in NF1 patients. Given the potential for impairment in multiple brain areas, it is particularly important to examine visuospatial impairments by carrying out imaging studies in the NF1 patient population, rather than extrapolating from imaging studies carried out in the general population. Ultimately, findings from human studies can be integrated with results from animal models to better understand cognitive symptoms associated with NF1 and to identify potential treatments.

4.19.11 Developmental Aspects of NF1

During early brain development, experience and skill acquisition shapes normal organization of neuronal networks. Genetic disorders such as NF1 are present from birth and therefore may affect early development. NF1 mutations impair experience-dependent plasticity in the adult (LTP learning) and so may also cause abnormal organization of neuronal networks early in development.

Early in development, the brain goes through a series of sensitive periods, windows of time during which experience-dependent learning has a particularly strong impact on the organization of neural networks – and therefore behavior. It is thought that during these sensitive periods, experience is more likely to induce plasticity and can shape the physical organization and connectivity of neural networks. After the sensitive period, later learning and plasticity has a lower probability of induction and is constrained by the networks established during development (Knudsen, 2004; Hensch, 2004) Sensitive periods are most easily demonstrated in primary sensory processes such as visual acuity, which depends on early binocular visual experience. Sensitive periods for complex sensory processes depend on acquisition of more simple processing abilities. For example, the visual system progresses through several sensitive periods, with the development of higher-order functions such as motion detection having a very short sensitive period that occurs after the normal development of visual acuity. Sensitive periods also occur for very complex functions such as language, music, and social interaction. For each of these functions, the ability to acquire and develop new skills throughout life is shaped by early experience during the sensitive period. Development of language skills requires early exposure to language, as is demonstrated by the limited proficiency often seen in languages learned later in life. Normal social interactions later in life require early exposure to healthy social relationships in the home and are dramatically influenced by inappropriate social interaction during this time (Knudsen, 2004; Hensch, 2004; Johnson, 2005).

It is currently unknown whether patients with NF1 have abnormal sensitive periods. However, the $NF1^{+/-}$ mice demonstrate impairments in physiological properties that are thought to be important to sensitive-period regulation. For example, work in primary cortical areas such as visual and barrel cortices in rodent has demonstrated the importance of the maturation and activity of inhibitory networks in modulating cortical sensitive periods. As local inhibitory networks mature and become increasingly active, local excitation induced by visual experience becomes less effective at causing plasticity. Therefore, the maturation and increased activity of inhibitory cortical networks is thought to contribute to the closure of sensitive periods (Fagiolini and Hensch, 2000; Hensch, 2005). Consistent with this hypothesis, tonic increases in GABAergic activity

correlate with the closure of a sensitive period in the visual cortex which is important for visual acuity. Expression of cortical BDNF at abnormally early time points in a mouse model leads to accelerated maturation of inhibitory networks during development and earlier closure of a sensitive period (Huang et al., 1999). In NF1, hyperactivity of the ras pathway may similarly lead to early, constitutive increases in signaling downstream of BDNF receptors, to enhancements of GABAergic tone, and consequently to early closure of sensitive periods. This hypothesis can be tested with animal models of NF1. It is important to determine whether NF1 alters the properties of sensitive periods because it may be critical to treat patients during these periods.

Developmental aspects of NF1 are important to explore; however, mounting evidence suggests that cognitive symptoms of NF1 can be treated even in the adult. The ongoing effects of *Nf1* mutation in the mature brain (higher ras signaling and subsequent increased inhibition) drive the behavioral impairments seen in the adult $NF1^{+/-}$ mouse. Indeed, normalizing ras levels in the adult $NF1^{+/-}$ mouse leads to rescue of attention and learning deficits. Further, current pharmacologic treatments are successful in treating ADHD symptoms in children and adults with NF1. The effectiveness of these interventions in treating symptoms of NF1 in the adult brain emphasizes the importance of neurofibromin as a modulator of neuronal function in the mature brain, independent of its effects during development.

4.19.12 Mechanism-Driven Treatment: Pharmaceutical Targets for Treatment of Neurofibromatosis

Hyperactivation of the ras pathway as a result of *Nf1* mutation has been shown to be a critical pathology causing learning and behavioral deficits in the NF1 mouse model. Downregulation of ras activity in the CNS, therefore is an important therapeutic goal (Weiss et al., 1999).Targeting ras signaling can potentially reverse the functional effects of *Nf1* mutation at its direct downstream effector. Therapeutic interventions that manipulate the ras pathway have the potential to specifically treat NF1 symptoms with limited disruption of behaviors and cognitive processes that are unaffected in NF1.

Activation of ras requires its association with the membrane. This association occurs through isoprenyl groups that are added to the ras protein following translation. Isoprenyl groups, including farnesyl groups, have a lipid moiety that can insert into the membrane lipid bilayer. This tethers ras to the plasma membrane, where it can be activated (Bernards and Settleman, 2004). Isoprenyl groups are synthesized as a byproduct of the cholesterol synthesis pathway (Casey et al., 1989; Weiss et al., 1999). A class of therapeutics, the statins, inhibits the cholesterol synthesis pathway by inhibiting its rate limiting enzyme, HMG-CoA reductase. In addition to inhibiting cholesterol synthesis, statins lower production of isoprenyl groups. This lowers the levels of ras at the plasma membrane (Mendola and Backer, 1990; Xu et al., 1996). The statins are a relatively safe drug class, which is currently widely prescribed to people as a cholesterol-lowering agent (Corvol et al., 2003). This makes the statin class an attractive candidate for treatment of NF1.

Studies of the effect of statins in $NF1^{+/-}$ mice have been promising. Indeed, statins can normalize the total levels of active ras in the hippocampus and neocortex of $NF1^{+/-}$ mice. Statin treatment also normalizes activation of downstream targets of ras, such as p-MAPK. Importantly, adult $Nf1^{+/-}$ mice that were given a brief treatment of lovastatin, a statin that penetrates the blood–brain barrier (Duggan et al., 1989; Botti et al., 1991), performed normally in behavioral tests of attention and spatial learning. Lovastatin also reversed the deficits in hippocampal plasticity seen in the $Nf1^{+/-}$ mice. These effects of lovastatin were seen at a dose which did not affect ras/MAPK signaling in wild-type controls (Li et al., 2005), demonstrating that ras/MAPK signaling in NF1 may be more sensitive to the effects of statins. Heightened sensitivity to lovastatin in $NF1^{+/-}$ mice is an important characteristic for a potential therapeutic. This suggests that despite their wide action spectrum, statins may be used at low doses to selectively improve specific disease-causing abnormalities in biochemical signaling.

These results demonstrating the effectiveness of lovastatin in treating $NF1^{+/-}$ mice have led to the initiation of clinical trials. Lovastatin and simvastatin are currently being tested for safety and efficacy in several NF1 patient populations. Statin clinical trials for NF1 are an exciting example of rational pharmacologic therapy being designed to target the functional effects of a disease-causing mutation. While such therapeutics are being developed, other pharmaceuticals specific to certain cognitive symptoms are currently being used successfully in children with NF1.

Methylphenidate can be used to treat ADHD symptoms of NF1. In fact, patients with NF1 respond to much lower doses than are used to treat ADHD not associated with NF1 (Mautner et al., 2002). The effect of treatment on attention deficits was assessed quantitatively in children with ADHD compared to children with comorbid NF1 and ADHD using the Test of Variables of Attention (TOVA). The TOVA is a computerized test that measures sustained attention and impulsivity. Improvements in general cognition as well as academic and social performance by methylphenidate treatment were assessed using the child behavioral checklist (CBCL). This test is based on a standardized series of questions that elicit feedback from the child with ADHD, their parents, and their teachers. The CBCL is used to assess effects of attention deficit relevant to function in daily life (Palumbo and Lynch, 2006). In children with NF1 and ADHD, low-dose methylphenidate treatment improved TOVA scores immediately and improved CBCL scores after 1 year of treatment (Mautner et al., 2002). While the mechanism of methylphenidate activity in ADHD is unclear, methylphenidate is known to modulate excitability of prefrontal inhibitory networks (Andrews and Lavin, 2006). Inappropriate, increased inhibition is critical to some of the behavioral deficits seen in the NF1 mouse model. Therefore, it is possible that the ability of methylphenidate to regulate inhibitory networks is important to its effectiveness in treating ADHD associated with NF1.

4.19.13 Implications for Other Learning Disabilities

The approach to NF1 taken here has attempted to integrate multidisciplinary aspects of NF1 research in order to build a more complete framework for understanding mechanisms of disease. As NF1 is a single-gene disorder, all patients with the disease can be identified and the symptoms of the disease fully described using carefully carried out neuropsychological batteries. In NF1, cognitive symptoms include learning deficits, attention and planning impairments, and visuospatial impairment. Insights into the neurosystems that mediate these cognitive functions are drawn from functional imaging studies and basic neuroscience. Particularly, dysfunction in hippocampus, prefrontal cortex, and parietal cortex may contribute to the cognitive symptoms associated with NF1. Importantly, animal models of the disease have been utilized to study these systems in a manner that integrates genetics, biochemistry, physiology, and behavior. This approach has yielded powerful insights into mechanisms of pathology in NF1. In mouse models of NF1, increased inhibitory tone was found to cause deficits in hippocampal plasticity required for behavioral learning. These increases in inhibitory tone were caused by constitutively increased activity of the ras signaling pathway, which is normally negatively modulated by the *Nf1* gene product, neurofibromin. These insights into the neuronal mechanisms of NF1 have led to exciting prospects for therapeutic intervention using statins to decrease ras pathway activity. In NF1, integration of current genetics and neuroscience with psychology and medicine has allowed a unified approach to cognitive disease and successfully identified potential biochemical targets that can restore cognitive health.

As this integrative approach is applied to other diseases affecting cognitive function, it will hopefully expand our understanding of critical physiological processes and systems that mediate cognition. Some of the genes mutated in these diseases are involved in similar pathways to those modulated by NF1. For example, Coffin-Lowry syndrome is an X-linked disorder involving severe mental retardation caused by disruption of the gene encoding Rsk2, a ribosomal S6 serine-threonine kinase-2. Rsk2 lies downstream of pMAPK, the target of ras which is upregulated in $Nf1^{+/-}$ mice. Rsk2 activation by ras/pMAPK leads to CREB activation. Neurofibromin and Rsk2 are both part of the ras signaling pathway, with neurofibromin being a negative regulator of the pathway, while Rsk2 is a downstream target that connects ras activity to CREB activation of transcription. In a mouse model made by disruption of Rsk2, loss of CREB activation in the hippocampus caused severe learning deficits (Weeber and Sweatt, 2002). It will be interesting to compare the effects of *rsk2* and *Nf1* mutations in different cell types and systems and on behavior and physiology. Modification of GABA function has also been implicated as a mechanism of cognitive dysfunction by other genetic neurodevelopmental disorders. Abnormal inhibition was found in a mouse model of Down syndrome that shows hippocampal LTP deficits (Kleschevnikov et al., 2004; Costa and Grybko, 2005) and spatial learning impairments (Reeves et al., 1995). Comparison of the physiology and systems level pathology seen in Down syndrome and NF1 may yield interesting insights about the contribution of inhibition to multiple cognitive processes.

As these data accumulate, a synthesized picture will gradually unfold of the critical molecular pathways and systems level interactions that underlie normal and abnormal cognitive function.

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4.20 The NMDA Receptor

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4.20.1 Introduction

In 1983, Graham Collingridge made the breakthrough discovery that induction of tetanus-induced forms of long-term potentiation (LTP) are blocked by blockade of a specific subtype of glutamate receptor, the *N*-methyl-D-aspartate (NMDA) receptor (Collingridge et al., 1983). Collingridge's fascinating discovery was based on the observation that the glutamate analog aminophosphonovaleric acid (APV), an agent that selectively blocks the NMDA subtype of glutamate receptor, could block LTP induction while leaving baseline synaptic transmission entirely intact.

This was the first experiment to give a specific molecular insight into the mechanisms of LTP induction. The properties of the NMDA receptor that allow it to function in this unique role of selectively triggering long-term synaptic potentiation, and indeed memory (Morris, 1989; see **Figure 1**), are important, and in this chapter I will present a detailed analysis of regulation of the NMDA receptor.

The NMDA receptor is both a glutamate-gated channel and a voltage-dependent one. The simultaneous presence of glutamate and a depolarized membrane is necessary and sufficient (when the co-agonist glycine is present) to gate the channel (**Figure 2**). Pairing synaptic stimulation with membrane depolarization provided via either alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-subtype glutamate receptors or neuronal action potential firing (plus the low levels of glycine always normally present) opens the NMDA receptor channel. Channel gating in this fashion leads

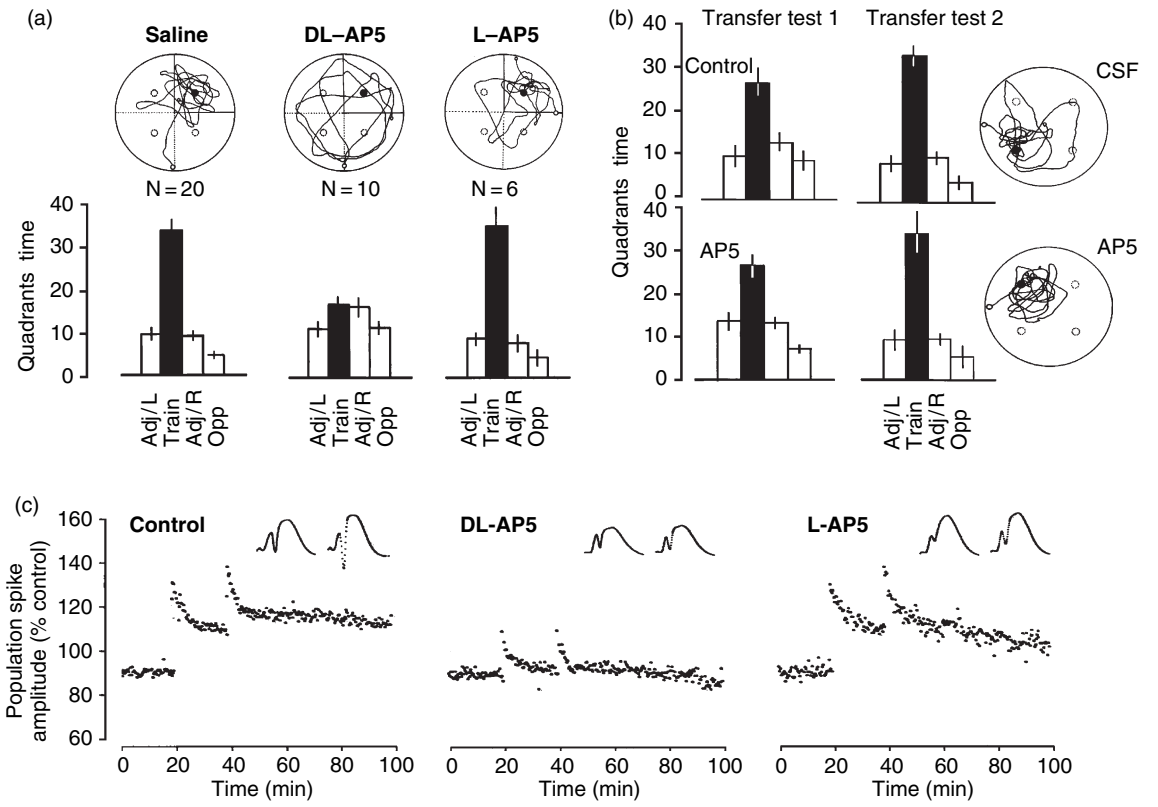


Figure 1 The NMDA receptor antagonist D-AP5 (i.e., APV) blocks learning in the Morris water maze. These data are from the landmark paper by Richard Morris (Morris, 1989), demonstrating that infusion into the central nervous system of DL-AP5 (an active mixture) but not the control, inactive enantiomer L-AP5, blocks learning in the water maze task. (a) Pretraining infusion of DL-AP5 blocks learning of a spatially selective search strategy for locating the hidden platform. Adj/L, adjacent left of training quadrant; Adj/R, adjacent right of training quadrant; Train, training quadrant; Opp, opposite from training quadrant. (b) Posttraining blockade of NMDA receptors does not affect memory recall. (c) The same infusion protocol leads to effective blockade of NMDA receptor-dependent LTP in the dentate gyrus.

to the induction of long-term changes in synaptic strength and contributes to long-term memory formation in vivo.

The NMDA receptor is a calcium channel and its gating leads to elevated intracellular calcium in the postsynaptic neuron. It is this calcium influx that triggers lasting changes in synaptic function, and indeed many subsequent chapters in this volume deal with the various processes this calcium influx triggers. It is important to remember that it is not necessarily the case that every calcium molecule involved in synaptic plasticity and memory formation actually comes through the NMDA receptor. Calcium influx through voltage-gated calcium channels in the membrane and calcium released from intracellular stores are also involved, but for this chapter we will focus exclusively on the NMDA receptor.

The gating of the NMDA receptor/channel involves a voltage-dependent Mg^{++} block of the channel pore. Depolarization of the membrane in which the NMDA receptor resides is necessary to drive the divalent Mg^{++} cation out of the pore, which then allows calcium ions to flow through. Thus, the simultaneous occurrence of both glutamate in the synapse and a depolarized postsynaptic membrane is necessary to open the channel and allow LTP-triggering calcium into the postsynaptic cell.

These properties, glutamate dependence and voltage dependence, of the NMDA receptor allow it to function as a coincidence detector. This is a critical aspect of NMDA receptor regulation and this allows for a unique contribution of the NMDA receptor to information processing at the molecular level. Using the NMDA receptor, the neuron can trigger a unique

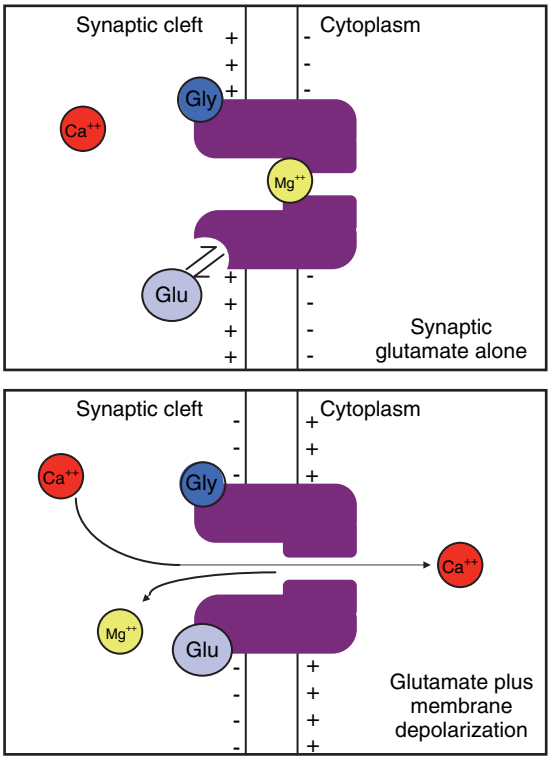


Figure 2 Coincidence detection by the NMDA receptor. The simultaneous presence of glutamate and membrane depolarization is necessary for relieving Mg^{++} blockade and allowing calcium influx. Figure by J. David Sweatt and Sarah E. Brown.

event, calcium influx, specifically when a particular synapse is both active presynaptically (glutamate is present in the synapse) and postsynaptically (when the membrane is depolarized).

This confers a computational property of associativity on the synapse. This attribute is nicely illustrated by pairing LTP where low-frequency synaptic activity paired with postsynaptic depolarization can lead to LTP (*See* Chapter 4.16). The associative property of the NMDA receptor allows for many other types of sophisticated information processing as well, however. For example, activation of a weak input to a neuron can induce potentiation, provided a strong input to the same neuron is activated at the same time (Barrionuevo and Brown, 1983). These particular features of NMDA receptor regulation have stimulated a great deal of interest as they are reminiscent of classical conditioning, with depolarization and synaptic input roughly corresponding to unconditioned and conditioned stimuli, respectively.

The associative nature of NMDA receptor activation allows for synapse specificity of the induction of long-term changes in synaptic strength as well. For example, if one pairs postsynaptic depolarization with activity at one specific set of synaptic inputs to a cell, while leaving a second input silent or active only during periods at which the postsynaptic membrane is near the resting potential, then selective potentiation of the paired input pathway occurs.

Thus we see that the NMDA receptor is a critical molecular locus for triggering lasting changes, which has unique biophysical properties that allow it to perform a coincidence detection role in the neuron. For these intriguing reasons, the most-studied molecule in the short history of molecular studies of learning and memory is the NMDA subtype of glutamate receptor.

4.20.1.1 Structure of the NMDA Receptor

The NMDA receptor is a glutamate-gated cation channel and as such is a multisubunit transmembrane protein. Current models hypothesize that it is a tetrameric hetero-oligomeric protein with more than one glutamate binding site. It is of course voltage dependent and this arises from the voltage-dependent Mg block of the pore that we discussed above. The protein has binding sites for zinc, polyamines, and glycine (a co-agonist necessary for activity).

Abundantly expressed individual subunits of the receptor are named NR1, NR2A, NR2B, NR2C, and NR2D (the somewhat unusual nomenclature arose for historical reasons related to different groups that cloned the first NMDA receptor subunits). One functional NMDA receptor comprises one or more NR1 subunits plus one or more NR2-type subunits. The NR2 subunits determine the calcium permeability of the channel and can influence the voltage dependence of its activation, kinetics of opening, and other biophysical properties. NR1 and NR2A and 2B are phosphorylated at a number of different sites: NR1 by protein kinase C (PKC) and PKA, NR2A by cyclin-dependent kinase 5, and NR2A and NR2B by various tyrosine kinases such as src and fyn.

Indeed, the NMDA receptor is subject to a wide variety of direct modulatory influences, some of which are listed in **Table 1**. Moreover, recent work by Grant and colleagues has shown that the NMDA receptor is in fact a large multiprotein complex (Husi et al., 2000; Husi and Grant, 2001). This complex includes a striking representation of many different types of scaffolding proteins and signal transduction molecules (see **Figure 3**). In fact, the NMDA receptor

Table 1 Direct modulators of the NMDA receptor

Modulator	*Mechanism*	*Effect*
Src family tyrosine kinases (src, fyn)	Tyrosine phosphorylation, loss of Zn inhibition	Enhancement
Scaffolding proteins		
Receptor for activated C kinase	Binding	Inhibition
Polysynaptic density-95	Scaffolding	Modulation
Protein kinase C	Ser/thr phosphorylation (direct)	Enhancement
	Src activation (indirect)	Enhancement
Protein kinase A/protein phosphatase 1/Yotiao	Phosphorylation	Enhancement
	Dephosphorylation	Inhibition
Cyclin-dependent kinase 5	Ser/thr phosphorylation	Enhancement
Nitric oxide/reactive oxygen species	Sulfhydryl nitrosylation Or oxidation	Inhibition
Polyamines (e.g., spermine, spermidine)	Direct binding to a modulatory Site	Augmentation
Casein kinase II	Ser/thr phosphorylation Modulation of polyamine effects	Enhancement

Molecule	Mr (kD)
Glutamate receptors	
NR1	120
NR2A	180
NR2B	180
GluR6 + 7	117
mGluR1a	200
Scaffolding and adaptors	
PSD-95	95
ChapSyn110/PSD-93	110
Sap102	115
GKAP/SAPAP	95-140
Shank	200
Homer	28/45
Yotiao	200
AKAP150	150
NSF	83
PKA	
PKA catalytic subunit	40
PKA-R2β	53
PKC	
PKCβ	80
PKCγ	80
PKCε	90
CaM kinase	
CaM kinase II β	60
phosph-CaM kinase	60

Molecule	Mr (kD)
Phosphatases	
PP1	36
PP2A	36
PP2B (calcineurin)	61
PPs	50
PTPID/SHP2	72
Tyrosine kinases	
Src	60
PYK2	116
MAP kinase pathway	
ERK (pan ERK)	42/44
ERK1	42/44
ERK2	42
MEK1	45
MEK2	46
MKP2	43
Rsk	90
Rsk-2	90
c-Raf1	74
Small G proteins and modulators	
Rac1	21
Rap2	21
SynGAP	10,12,35,60
NF1	60,101

Molecule	Mr (kD)
Other signaling molecules	
Calmodulin	15
nNOS	155
PI3 kinase	85
PLCγ	130
cPLA2	110
Citron	183
Arg3.1	55
Cell adhesion and cytoskeletal proteins	
N-cadherin	150
Desmoglein	165
β-Caternin	92
LI	200
pp120cas	120
MAP2B	280
Actin	45
α-actinin 2	110
Spectrin	240/280
Myosin (brain)	205
Tubulin	50
Coractin	80/85
CortBP-1	180/200
Clathryn heavy chain	180
Dynamin	100
Hsp-70	70

Figure 3 Summary of molecular composition of the NMDA receptor supramolecular complex. The component molecules and their molecular weights are listed. Adapted from Husi H, Ward MA, Choudhary JS, Blackstock WP, and Grant SG (2000) Proteomic analysis of NMDA receptor-adhesion protein signaling complexes. *Nat. Neurosci.* 3: 661–666, with permission from Elsevier; and Husi H and Grant SG (2001) Proteomics of the nervous system. *Trends Neurosci.* 24: 259–266, with permission from Elsevier.

supramolecular complex includes a number of proteins whose function has been directly implicated in human learning, including neurofibromatosis type 1 protein (NF1), PKA, raf-1, mitogen and extracellular signal-related kinase (MEK), extracellular signal-regulated protein (ERK), and ribosomal S-6 kinase type 2 (RSK-2). These gene products have all directly or indirectly been implicated in human learning, as they are associated with various types of human mental retardation syndromes: as was commented upon by Grant and colleagues in their report, the presence of gene products linked to human mental retardation within the NMDA receptor complex was intriguing, and consistent with a role for this complex in human cognition.

Given the dozens of individual molecular events that have been reported as being involved in NMDA receptor regulation in the literature, how can one begin to organize this immense molecular system into a coherent picture? In order to do this, in this chapter regulation of the NMDA receptor will be broken down into the following three basic components:

1. Mechanisms upstream of the NMDA receptor that directly regulate NMDA receptor function,
2. Mechanisms upstream of the NMDA receptor that control membrane depolarization, and
3. The components of the synaptic infrastructure that are necessary for the NMDA receptor, and the associated synaptic signal transduction machinery, to function normally.

These various components of the NMDA receptor regulatory machinery are schematized in **Figure 4**. This remainder of this chapter will fill in the molecular details necessary to flesh out this general model.

4.20.2 NMDA Receptor Regulatory Component 1: Mechanisms Upstream of the NMDA Receptor that Directly Regulate NMDA Receptor Function

The NMDA receptor is a biochemical signal integrator. Its capacity for signal integration and coincidence detection is not limited to determining the simultaneous presence of glutamate and depolarization. It senses biochemical signals as well, that are used in computing the degree of calcium influx. This section will describe many of the biochemical mechanisms known to directly regulate the NMDA receptor. In general, the discussion will be limited to those processes which have been directly implicated in LTP induction, memory formation, or both.

These biochemical processes generally are not all-or-none like the glutamate/depolarization mechanism, but rather serve to modulate the magnitude of postsynaptic calcium influx. The section titled 'NMDA receptor regulation component 2: Mechanisms upstream of the NMDA receptor that control membrane depolarization' will describe biochemical processes that are used to control the NMDA receptor in an all-or-none fashion, indirectly through controlling the membrane potential.

As described, the NMDA receptor is a coincidence detector, but in addition the NMDA receptor is also a temporal integrator. These temporal integration mechanisms operate on a longer time frame and are limited to biochemical processes, as opposed to biophysical processes. The time frame in which the NMDA receptor can detect the simultaneous presence of depolarization and glutamate is of course quite limited because the membrane depolarization

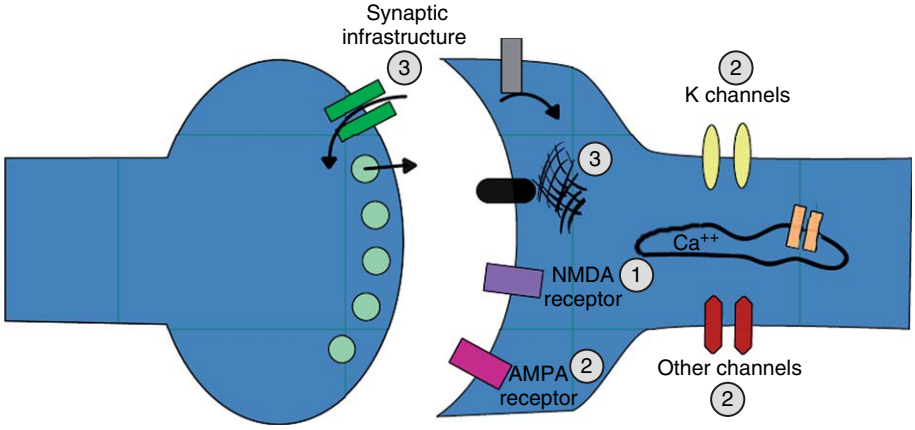


Figure 4 LTP induction machinery. Schematic diagram summarizing examples of the three different components of NMDA receptor regulation discussed in this chapter. See text for explanation. Figure by J. David Sweatt and Sarah E. Brown.

is so brief. In contrast, a biochemical signal such as elevation of a second messenger or increased protein kinase activity has a much longer half-life. Thus a temporal integration mechanism on the seconds (or longer) timescale must use these types of processes. The capacity of the NMDA receptor to be modulated by protein kinases and other messenger molecules allows for this sort of temporal integration.

4.20.2.1 Kinase Regulation of the NMDA Receptor

One of the oncogene products produced by the Rous sarcoma virus, which also has a homolog in the mammalian genome, is the tyrosine kinase src. Src family tyrosine kinases such as src and fyn directly phosphorylate the NMDA receptor (Raymond et al., 1994; Suzuki and Okumura-Noji, 1995), increasing calcium flux through the receptor. Tyrosine phosphorylation of the NMDA receptor increases current flow through the ion channel by reducing a tonic, zinc-dependent inhibition (Zheng et al., 1998).

It is important to bear in mind that there are a number of different mechanisms that can cause increased current flow through a ligand-gated ion channel:

1. The probability that the channel will open can be increased, which is referred to as increased channel open probability.
2. The conductance, that is, the rate at which ions flow through the channel, can be increased.
3. The number of channels in the membrane can increase.
4. The affinity of the channel for its ligand can increase, a mechanism that of course can only operate at subsaturating ligand concentrations.

This chapter will not go into detail on which of these mechanisms is involved in channel modulation, except where the mechanism is directly relevant to the molecular mechanisms that are involved (for example, increased membrane insertion of a channel implies the involvement of specific molecular processes). In addition, in many cases the specifics are not known or the channel modulation involves multiple mechanisms. As one example, src modulation of the NMDA receptor at a minimum involves increased channel open probability.

Protein tyrosine kinase phosphorylation of the NMDA receptor may be required for LTP induction, and at a minimum this mechanism serves an important modulatory role controlling the likelihood of LTP induction (Lu et al., 1998). The activities of Src and Fyn in neurons are controlled by a number of upstream signal transduction cascades. One important regulator of Src is the focal adhesion kinase (FAK) CAKbeta, also known as pyk2, and this cascade has been shown to be involved, through Src, in regulating LTP induction (Huang et al., 2001). In addition, the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) cascade and the PKC cascades, which will be discussed below, also can activate Src family kinases and these pathways may also modulate NMDA receptor function and LTP induction via Src (Grosshans and Browning, 2001). Dephosphorylation of the src/fyn sites on NMDA receptors likely occurs through the action of the tyrosine phosphatase STEP.

A number of interesting cell surface receptors modulate NMDA receptor function, and thus potentially LTP induction, acting through the src cascade (see **Figure 5**). The ephrins, which have been mostly studied in the context of nervous system development, modulate NMDA receptors in cultured neurons (Takasu, 2002). Ephrin B2, acting through its receptor EphB2, activates src and modulates NMDA receptors via this mechanism. Genetic deletion of EphB2 leads to an attenuation of LTP in area CA1 (Grunwald et al., 2001; Henderson et al., 2001). Apolipoprotein E receptors in hippocampus also modulate LTP induction via a src/NMDA receptor pathway (Weeber et al., 2002). Finally, the *obese* gene product leptin acts through its cell surface receptor and a phosphatidyl-inositol-3 (PI3)-kinase/MAPK/src pathway to modulate NMDA receptors and LTP induction in the hippocampus (Shanley et al., 2001). Thus, the Src/Fyn pathways serve an important role in funneling cell surface signals to the NMDA receptor itself, modulating its activity and regulating LTP induction.

Src tyrosine kinase potentiation of NMDA receptors is also subject to a variety of other influences. RACK1 (receptor for activated C kinase 1) promotes formation of a Fyn/RACK1/NR2B complex that actually inhibits fyn phosphorylation of the NMDA receptor and diminishes current through the receptor (Yaka et al., 2002). Also, the postsynaptic density (PSD) core protein PSD-95 modulates src phosphorylation of NMDA receptors (NMDARs) and src potentiation of NMDAR currents appears to require the presence of PSD-95 (Liao et al., 2000).

PKC not only can act indirectly through Src to modulate the NMDAR, but PKC also can also directly phosphorylate the receptor on serine/

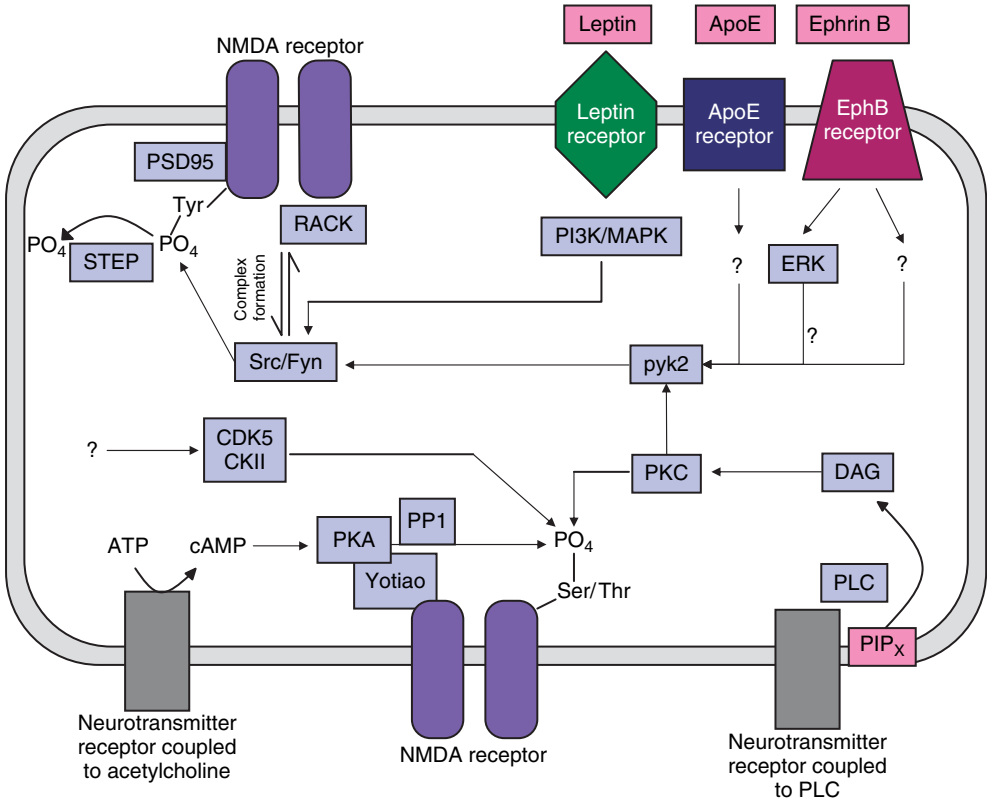


Figure 5 Receptor modulation of the NMDA receptor by the Src, PKA, and PKC cascade. These kinase cascades are represented here in relation to modulation of NMDA receptors, which could potentially lead to regulating LTP induction. See text for discussion and definitions. Figure by J. David Sweatt and Sarah E. Brown.

threonine residues and affect its function (Logan et al., 1999; Liao et al., 2001). Phosphorylation of the NMDAR by PKC causes increased calcium flow through the receptor (Ben-Ari et al., 1992). The potential import of this is quite straightforward: any cell surface receptor coupled to a phospholipase C cascade can modulate the likelihood of LTP induction through direct regulation of the NMDA receptor complex (see **Figure 5**).

The cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) also can augment NMDA receptor function, although the mechanism is complex and not entirely worked out (Westphal et al., 1999). PKA binds to the NMDAR via an associated protein, Yotiao (Yotiao is a specific isoform of A kinase anchoring protein, a category of protein which we will discuss again in section 4.20.3 of this chapter). Yotiao binds both PKA and protein phosphatase 1 (PP1) to the NMDA receptor, and when all three are bound together the PP1 activity predominates and keeps the NMDA receptor phosphorylation (and activation) low. PKA activation by cAMP leads to enhancement of NMDA currents, although it is not entirely clear if this is due to PKA phosphorylation of the NMDA receptor, loss of tonic dephosphorylation by PP1, or both. Again, in the context of the hippocampal pyramidal neuron this mechanism represents a basis for any neurotransmitter receptor coupled to adenylyl cyclase to be able to modulate NMDA receptor function and the induction of LTP.

The cyclin-dependent kinases (CDKs) are key regulators of cell division, controlling progression through the cell cycle. However, this role is of course not germane to understanding the function of nondividing neurons in the adult central nervous system (CNS). However, one cdk isoform, cdk5, is selectively expressed in postmitotic neurons and functions in regulating neuronal migration and neurite outgrowth in development. Moreover, recent work has shown that this kinase is involved in synaptic plasticity and learning in adult animals (Fischer et al., 2002). Specifically, inhibition of cdk5 blocks NMDAR-dependent LTP in area CA1 and blocks contextual fear conditioning. One possible mechanism for this

effect is cdk5 regulation of NMDA receptor function, as cdk5 phosphorylates the NR2A subunit and cdk5 inhibitors reduce NMDA-induced currents in hippocampal neurons (Li et al., 2001). Thus, cdk5 may modulate NMDA receptor function in a manner reminiscent of src, PKC, etc. The mechanisms controlling cdk5 regulation in hippocampal pyramidal neurons have yet to be worked out.

4.20.2.2 Redox Regulation of the NMDA Receptor

Modulation of the NMDAR is of course not restricted to posttranslational modifications involving phosphorylation. An interesting and novel type of regulation that is gaining increased attention is redox modulation of protein function. In the context of NMDAR function, there are two specific examples of this type of regulation, both of which elicit inhibition of NMDAR function. The reactive nitrogen species nitric oxide (NO), a free radical, can react with sulfhydryl moieties in cysteine side chains, a reaction leading to S-nitrosylation of the side chain. This reaction occurs in NR2A subunits at reasonably low levels of free NO and leads to decreased channel opening (Choi et al., 2000). A second example of redox regulation of NMDA receptors involves reactive oxygen species (ROS) such as superoxide and peroxynitrite, the product of the reaction of superoxide plus NO (Choi and Lipton, 2000). ROS inhibition of the NMDAR likely occurs via cysteine oxidation in a fashion reminiscent of the effects of NO, although the mechanisms of this effect are not clear at present.

The physiologic role of NO and ROS inhibition of NMDAR function is not clear. One interesting speculation is that oxidative inhibition of NMDARs might serve to lock the synapse in a particular state after plasticity had been triggered, or the mechanism might serve as a basis for inhibitory crosstalk limiting the capacity of a synapse to undergo LTP.

4.20.2.3 Polyamine Regulation of the NMDA Receptor

Finally, polyamine compounds such as spermine, spermidine, and putrescine can modulate NMDAR function. Polyamines are synthesized normally in cells and are essentially long aliphatic chains with several amino moieties covalently attached. Polyamines have diverse modulatory effects on NMDA receptors *in vitro* and *in vivo*, but one effect that they have is augmentation of NMDAR function. This effect is through the unusual mechanism of relief of tonic proton inhibition of the channel (Traynelis et al., 1995, Gallagher et al., 1997). Polyamine co-application with NMDA leads to an enhancement of NMDA-induced synaptic potentiation in area CA1. Casein kinase II (CKII), a calcium-independent protein kinase whose function has not been widely investigated in the CNS, augments NMDAR function by acting in concert with polyamine binding to the receptor intracellular domain (Lieberman and Mody, 1999). The role of this mechanism in LTP induction in the intact cell is unknown. However, CKII is activated by LTP-inducing stimulation (Charriaut-Marlangue et al., 1991) and evidence exists suggesting activity-dependent increased polyamine synthesis in the hippocampus (Ingi et al., 2001). These mechanisms might serve a role in temporal integration with repeated stimulation or in setting a baseline likelihood of LTP induction.

4.20.3 NMDA Receptor Regulatory Component 2: Mechanisms Upstream of the NMDA Receptor that Control Membrane Depolarization

As was discussed in Chapter 4.16, recent discoveries have highlighted the importance of mechanisms for controlling membrane depolarization in LTP induction. Although the potential importance of modulating the level of membrane potential in regulating LTP induction was first pointed out in 1987 (Byrne, 1987), the recent emphasis was catalyzed by the discovery of back-propagating action potentials and of their involvement in providing the depolarization of the synaptic membrane necessary for LTP induction (Stuart and Sakmann, 1994; Magee and Johnston, 1995; Spruston et al., 1995). A second important development was the emergence of the silent synapse model of LTP induction, wherein there are synapses that contain NMDA receptors but no AMPA receptors. Obviously, in the silent synapse model the membrane depolarization necessary for NMDA receptor activation cannot come from local AMPA receptors, but must be propagated via the membrane from a distal site. Taken together, these two considerations bring into focus the necessity of understanding the mechanisms that control the electrical properties of the dendrite and dendritic spines, and their role in regulating the NMDA receptor through controlling the depolarization envelope that the receptor experiences.

Table 2 lists a number of the important molecules contributing to regulation of membrane depolarization in neuronal dendrites. Progress in this area has been greatly facilitated by relatively recent technical advances that allow direct cell-attached patch recording from the distal dendritic regions of CA1 pyramidal neurons. These studies have identified a number of relevant membrane currents that control dendritic membrane depolarization and excitability, and in most cases there are reasonable hypotheses about the molecules underlying these currents. However, keep in mind that linking a specific molecule with a specific ionic current involves some degree of speculation at present.

Finally, please note that in the next section, wherever possible, the term current will be used to refer to an entity identified in physiology experiments, and use the term channel to refer to specific molecules. Also, in some cases the channel molecules can be referred to by their own specific subtype names (e.g., Kv4.2).

4.20.3.1 Dendritic Potassium Channels

4.20.3.1.1 A-type currents

A-type, or voltage-dependent, rapidly inactivating K^+ channels localized in the dendrites of hippocampal pyramidal neurons play a critical role in shaping the local electrical responses of the dendritic membrane and dendritic tree. The functions of A-type channels in general are to repolarize the membrane after an action potential, contribute to the resting membrane potential (modestly), and regulate firing frequency. Johnston and coworkers have proposed a model in which A-type channels in distal dendrites of the hippocampus are critical regulators of back-propagating action potentials, regulating LTP induction through controlling voltage-dependent NMDA receptor activation (Hoffman et al., 1997; Yuan et al., 2002; Watanabe et al., 2002).

Moreover, this type of regulatory mechanism is subject to modulation by cellular signal transduction cascades. Activation of PKA or PKC shifts the activation curve of A-type K^+ currents recorded in hippocampal area CA1 dendrites (Hoffman et al., 1997). The voltage-dependence of their activation is shifted in the depolarizing direction, leading to increases in dendritic excitability and increased back-propagating action potentials in dendrites. More recent work has shown that the alterations in A-current voltage dependence caused by application of PKA, PKC, or β-adrenergic receptor activators are secondary to activation of ERK/MAPK (Yuan et al., 2002; Watanabe et al., 2002). Overall, these observations indicate that K channel regulation of dendritic membrane properties is regulated by cell surface neurotransmitter receptors coupled to ERK activation. The implication of this, as will be discussed in more detail, is that neuromodulation of K channel function could serve a critical role in controlling action potential back-propagation and local membrane electrical properties. This mechanism would then allow indirect but critical control over the membrane depolarization necessary for NMDA receptor activation.

What is the molecular basis for this regulation of voltage-dependent K channel function? The A-type potassium channel pore-forming subunit Kv4.2 is localized to subsynaptic compartments of dendrites in CA1 pyramidal neurons and is likely the pore-forming

Table 2 Mechanism upstream of the NMDA receptor involved in membrane depolarization

Ionic current	*Molecules involved*	*Role*	*Mechanisms of modulation*
K currents			
Voltage-dependent	Kv4.2 (and Kv4.3)	Limit bAPs Limit EPSP magnitude	ERK, PKA, CaMKII
"A" currents			
"H" currents	NCN channels (HCN)	Regulate excitability	Cyclic nucleotides (direct)
Na currents			
AMPA receptors	GluR1, GluR2 aka GluR-A,B	Depolarize membrane	PKA, CaMKII, PKC
Voltage-dependent Na^+ currents	Na(v)1.6, 1.1, 1.2	AP propagation	PKC (decreased inactivation)
Ca currents	? – Likely many	AP propagation (hypothetical)	PKA
Cl currents			
GABA receptors	All GABA-A receptor subunits	AP firing Excitability	Numerous

bAPs, back-propagating action potentials; EPSP, excitatory postsynaptic potential.

subunit of dendritic A-type channels in these regions. Moreover, Kv4.2 is a substrate for ERK in hippocampal pyramidal neurons (Adams et al., 2000), and activation of PKA and PKC, as well as stimulation of β-adrenergic receptors, leads to ERK activation and Kv4.2 phosphorylation by ERK in hippocampal area CA1 (Yuan et al., 2002). Furthermore; modulation of A currents by PKA, PKC, and β-adrenergic receptors are secondary to ERK activation, and this mechanism is a basis for controlling back-propagating action potentials in pyramidal neuron dendrites.

Thus, one contemporary hypothesis is that ERK phosphorylation of Kv4.2, the K^+ channel pore-forming subunits likely to mediate A currents in hippocampal dendrites, decreases the probability of channel opening or the number of channels in the membrane. Once these channels in a particular region of a dendrite are rendered nonfunctional due to phosphorylation, the ability of a back-propagating action potential to invade that particular dendrite increases. This will allow, or increase the likelihood of, NMDA receptor activation and Ca^{2+} influx locally, and thus control the induction of LTP at that synapse.

Available data suggest the particular importance of this mechanism in theta-type LTP induction protocols (Winder et al., 1999; Watabe et al., 2000). Theta-frequency stimulation causes complex spike bursting in area CA1 cells, which can back-propagate into the dendrites and depolarize synapses. Several groups, including the laboratories of Kandel, Winder, and O'Dell, have shown that blocking ERK activation in mouse area CA1 blocks not only the complex spike bursting seen with the theta-frequency stimulation protocol, but also the LTP that is so induced (Winder et al., 1999; Watabe et al., 2000). It seems likely that blocking ERK in these experiments decreases the phosphorylation of Kv4.2, leading to an increase in the probability of current flux through these channels. Moreover, Winder's group has found that β-adrenergic receptor-mediated modulation of LTP induced with theta-frequency stimulation is blocked by inhibitors of ERK activation. Again, these findings are consistent with a model wherein ERK regulation of membrane electrical properties, via control of Kv4.2 channels, regulates back-propagating action potentials and controls NMDA receptor activation.

4.20.3.1.2 The H current

The H in H current stands for hyperpolarization, and this is a cation-mediated (sodium + potassium) current that is partially active at the resting membrane potential and that is further activated with membrane hyperpolarization. Thus, the channels underlying the H current are voltage-dependent, hyperpolarization-activated channels. They also are directly gated by cyclic nucleotides (cAMP and cyclic guanosine monophosphate (cGMP)), which open the channels, but there also is good evidence that some of the enhancing effects of cAMP on Ih are mediated by PKA-dependent phosphorylation. The net effect of H channel function in dendrites is to dampen membrane excitability like other potassium channels do, at least in CA1 pyramidal neurons. However, they dampen membrane excitability without significantly attenuating the peak depolarization of back-propagating action potentials. Because they are hyperpolarization activated, they shut down when the membrane is strongly depolarized: this means that they do not significantly affect peak depolarization.

As a first approximation, enhancing H channel function can be thought of as leading to sharpening of the back-propagating action potential, narrowing the window of membrane depolarization (Magee, 1998). H channels are open and counteracting membrane depolarization when the membrane is modestly depolarized, but inactive when the membrane is at the peak of the action potential. This effect might play an important role in timing-dependent plasticity mechanisms, restricting the time frame over which associative actions such as NMDA receptor activation might occur. A second, more tonic effect of augmenting H channels is to limit the ability of modest depolarization to penetrate a dendritic region. Overall, one can think of H channels as a cyclic nucleotide/PKA-dependent filtering mechanism for limiting membrane depolarization, a mechanism for enhancing the signal-to-noise ratio for depolarization-dependent associative events. cAMP/PKA, acting through H channels, might serve to assure that associative events such as NMDA receptor activation occur only in the presence of sufficiently robust electrical signals.

4.20.3.2 Voltage-Dependent Sodium Channels (and Calcium Channels?)

As in axons, propagation of action potentials along dendrites depends on voltage-gated sodium channels. In situations where back-propagating action potentials provide the depolarization necessary for NMDA receptor activation, this effect is of course dependent on the function of voltage-gated sodium channels.

Work from Colbert and colleagues has demonstrated that this is a potential site of plasticity for the regulation of LTP induction (Colbert and Johnston, 1998). Specifically, PKC can regulate the rate of inactivation of sodium channels in pyramidal neuron dendrites, a mechanism allowing PKC control of the extent of action potential back-propagation. Mechanistically, PKC decreases the extent of sodium channel inactivation, allowing for repetitive action potentials (where Na channel inactivation is relevant) to more effectively penetrate the dendrites (Spruston et al., 1995; Tsubokawa, 2000). By this mechanism PKC activation can promote the induction of LTP.

Back-propagating action potentials also activate voltage-dependent calcium channels in the dendritic membrane (Spruston et al., 1995; Andreasen and Nedergaard 1996). This is a critical source of dendritic calcium influx, but under certain conditions voltage-gated calcium channels (VGCCs) can contribute to the action-potential-associated membrane depolarization as well. In various experimental circumstances, VGCCs can even propagate a dendritic calcium spike, a regenerative action potential mediated by calcium flux across the membrane. Thus, modulation of calcium channels, aside from being a mechanism for regulating calcium influx, also can theoretically contribute to controlling local membrane depolarization.

4.20.3.3 AMPA Receptor Function

Three chapters in this volume (*See* Chapters 4.28, 4.29) will cover in detail a variety of mechanisms by which protein kinases augment AMPA receptor function. This is one of the principal mechanisms by which synaptic strength is enhanced during the expression of early-phase LTP (E-LTP). However, it should not escape our attention that these mechanisms could play a critical role in the induction of LTP as well. In this context, it is important to note that Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), PKC, and PKA all enhance AMPA receptor function; any cell surface receptor or calcium influx process that leads to activation of these kinases could influence the likelihood of LTP induction through augmenting AMPA receptor membrane depolarization.

This amplification of AMPA receptor function could be important in several contexts. First, AMPA receptors provide the initial depolarization of the membrane that brings the cell to threshold for firing an action potential. An augmentation of AMPA receptor function means that for any given level of glutamate at the synapse there is greater depolarization, at least until the concentration of glutamate reaches a saturating level. Thus, the cell is more likely to reach threshold for firing an action potential, and thus is more likely to trigger NMDA receptor activation. In non-silent synapses where AMPA receptors and NMDA receptors are both present, AMPA receptor augmentation will directly lead to greater local membrane depolarization and enhanced NMDA receptor function. This is an appealing model in the context of second messenger-coupled neurotransmitter receptors modulating LTP induction.

Second, enhanced AMPA receptor function can serve as a temporal integration mechanism. Protein kinase activation and substrate phosphorylation typically are much longer lasting (relatively) than glutamate elevation at the synapse. Depolarization-associated calcium influx or second messenger elevation can set in motion a positive feedback mechanism over time, such that subsequent stimulation of AMPA receptors is enhanced relative to an initial response. This could occur via calcium-activated kinases phosphorylating AMPA receptors or even through AMPA receptor insertion into the membrane. Although this idea is quite speculative at this time, these mechanisms nicely fit the classical definition of a temporal integration system.

4.20.3.4 GABA Receptors

As described in Chapter 4.18, gamma-aminobutyric acid-gated chloride ion channels (GABA-A receptors) control numerous processes relevant to the triggering of LTP. As these processes were described in Chapter 4.18 in the context of hippocampal circuit information processing mechanisms, they will not be covered here. Moreover, at present the role of GABA-A receptors has largely been studied in the context of controlling the likelihood of action potential firing and membrane depolarization in the cell body region, as opposed to local control of the membrane electrical properties in the distal dendritic regions. While it is clear that dendritic GABA-A receptor activation could play a powerful role in controlling NMDA receptor activation locally, at present the specific molecular mechanisms that might operate in the plasticity of this system have not been well defined.

4.20.4 NMDA Receptor Regulation Component 3: The Components of the Synaptic Infrastructure that Are Necessary for the NMDA Receptor and the Synaptic Signal Transduction Machinery to Function Normally

The components of the synaptic infrastructure, such as scaffolding proteins, cytoskeletal proteins, and cell surface adhesion molecules (**Table 3**), are now coming to be appreciated as important signaling components in the cell, which respond rapidly and with great variety to extracellular and intracellular signals. However, placing them in a scheme for NMDA receptor regulation is fairly speculative at this point, and in some cases the most that can be said is that they are known to interact with other proteins known to be involved in NMDA receptor regulation. With these caveats in mind, what follows is a brief listing of the more notable components of the synaptic infrastructure that have been implicated as potentially contributing to NMDA receptor regulation. While this section will delve into several specific categories of molecules, it is important to keep in mind that the overall take-home message of this section is that the NMDA receptor does not function in isolation. It is a component of a richly complicated physical structure that is itself subject to regulation.

4.20.4.1 Cell Adhesion Molecules and the Actin Matrix

A prominent category of synaptic adhesion molecules is the integrins (Martin et al., 2002). Integrins are cell surface molecules that transduce signals from the extracellular matrix to the inside of the cell. They are single-transmembrane-domain proteins that usually function as heterodimers of alpha and beta subunits. Knockout mice deficient in alpha5 and beta3 integrin exhibit hippocampus-dependent learning deficits and deficits in NMDA receptor-dependent LTP in area CA1, as is described in more detail in Chapter 4.32 by Davis and colleagues.

Table 3 Components of the synaptic infrastructure necessary for NMDA receptor function

Component	*Targets*	*Role*
Cell adhesion molecules		
Integrins	src, rho, rac, ras/MAPKs	Transmembrane signaling Interactions with extracellular matrix NMDAR regulation
	MLCK, FAK?	Spine morphology?
Syndecan-3	fyn, NMDAR	Signaling from matrix heparan sulfates to the NMDA receptor
N-cadherin	Other cadherins, Cytoskeleton	Spine morphology? Pre–post adhesion?
Actin cytoskeleton/associated proteins		
Rho	Membrane–cytoskeleton interactions	Regulate synaptic structure
Cdk5	NMDA receptor	Increase NMDA receptor function
Filamin	K channels	K channel localization
Presynaptic processes		
Glutamate release	Synaptic glutamate	NMDA receptor activation
Glutamate re-uptake	Synaptic glutamate	Limiting NMDA receptor desensitization
Anchoring/interacting proteins		
PSD-95	Receptors signal transduction mechanisms nNOS, SynGAP, GKAP	Postsynaptic organization
RACK1/fyn	NMDA receptor	Direct regulation of NMDA receptor
Shank/HOMER	Metabotropic receptors	Effector localization, cytoskeleton
GRIP	AMPA receptors, PICK-1/PKC	Postsynaptic organization
AKAP	PKA, PP2B	Kinase and phosphatase localization
CaMKII	Signal transduction	Regulate likelihood of LTP induction

Integrins interact with a wide variety of intracellular effectors, three categories of which are clearly important to keep in mind in terms of LTP induction in general and regulating NMDA receptor function specifically (see **Figure 6**). First, integrins couple to src activation in many cells, and as we discussed in the section titled 'NMDA receptor regulatory component 1: Mechanisms upstream of the NMDA receptor that directly regulate NMDA receptor function,' this is a mechanism for directly augmenting NMDA receptor function. Second, integrins couple to ras and via this mechanism can lead to ERK activation: this might play a role in K channel regulation (and regulating other effectors), as was discussed in the section titled 'NMDA receptor regulatory component 2: Mechanisms upstream of the NMDA receptor that control membrane depolarization.' Finally, the prototype function of integrins is in regulating the actin cytoskeleton. This potential role of integrins has taken on especial significance given findings by a number of laboratories, principally among them Lisman's and Lynch's, that normal dynamic regulation of the actin cytoskeleton is necessary for LTP induction. Exactly how the actin cytoskeleton regulates LTP induction is unclear at present, but there are many examples of candidate effector mechanisms that will be discussed in the remainder of this section.

Integrin regulation of the actin cytoskeletal matrix is complex. One principal role is linking the extracellular matrix to sites of actin matrix adhesion on the cytoplasmic side of the membrane. Integrin cytoplasmic tails bind to alpha-actinin and talin, which in turn recruit actin-binding proteins, such as vinculin, to the complex. This complex serves to anchor the cytoskeleton to the perisynaptic plasma membrane and synaptic zone.

Integrins also regulate the small G proteins rho (ras homolog, first identified in *Aplysia*) and rac, which regulate actin dynamics; this dynamic regulation of the actin matrix may contribute to activity-dependent changes in spine morphology. Consideration of integrin regulation of rho activity is especially appealing in this context because the classic role of rho is in regulating actin–myosin-based movement through activating myosin light-chain kinase. Another potential

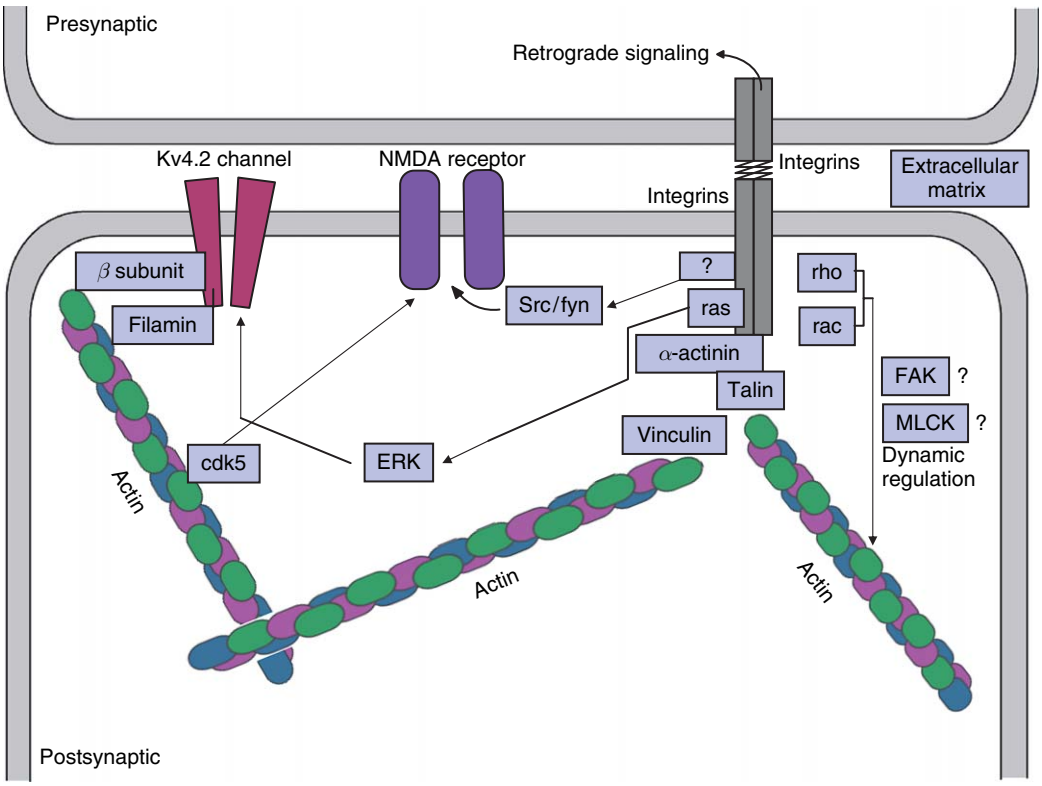


Figure 6 Interactions among integrins and intracellular effectors that regulate NMDA receptor function. See text for discussion and definitions. Figure by J. David Sweatt and Sarah E. Brown.

integrin effector in this context is FAK, which also serves to control cell morphology via the actin cytoskeletal matrix in many cells.

The actin cytoskeletal matrix may also contribute to NMDA receptor regulation in fairly direct ways. For example, actin microfilaments can serve as anchors for signaling components that affect the NMDA receptor directly. One example of this is actin filaments serving as the anchor for cdk5, which, as was discussed earlier, can phosphorylate and activate the NMDA receptor. Also, A-type potassium channels interact with the actin-binding protein filamin via their cytoplasmic c-terminal domain, and potassium channel beta subunits couple these channels to the actin cytoskeleton as well. These interactions certainly help localize A-channels appropriately in the dendritic spine. Perhaps more importantly, disruption of these interactions can cause attenuation of potassium channel function, and as we discussed above, A-channel inhibition promotes increased membrane excitability and enhanced NMDA receptor function.

An additional transmembrane, extracellular-matrix-binding protein that has been directly implicated in LTP induction is Syndecan-3. Inhibition of this molecule using various approaches leads to deficits in LTP (Lauri et al., 1999). Syndecan-3 binds heparan sulfates in the extracellular space, which are components of the glycosaminoglycan family of molecules present there. Syndecan-3 associates with the tyrosine kinase fyn, which might regulate NMDA receptor activity through direct tyrosine phosphorylation. The cell adhesion molecules L1 and N-CAM have also been implicated in the expression of LTP in some studies; however, recent results from knockout mice have suggested that loss of these molecules does not lead to LTP deficits (Luthl et al., 1994; Holst et al., 1998; Bliss et al., 2000). Finally, the N-cadherin subtype of cell adhesion molecule has been implicated in LTP induction and maintenance (see Huntley et al., 2002, and Chapter 4.31 for reviews). A likely role for the cadherins is in stabilizing strong connections between the presynaptic and postsynaptic membranes, although like other cell adhesion molecules, the cadherins also interact with and can regulate the actin cytoskeleton.

4.20.4.2 Presynaptic Processes

It is a statement of the obvious that any presynaptic process that regulates glutamate release can impinge upon NMDA receptor function, through controlling the level of synaptic glutamate that is attained. A number of specific examples of this type of mechanism are discussed in Chapter 4.33, so I will not reiterate the details here. However, please note that these are potentially relevant mechanisms for regulating NMDA receptor function indirectly.

4.20.4.3 Anchoring and Interacting Proteins of the Postsynaptic Compartment

4.20.4.3.1 Postsynaptic density proteins

The postsynaptic density is a multiprotein assembly that is the organizing center for many receptors and effectors, and the cytoskeleton, in the postsynaptic compartment (Sheng and Pak, 2000; Sheng, 2001).

PSD-95 is a protein enriched in the postsynaptic density and a prominent player in this context (Migaud, 1998). Identification of this protein by Mary Kennedy helped launch great progress in our understanding of the molecular basis for the organization of the complex postsynaptic infrastructure. PSD-95 binds to NMDA receptors (specifically the NR2 subunit) postsynaptically and serves as a multidomain anchoring protein for a large number of scaffolding and structural proteins postsynaptically (see **Figure 7**). PSD-95 helps anchor nitric oxide synthase (NOS), localizing this source of the reactive nitrogen species NO. PSD-95 also binds the ras GTPase (GTP: guanosine triphosphate) activating protein synGAP, whose function is still under investigation but which may regulate the ras/ERK cascade locally at the synapse. PSD-95 also anchors the cytoskeleton through its interactions with a protein termed SPAR, which is a GTPase activating protein (GAP) for rap and also an actin-interacting protein in its own right. Rap helps control the cytoskeleton indirectly through its target signal transduction processes such as the MAPK cascades.

PSD-95 also binds to proteins that seem to be more explicitly structural, such as the scaffolding protein Shank. Shank is another multidomain molecule that links the PSD-95-binding protein GKAP to the actin skeleton through cortactin. Shank also binds to HOMER, a metabotropic receptor-binding protein. Thus, via Shank and HOMER, group I metabotropic receptors coupled to phospholipase C can be localized near the NMDA receptor. HOMER also binds the inositol triphosphate (IP3) receptor, which may help localize the endoplasmic reticulum close to the NMDA receptor, allowing for proximity of intracellular calcium release mechanisms to the

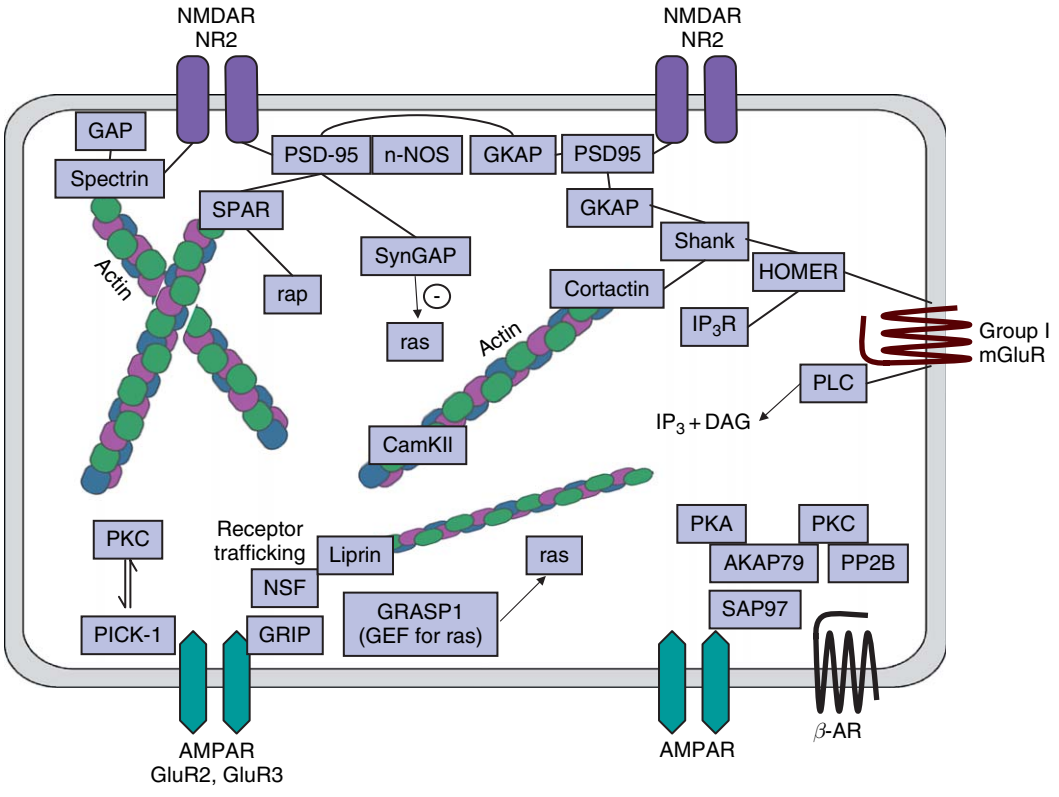


Figure 7 PSD-95 as an anchoring protein for NMDA receptors. See text for explanation. Figure by J. David Sweatt and Sarah E. Brown.

principal pathway for extracellular calcium influx, the NMDA receptor.

Genetic deletion of PSD-95 leads to striking alterations in LTP in area CA1: LTP is enhanced (Migaud et al., 1998). This is associated with learning deficits. It is unclear how the loss of PSD-95 leads to the augmentation of LTP; interpretation of the result is difficult because of the many complex roles of PSD-95 at the synapse, as outlined earlier. Overall, the data suggest that PSD-95 is somehow altering the threshold for LTP induction, a result consistent with the idea that PSD-95 and its associated proteins serve as dynamic modulators of NMDA receptor function.

4.20.4.3.2 Additional direct interactions with the NMDA receptor

The NMDA receptor NR1 and NR2 subunits also bind spectrin, the actin-binding protein. This may serve as an additional cytoskeleton anchoring site postsynaptically. Moreover, this interaction is subject to regulation by phosphorylation: tyrosine phosphorylation of NR2B leads to decreased interactions of spectrin with the receptor, and NR1 interaction with spectrin is modulated by serine/threonine phosphorylation. However, the role of these effects in synaptic plasticity and memory formation is not clear at this point. Finally, as described in the section titled 'Kinase regulation of the NMDA receptor,' the scaffolding protein RACK1 promotes formation of a Fyn/RACK1/NR2B complex that actually inhibits fyn phosphorylation of the NMDA receptor and diminishes current through the receptor (see **Figure 5**). Also, PSD-95 modulates src phosphorylation of NMDARs and src potentiation of NMDAR currents appears to require the presence of PSD-95.

Consideration of the complicated structure and regulation of the postsynaptic density complex highlights the importance of thinking of the entire postsynaptic domain as a large functional unit. The NMDA receptor is embedded in a dynamic multiprotein complex that it regulates and in turn that regulates it. While many details of the structural components of the PSD complex are still being worked out and their roles in LTP induction and memory formation are being actively investigated,

it is clear that disrupting one or more of the cogs in this machine can lead to disruption of the proper function of the NMDA receptor.

4.20.4.3.3 AMPA receptors

AMPA receptors of course provide the initial depolarization, either locally or distally in the neuron, that ultimately results in NMDA receptor activation. As such, alterations in the AMPA receptor protein or its associated interacting proteins can lead to loss of proper regulation of NMDA receptor activation. However, in this section we will focus on the AMPA receptor as a structural component of the synapse.

AMPA receptors, like NMDAR, also reside postsynaptically but are in much more of a state of flux than NMDA receptors. In fact, the average half-life for an AMPA receptor in the postsynaptic membrane is 15 min. Also, AMPA receptor membrane insertion can be activity-dependent. Thus the AMPA receptor should probably not be thought of like the NMDA receptor: the NMDA receptor likely serves a frankly structural role in addition to its function as a ligand-gated ion channel, while the AMPA receptor is more peripherally associated with the PSD (see Passafaro et al., 2001).

The AMPA receptor binds at least two structural proteins: PICK-1, which binds PKC, and GRIP (see Sheng and Pak, 2000). GRIP is a multidomain scaffolding protein that likely functions in AMPA receptor trafficking. GRIP also binds to GRASP1, a guanine nucleotide exchange factor for ras (see Sweatt, 2001; Vetter, 2001); the functional role of GRASP1 at the synapse is unclear at present. AMPA receptors can also bind NSF, a vesicle-associated protein that may also be involved in receptor membrane insertion is a fashion reminiscent of its role presynaptically in neurotransmitter vesicle fusion.

AMPA receptors also bind the A kinase anchoring protein AKAP79, an interaction that appears to be mediated by the PSD-95 homolog SAP-97 (Coghlan et al., 1995; Colledge, 2000; Dodge and Scott, 2000). As the name implies, AKAPs bind and localize PKA through interacting with the regulatory subunits of the kinase. The general role of AKAPs is to help localize PKA near relevant targets such as the AMPA receptor postsynaptically. The story is actually more complicated than that, as AKAP79 in the hippocampus also binds and localizes a protein phosphatase, PP2B (aka calcineurin). As a first approximation, it is useful to think of proteins such as AKAPs serving a role to increase the signal-to-noise ratio for signal transduction, localizing kinases close to their substrates to increase the efficacy of phosphorylation, but also localizing phosphatases to those same substrates in order to keep their basal phosphorylation low and to allow for rapid reversal of phosphorylation events once the kinase activation is over (Dodge and Scott, 2000). AKAP79 may also serve specifically to localize the calcium-sensitive phosphatase PP2B to the AMPA receptor in order to facilitate calcium-dependent AMPA receptor dephosphorylation and downregulation (Tavalin et al., 2002).

4.20.4.3.4 Ca^{2+}/calmodulin-dependent protein kinase II

CaMKII is highly enriched at the postsynaptic density complex. This enrichment occurs through CaMKII binding to the actin cytoskeleton, and the anchor for the cytoskeleton is the NMDA receptor, as we have discussed extensively. Thus, one purpose of the NMDA receptor/PSD-95/cytoskeleton complex is to help localize CaMKII to the PSD domain. This keeps a critical effector of the NMDA receptor, CaMKII, tightly bound and localized for effective responsiveness to NMDA receptor activation. Interaction of CaMKII with the PSD also can be regulated by CaMKII autophosphorylation; this is discussed in more detail in Chapter 4.23.

While the various scaffolding proteins, PSD-95, etc., that have been discussed are involved upstream of the NMDA receptor, regulating its function, CaMKII is downstream of the NMDA receptor. However, it is listed as a component of the synaptic infrastructure necessary for proper NMDA receptor function because it is such an important and direct target of the NMDA receptor; in essence loss of CaMKII function may functionally translate as equivalent to loss of NMDA receptor function. In addition, CaMKII binding to the PSD complex may play a structural role in concert with the actin cytoskeleton to serve as part of the infrastructure necessary for the NMDA receptor to function appropriately.

4.20.5 Summary

This discussion makes clear that the NMDA receptor does not reside in isolation in the membrane. In fact, Seth Grant's laboratory has clearly established that the NMDA receptor is the anchor for a large multiprotein

complex of structural proteins and signal transduction components (see **Figure 3**). This consideration serves as an important caveat for interpreting results from NMDA receptor knockout mice. This apparently clean experimental manipulation, wherein the NMDA receptor is entirely lost, likely results in a large number of secondary effects on molecules normally associated with the NMDA receptor postsynaptically. In fact, experiments using various deletion mutants missing the cytoplasmic anchoring domains of the NMDA receptor have allowed dissection of the role of the NMDA receptor as a scaffolding protein versus its role as a ligand-gated ion channel (Sprengel et al., 1998). Deletion of the intracellular domain of the NMDA receptor appears to be sufficient to account for essentially all of the physiologic and behavioral deficits observed in NMDA receptor knockout mice. An important take-home message of this finding is that the role of the NMDA receptor as a component of the PSD infrastructure is just as important as its role as a ligand-gated ion channel.

In this chapter, we have discussed three major categories of molecular components and processes that are involved in NMDA receptor regulation. It is very important not to think of these in isolation from each other: they are functional categories to help organize the complex biochemical machinery of NMDA receptor regulation, not compartmentalized biochemical processes in the cell. Thus, we need to begin to think of the NMDA receptor as an immensely complicated information processing machine. It integrates a plethora of biochemical signals and computes, based on a number of molecular inputs, whether to trigger a lasting molecular change. The interactions of these various processes are what allow the NMDA receptor to serve in its role as a molecular decision-maker, and allow for at least part of the necessary sophistication required for deciding when to trigger memory formation in the animal in vivo.

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4.21 Second Messengers: Calcium and cAMP Signaling

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4.21.1 Introduction

Second messenger systems provide incredibly powerful mechanisms for the transformation of stimuli from merely electrical and biochemical signals into stable, long-lasting, and memory-specific neuronal representations. The brain uses the second messengers, calcium and cyclic adenosine monophosphate (cAMP), to effectively transmit synaptic stimulation into stored information. The use of second messengers for this process provides several benefits. First of all, the primary message does not necessarily have to enter into the cell, a process that can be energy-consuming and temporally restrictive because of the rapidity with which neurons must respond to stimuli. Secondly, second messengers are typically small molecules that can easily diffuse within the cell. Calcium and cAMP fit these criteria. These messengers allow amplification of a primary signal as well as the integration of multiple signaling pathways at different locations within the cell. This chapter focuses on the ways in which neurons use second messenger systems, with particular emphasis on calcium and cAMP, in order to provide localized, amplified, and specific responses to memory-inducing stimuli. In this chapter, we illustrate the ways in which second messenger systems converge to produce long-term changes in synaptic plasticity.

4.21.2 The Role of Calcium in Synaptic Plasticity and Memory Processing (Introduction)

Hebbian plasticity, thought to be the basis for memory encoding, depends on the generation of a localized calcium pool that is large enough to cause presynaptic neurotransmitter release. In turn, the presynaptic release must be sufficient to evoke a postsynaptic response that is strong enough to cause

persistent modifications at synapses. Calcium signals of this magnitude can be generated by a number of mechanisms, including entry through voltage-gated calcium channels (VGCCs) or ligand-gated channels. An additional source of calcium comes from intracellular calcium stores including the endoplasmic reticulum (ER). Calcium signals affect the activity of many proteins including kinases, phosphatases, and membrane receptors. The calcium-binding protein calmodulin (CaM) mediates many of calcium's effects in the cell and plays a central role in the convergence of cAMP and calcium signaling.

4.21.2.1 Mechanisms of Generating a Calcium Signal

Neurons use a variety of mechanisms to generate localized calcium signals that are large enough to generate long-term plasticity. Cytosolic free calcium concentrations (less than 100 nM) are much lower than in the extracellular space, and therefore calcium influx at the membrane is driven by a 1000-fold concentration gradient. The primary mechanisms for calcium entry into neurons are *N*-methyl-D-aspartate (NMDA) receptors (*See* Chapter 4.20) and calcium channels, of which multiple types are expressed throughout the brain. When activated by glutamate, NMDA receptors produce localized calcium increases at the synapse. However, calcium passed via NMDA receptors is not always sufficient to drive long-term potentiation (LTP) or the downstream transcriptional and translational events required for long-term memory. For example, tetanus-induced long-lasting LTP (L-LTP) in area CA1 of the hippocampus can be expressed even in the presence of NMDA receptor inhibitors. This expression instead depends on calcium entry through L-type calcium channels (Impey et al., 1996). L-type calcium channels are one of several VGCCs expressed in neurons. Other VGCCs are the P/Q-, N-, L-, R-, and T-type channels. VGCCs are composed of four repeating pore proteins, each containing six transmembrane regions. One of these transmembrane domains contains several positively charged residues, the presence of which permits rapid activation of the channel in response to changes in membrane potential (**Figure 1**).

Calcium entry through calcium channels activates nearby potassium channels that release potassium from the cell, propagating a cascade of VGCC activation within the vicinity of the original calcium signal. Although L-type channels are distributed primarily (though not exclusively) in proximal dendrites, N- and P/Q-type calcium channels are found at presynaptic terminals and participate in neurotransmitter release after strong depolarization. That there are at least some cases in which LTP occurs in spite of NMDA receptor blockade, indicating that the calcium signal is input specific (Grover and Teyler, 1990; Moosmang et al., 2005). Deviant temporal input at neurons can also produce different forms of LTP, probably because of the localization of the calcium source and the magnitude of the calcium signal.

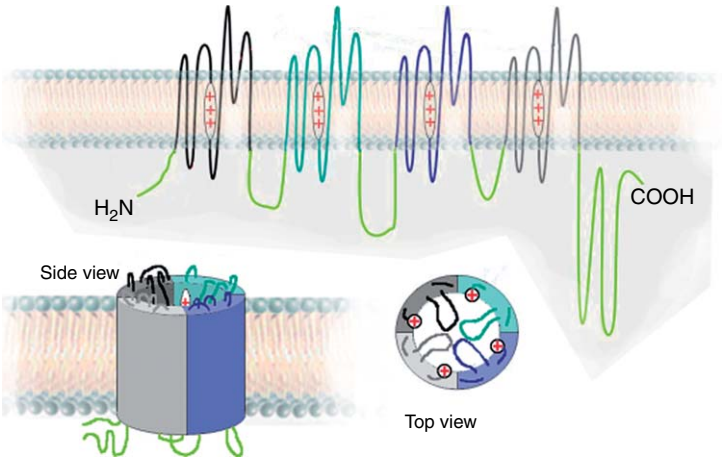


Figure 1 Voltage-gated calcium channel (VGCC) structure. VGCCs are composed of four repeating pore domains. Each pore domain consists of six transmembrane segments, the fourth of which acts as a voltage sensor, allowing rapid opening of the channel pore in response to changes in membrane voltage. Original figure, courtesy of T. Jacob; revised image courtesy of D. Trapp. Adapted from Jacob T, Olfaction: A tutorial on the sense of smell and taste – A brief tutorial, School of Biosciences, Cardiff University, UK, with permission from D. Trapp.

Not all of the calcium signal originates from extracellular sources. Inositol-1,4,5-triphosphate (IP3), derived from phosphatidyl inositol bisphosphate by phospholipase C (PLC), mediates the release of calcium from intracellular stores. Levels of IP3 increase when receptors coupled to PLC (e.g., metabotropic and glutamatergic receptors) are activated (Ross et al., 2005). Once produced and mobilized, the large diffusion constant of IP3 as well as the distribution of IP3 receptors (IP3R) within the ER provide input-specific IP3 receptor binding and calcium release from the ER. Although IP3 functions more globally than calcium because of its large diffusion constant, IP3-mediated diffusion is not the only way for intracellular calcium to participate in synaptic plasticity. ER calcium is also controlled by calcium-sensitive ryanodine receptors (RyR) localized at the ER membrane. Which ER receptors get activated appears to be input specific. For example, activation of cholinergic neurons via adenosine involves IP3-induced release of ER calcium, whereas RyRs remain nonparticipants (Basheer et al., 2002). The contribution of these ER receptors to LTP and memory formation remains elusive. Knockout animals of the RyR type three receptor actually display a facilitation of hippocampal CA1 LTP that is not blocked by NMDAR inhibition, suggesting that the calcium release from the ER may actually inhibit LTP in area CA1 (Futatsugi et al., 1999). IP3R knockout animals are reported to have a similar enhancement in LTP at hippocampal mossy fiber synapses (Itoh et al., 2001), underscoring the unique function of distinct calcium pools in plasticity.

4.21.2.2 Calcium Effector Molecules

The localization and strength of the calcium signal can determine the fate of a synapse, for example, by favoring LTP or long-term depression (LTD). This can be accomplished by direct or indirect activation or inhibition of effector proteins. The effects of calcium on target proteins are often mediated through the high-affinity calcium-binding protein, CaM. CaM, when bound to calcium, binds to and activates a number of enzymes that are critical for long-term synaptic plasticity. The following sections discuss some of the calcium-sensitive proteins that are central to synaptic plasticity.

4.21.2.2.1 Calcium-activated proteins

Calcium activates many proteins including specific adenylyl cyclases, calcium/phospholipid-dependent protein kinases (PKCs), the Ca^{2+}/calmodulin-dependent protein kinases (CAMKs), phosphodiesterases, and phosphatases. Calcium stimulation of these enzymes is mediated by the Ca^{2+} binding protein, CaM. In the 1960s, CaM was first discovered as an activator of cyclic 3′5′-nucleotide phosphodiesterase (PDE). CaM is especially abundant in the brain. CaM is a 17-kD protein that contains no intrinsic enzyme activity but, rather, functions to regulate a wide variety of enzymes in the presence of Ca^{2+}. Containing the prototypical calcium-binding E-F motifs, calmodulin contains four binding sites for calcium. As a result of heterotropic positive cooperativity, enzymes respond to Ca^{2+} with greatly different sensitivities. Free energy coupling between CaM and its different binding proteins varies, allowing Ca^{2+}/CaM effector molecules to respond with slightly different kinetics to increases in free Ca^{2+} (Xia and Storm, 2005). CaM's influence on neuronal functions is widespread, with protein partners that include the calcium-sensitive adenylyl cyclases 1 and 8, protein kinases, calcineurin, nitric oxide synthase (NOS), Ca^{2+} channels, RASGRF1 (a mitogen-activated protein kinase [MAPK] upstream activator), neuromodulin, and neurogranin. **Figure 2** outlines some of the proteins that control free CaM within in the cell. The proteins neuromodulin, neurogranin, and regulator of calmodulin signaling (RCS) are all proteins that determine the levels of free CaM in the resting neuron. Neuromodulin, a neuronal-specific binding protein for CaM, is inhibited from binding to calmodulin in the presence of calcium. In addition, PKC phosphorylation of neuromodulin blocks CaM binding.

The serine/threonine protein kinase C family is highly expressed in brain and contains several isoforms that are responsive to Ca^{2+}. The PKC family of proteins is composed of 11 isozymes that make up three subclasses: the conventional, novel, and atypical groups. Ca^{2+} and diacylglycerol both activate the alpha, beta, and gamma members, all conventional isoforms of PKC, but the novel and atypical groups appear to be calcium-insensitive. When not associated with the plasma membrane, PKC binds Ca^{2+} very inefficiently. PKC can bind at least eight calcium ions per protein (Bazzi and Nelsestuen, 1990) in the presence of a phospholipid bilayer. Translocation of PKC from the cytosol to the membrane occurs following certain types of hippocampus-dependent training, presumably a requirement for interaction and modification of synaptic proteins at relevant circuit synapses (Bank et al., 1989). Although the temporal aspects of this translocation are debated, disruptions in PKC isoforms indicate the importance

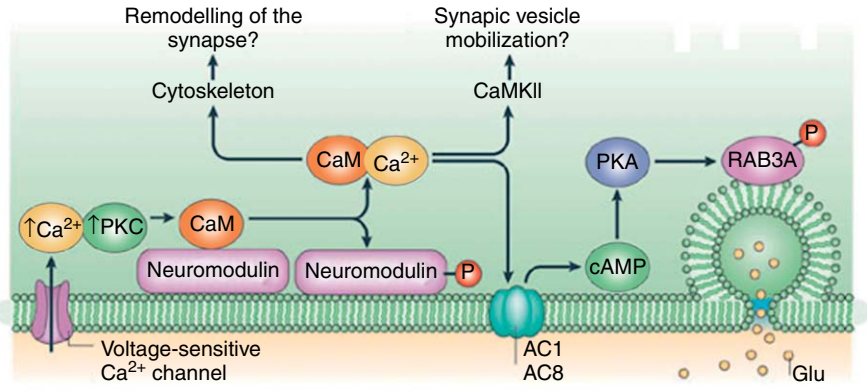


Figure 2 Neuromodulin participation in presynaptic events. Neuromodulin controls the amount of presynaptic calmodulin (CaM) available for calcium binding. Calcium activation of calcium/phospholipid-dependent protein kinase (PKC) causes a PKC-dependent phosphorylation of neuromodulin and the subsequent release of CaM results in direct phosphorylation of the small G protein, Rab3A, which is throught to assist in facilitation of presynaptic vesicle release. Reproduced from Xia Z and Storm DR (2005) The role of calmodulin as a signal integrator for synaptic plasticity. *Nat. Rev. Neurosci.* 6: 267–276.

of this kinase family as calcium effectors in LTP and memory formation. PKC gamma knockout animals assayed for synaptic plasticity and memory perturbations show normal LTD and short-term plasticity; however, LTP is severely diminished except when the LTP tetanus is preceded by a low-frequency stimulus (Abeliovich et al., 1993a). This indicates that PKC gamma contributes to the induction, but not maintenance, of LTP. (*See* Chapter 4.22 for more on the role of the PKC family.)

A second group of Ca^{2+}-sensitive kinases that are central players in synaptic plasticity are the Ca^{2+}/CaM kinases, collectively known as CaM kinases (CAMK). These kinases consist of CAMK1–CAMKVI. Calcium/calmodulin-dependent protein kinase II (CAMKII) is ubiquitously expressed within the brain and is heavily localized to postsynaptic densities. The binding of Ca^{2+}/CaM to CAMKII regulates the activity of the kinase by allowing it to autophosphorylate its own inhibitory domain. This phosphorylation event causes CAMKII to function in a constitutively active state. Phosphorylated CAMKII appears to interact with the NR2B subunit of the NMDA receptors (Strack and Colbran, 1998), an association critical for the CAMKII-dependent LTP at Schaffer collateral-CA1 synapses (Barria and Malinow, 2005). (A thorough presentation of CAMKII in synaptic plasticity is addressed in Chapter 4.23.) In addition to activation of CAMKII, CaM also activates CAMK1, a cytosolic CAMK that probably affects synaptic strength by phosphorylating synapsin I. CAMKIV is also activated by Ca^{2+}/CaM and directly phosphorylates cAMP-responsive element binding protein (CREB). Activation of the predominantly nuclear CAMKIV is critical for late-phase LTP and also for consolidation of the hippocampus-dependent spatial memory (Kang et al., 2001). This correlates with the observations that CREB-induced transcription is critical for several forms of experience-dependent plasticity (Barth et al., 2000; Athos et al., 2002; Kida et al., 2002).

Although Ca^{2+}-activated kinases are critical mediators of the calcium signal, the calcium-sensitive adenylyl cyclases are central players in transmitting the Ca^{2+} message at activated synapses to downstream signaling pathways. There are ten adenylyl cyclases expressed in mammals, and at least three of these – AC1 and AC8, and AC3 – are strongly calcium sensitive, although some others have been reported to respond to calcium as well (Hanoune and Defer, 2001; Cooper, 2003). AC1 and AC8 are both calcium activated and highly expressed in the brain. Whereas AC1 is neurospecific, AC8 is expressed in several peripheral tissues in addition to the brain (Xia et al., 1993; Muglia et al., 1999). The neurospecific expression of AC1 makes it an excellent pharmacological target for increasing cAMP specifically in the CNS for treatment of memory and neurodegenerative disorders. AC1, which is considerably more sensitive to calcium than AC8, is activated half-maximally *in vitro* at approximately 150–200 nM free Ca^{2+}, levels that hover just above the resting intracellular calcium concentration in neurons. Calcium-sensitive adenylyl cyclases contain

a CaM-binding domain that mediates the calcium sensitivity of the enzyme (Wu et al., 1993). Mutation of this calmodulin-binding site strongly inhibits its calcium sensitivity. Because the second half of the chapter focuses on cAMP as a second messenger, the role of the adenylyl cyclases as mediators of the calcium signal is reviewed there in greater detail (see the section titled 'cAMP as a second messenger in plasticity and memory').

Calcium's effects on kinases and cyclases result in phosphorylation of target proteins and the production of cAMP, respectively. However, Ca^{2+}-modulated proteins also include phosphatases and cAMP hydrolyzing proteins. The Ca^{2+}-stimulated serine/threonine phosphatase calcineurin (also known as PP2B), for example, is the most calcium-sensitive of the phosphatases ($Kd = 0.1 - 1\,nM$) and the only calcium-sensitive phosphatase found in the brain (Mansuy, 2003). Calcineurin regulates synaptic plasticity both presynaptically (by restraining vesicle release) and postsynaptically, where it complexes with the postsynaptic density (PSD) and, in general, orchestrates the suppression of downstream signaling. Because of its calcium sensitivity and its association with the PSD, calcineurin can respond quickly to calcium influxes at the membrane and negatively regulate further calcium increases by dephosphorylating VGCCs, NMDA receptors, and AMPA receptors. Concomitant to inhibiting calcium influx at the membrane, calcineurin can also reduce inositol-induced calcium release (IICR) and calcium-induced calcium release (CICR) by negatively regulating both IP3 and RyRs. This negative regulation comes in the form of calcineurin-mediated dephosphorylation of PKC target sites on the receptors themselves (Mikoshiba, 1997; Bandyopadhyay et al., 2000). Mice with forebrain-specific calcineurin ablation have been engineered and tested for LTP and memory phenotypes. Animals without forebrain calcineurin display impaired LTD but normal LTP at Schaffer collateral-CA1 synapses. No deficits in several hippocampal-dependent memory tasks have been observed in these animals; however, working memory is impaired (Zeng et al., 2001). Although NMDA receptor function in calcineurin-deficient animals appears normal, it is hypothesized that an overall elevation in postsynaptic calcium and, perhaps, a deficit in AMPA receptor endocytosis might cause the observed LTD deficits. (For other mouse models with aberrant calcineurin expression, see **Table 1**.) Interestingly, calcineurin has recently been discovered to activate adenylyl cyclase activity (Chan et al., 2005), a property that may antagonize its other functions at the synapse.

4.21.2.2.2 Calcium-inhibited proteins

Not all calcium-sensitive proteins are activated by calcium. As mentioned earlier, the protein neuromodulin is one of a number of proteins inhibited by Ca^{2+}. Neuromodulin is expressed presynaptically and presides over the availability of free calmodulin by sequestering it at the membrane until PKC-mediated phosphorylation of neuromodulin releases calmodulin (Andreasen et al., 1983; Xia and Storm, 2005). Phosphorylation of neuromodulin by PKC occurs during LTP and probably functions presynaptically to allow maximal Ca^{2+}/CaM binding and vesicle release. Modulating postsynaptic CaM availability is the postsynaptically expressed protein neurogranin (also called RC3). Neurogranin is concentrated in dendritic spines and is also phosphorylated by PKC. Like neuromodulin, neurogranin is inhibited from binding CaM in the presence of Ca^{2+}.

Intracellular calcium levels must not be tonically elevated, however, or excitotoxicity can ensue. One way in which this can be prevented is the inhibition of calcium entry by calcium itself. An example of such inhibition is the calcium-mediated inhibition of NMDA receptor currents in hippocampal neurons (Legendre et al., 1993), a process that can be blocked by intracellular buffering agents. Under certain conditions, calcium inhibits further CICR by inhibiting ER-mediated calcium release. Specifically, both RyRs and IP3Rs can be inhibited by Ca^{2+}/CaM binding to the receptors. Although the situations in which this occurs are still somewhat unclear, CaM-mediated inhibition of intracellular calcium release appears to tightly control calcium concentrations, balancing the inhibition of release with ATP and phosphorylation-induced activation of these receptors (for a review, see Bardo et al., 2006). CaM binding is not the only way in which IP3 and RyR receptors can be inhibited by calcium, however. As already addressed, calcineurin-mediated dephosphorylation of PKC sites on these receptors provides a second mechanism by which neurons can regulate the optimal cytoplasmic Ca^{2+} concentrations required for synaptic plasticity and memory encoding.

4.21.2.3 Localization of the Calcium Signal

Efforts to determine the range of diffusion for intracellular calcium and other second messengers, such as IP3, produced evidence that calcium has a very

Table 1 Mouse genetic approaches to studying the role of calcium signaling in memory

Gene modified	*Alteration*	*Affected area of expression*	*Effect on plasticity*	*Effect on memory*
PKCβ[a]	Knockout	Global	Normal short-term and long-term plasticity	Deficits in cued and contextual fear
αCAMKII[b]	Knockout	Global	Deficit in STP, LTP and LTD	Deficits in spatial memory
αCAMKII$^{+/-}$[c]	Heterozygote	Global	Impaired cortical LTP	Deficient in remote memory maintenance
CAMKIV[d]	Transgenic, dominant negative	Forebrain	Deficits in L-LTP	Deficits in cued and contextual fear consolidation
NR2B[e]	Transgenic, overexpression	Forebrain	Enhanced LTP, lower threshold required	Enhanced auditory and contextual fear
AC1[f]	Knockout	Global	Decreased cerebellar LTP	Deficits in rotarod performance and spatial memory deficits in Morris water maze
AC1/AC8[f]	Double knockout	Global	Deficits in CA1 LTP	Deficient in hippocampus-dependent fear memory
RasGRF1[g,h]	Knockout	Global	Small impairment in Schaffer collateral LTP after two 100-Hz stimuli, severe impairments in LTD	Deficits in hippocampal but not amygdala-dependent long-term memory
RasGRF1/2[h]	Double knockout	Global	Impaired Schaffer collateral-CA1 theta burst LTP and LTD; deficits in L-LTP	No memory deficits reported
PKCγ[i]	Knockout	Global	Required for LTP induction, not maintenance	Mild hippocampal memory deficits
CaV1.2[j]	Transgenic, IQ domain mutation	Forebrain	No NMDAR-independent LTP	Deficient in spatial memory
Calcineurin (regulatory subunit CNB1)[k]	Knockout	Forebrain	LTD deficit, modest increase in LTP at CA1 synapses	Working, episodic memory impairments (eight-arm radial maze and delayed matching-to-place)
Calcineurin[l]	Transgenic, inducible inhibition	Forebrain	Enhanced one-train LTP and *in vivo* LTP in CA1	Enhanced spatial memory
Calcineurin (catalytic subunit CNAα)[m]	Transgenic, constitutively active	Forebrain	Deficits in PKA-dependent intermediate-LTP	Spatial memory deficits in Barnes maze
αCAMKII[n]	Transgenic, overexpression	Forebrain	Enhanced one-train LTP	Spatial memory deficits
AC1[o]	Transgenic, overexpression	Forebrain	Increased Schaffer collateral-CA1 LTP; 100-Hz, two-train LTP	Increased recognition memory
NR1[p]	Transgenic, tet-inducible; knockout	Forebrain, CA1 specifically	Deficits in CA1 LTP and LTD	Deficits in hippocampal memory consolidation
Neurogranin/RC3[q]	Knockout	Global	Reduced thresholds for LTP, but baseline transmission reduced	Deficits in Morris water maze acquisition, spatial learning deficits

STP, short-term potentiation, LTP, long-term potentiation, LTD, long-term depression; PKC, protein kinase C.
[a](Weeber et al., 2000); [b](Silva et al., 1992a,b); [c](Frankland et al., 2004); [d](Kang et al., 2001); [e](Tang et al., 1999; Wong et al., 1999); [f](Wong et al., 1999); [g](Giese et al., 2001); [h](Li et al., 2006); [i](Abeliovich et al., 1993a; Abeliovich et al., 1993b); [j](Moosmang et al., 2005); [k](Zeng et al., 2001); [l](Malleret et al., 2001); [m](Mansuy et al., 1998); [n](Wang et al., 2003); [o](Wang et al., 2004); [p](Krucker et al., 2002; Shimizu et al., 2000); [q](Krucker et al., 2002; Miyakawa et al., 2001).

low range of diffusion (particularly unbuffered calcium) compared with IP3. This makes calcium functional within restricted domains (Allbritton et al., 1992). The specificity generated by localized calcium pools allows transduction of synaptic signals that take place at postsynaptic densities far from the nucleus. Membrane pumps, the presence or absence of localized Ca^{2+}-binding proteins, and the stored calcium present in intracellular organelles all function to produce changes in local calcium concentrations. The smooth endoplasmic reticulum (SER) sequesters most of the intracellular calcium, a process mediated by sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pumps. SERCA pumps help maintain Ca^{2+} concentrations in the ER much higher than the intracellular Ca^{2+} concentrations. Because of this discrepancy between SER calcium and intracellular calcium, IICR and CICR provide neurons mechanisms to rapidly produce localized increases in Ca^{2+}. Because the SER membrane flexibly innervates even synaptic spine regions (Cooney et al., 2002), IICR and CICR can produce Ca^{2+} increases at synaptic densities.

Localization of the Ca^{2+} signal is extremely important and probably accounts for NMDA receptor-independent LTP versus NMDA-receptor-dependent LTP. For example, the temporal information in a 100- versus 200-Hz stimulation at Schaffer-collateral synapses in CA1 can shift LTP dependency from NMDA receptors to VGCCs. This type of temporal information may only be interpreted intracellularly through a second messenger such as Ca^{2+}, which, because of its limited diffusion, is restricted to local domains. Perhaps the Ca^{2+} signal generated by very rapid stimuli allows a sufficient number of VGCCs to be activated, in effect producing a Ca^{2+} concentration that overrides the Ca^{2+} signal provided by NMDA receptor activation. NMDA receptor-independent LTP has long been studied at the hippocampal mossy fiber inputs into the CA3 region. Here, NMDA receptor inhibition has been reported not to have any effect on mossy fiber LTP (Harris and Cotman, 1986). Similar reports of NMDA-independent LTP have been reported at Schaffer-collateral CA1 synapses (Impey et al., 1996; Moosmang et al., 2005) and also in the amygdala (Weisskopf et al., 1999).

4.21.2.3.1 Synaptic calcium

Among the Ca^{2+}-activated and -inhibited proteins, what effectors are critical for presynaptic vesicle release and long-lasting, input-specific, postsynaptic responses? Ca^{2+} influences both presynaptic properties such as vesicle release and postsynaptic properties, including activation of kinases that serve as moderators of membrane channels and receptors. Although Ca^{2+} affects the synapse in many ways, we review some of the central presynaptic and postsynaptic targets for calcium.

Synaptic vesicle release occurs within microseconds of the presynaptic action potential and is dependent on Ca^{2+} entry through VGCCs. The ways in which calcium moderates presynaptic terminal vesicle release are diverse and moderated by a number of presynaptic Ca^{2+} buffering agents including calretinin, calbindin D-28K, and parvalbumin. The distinct mobility, localization, solubility, and Ca^{2+}-sensitivity of these buffering proteins provide specific presynaptic responses in different areas of the brain and probably contribute to the type of synaptic connections made following specific stimuli. Of course, CaM also has specific roles in presynaptic activity. Ca^{2+} activation of PKC at presynaptic terminals allows phosphorylation of the presynaptic CaM-binding protein, neuromodulin. As already mentioned, this phosphorylation releases CaM for activation of its target proteins (see **Figure 2**).

Presynaptic increases in Ca^{2+} not only release CaM from its synaptic captor, neuromodulin, but lead to protein kinase A (PKA)-mediated vesicle mobilization through the activation of the Ca^{2+}-sensitive adenylyl cyclases. **Figure 2** pictorially demonstrates the cascade of molecular events leading from cAMP increases to facilitation of presynaptic neurotransmitter release. PKA directly phosphorylates RAB3A, a Ras-associated protein, which increases vesicle release probability. Rab3A is one of several presynaptic PKA targets; PKA phosphorylation of SNAP25 and its binding protein snapin probably sustains pools of primed and release-ready vesicles (Leenders and Sheng, 2005). Also effective in increasing presynaptic release is the expression of CAMKII, the inhibition of which blocks glutamate-stimulated boutons and miniature excitatory postsynaptic currents in neurons of the hippocampus (Ninan and Arancio, 2004). CAMKII is also thought to assist in vesicle release by phosphorylating synapsin I, a phosphorylation event that occurs and persists for several hours following LTP induction. It is hypothesized that synapsin I phosphorylation increases the number of vesicles available for release.

Because Ca^{2+} affects postsynaptic responses in numerous ways, we describe a few of the key postsynaptic Ca^{2+}-dependent events that are required for

long-term synaptic plasticity or memory formation. A central target for postsynaptic Ca^{2+} signal transduction is activation of the Ca^{2+}/CaM-sensitive CAMKII. CAMKII is thought to be the primary regulator of LTP while, in general, calcineurin determines the fate of a neuron for LTD. Once activated, postsynaptic CAMKII phosphorylates the AMPA receptor subunit GluR1, enhancing AMPA current conductance and, perhaps, translocation of the receptor to the membrane. A second way that calcium mediates AMPA receptor activation during LTP is by activating AC1 and AC8. This mechanism for LTP is commonly observed during early postnatal development. In this case, PKA phosphorylation of GluR4 is necessary for hippocampal LTP (Yasuda et al., 2003). While CAMKII activation affects ion channel activity, it can also function postsynaptically by downregulating proteins following calcium activation. For example, in olfactory sensory neurons, CAMKII phosphorylation of AC3 results in cAMP transients that allow sensitization in response to odorant stimulation. The interplay between PDE activation and CAMKII-induced AC3 inhibition are required for this process (Wei et al., 1998). Although both processes are calcium mediated, the temporal aspect of calcium activation is probably crucial for the olfactory responses in an animal.

The Ras-guanine nucleotide-releasing factors (RasGRF) – RasGRF1 and RasGRF2 – are Ca^{2+}/CaM-sensitive proteins that activate the small G proteins such as Ras and Rac and assist in the transmission of NMDAR calcium signals to activation of the Ras/MEK/MAPK family (see **Figure 5**). Glutamate activation of NMDA receptors causes a calcium-mediated binding of RasGRF1 to the NR2 subunit of NMDA receptors. RasGRF1 acts as a calcium sensor for NMDA receptors (Krapivinsky et al., 2003). RasGRF1 binding to NR2 causes activation of the MAPK pathway in the postsynaptic neuron and probably mediates LTD and, to some extent, LTP at Schaeffer collaterals in the hippocampus (Giese et al., 2001). Animals deficient in RasGRF1 expression have severe impairments in low-frequency stimulation-induced LTD (see **Table 1**).

Neurogranin, the postsynaptic member of the IQ domain family, is highly expressed in dendritic spines and modulates postsynaptic responses to calcium that are critical for postsynaptic plasticity. It is clear that neurogranin contributes to postsynaptic plasticity, as rodents that lack neurogranin expression have reduced thresholds for hippocampal LTP and are unable to undergo LTD in response to low-frequency stimulation probably because basal transmission is already depressed (Krucker et al., 2002). It is proposed that the neurogranin knockout animals have excess levels of free calmodulin present in the brain, and that therefore, greater sensitivity to calcium results in LTD at baseline calcium concentrations. However, when low-frequency stimuli that would normally cause LTD in wild-type animals are administered to neurogranin knockout hippocampal slices, LTP occurs.

4.21.2.3.2 Calcium's effects in the nucleus

The roles of Ca^{2+} in the nucleus during synaptic plasticity and memory processing are complex, often integrating several signaling cascades that converge in nuclear events. Like cytosolic effector specificity, the specificity of the Ca^{2+} signal is crucial for determining activity in the nucleus. For example, a Ca^{2+}-sensitive site (CaRE2) in the promoter of brain-derived neurotrophic factor (BDNF) can be activated by upstream regulatory factors 1 and 2 (USF1, USF2), but only in response to specific calcium signals, such as those generated through the L-type VGCCs. Glutamate activation of NMDA does not result in a Ca^{2+} signal sufficient to drive USF-activated BDNF gene expression (Chen et al., 2003). This distinction is critical in light of the fact that BDNF is upregulated transcriptionally during the consolidation of certain forms of hippocampus-dependent memory. Contributing to the complexity of Ca^{2+}-activated transcription are the different modes of activation by Ca^{2+}. For example, Ca^{2+} can activate transcription factors in the cytoplasm that then translocate to the nucleus, or it can induce translocation of proteins that activate transcription factors, such as CaM or CREB kinases. In fact, Ca^{2+}-activated CaM can physically translocate to the nucleus within seconds after neuronal activation (Mermelstein et al., 2001). Ca^{2+} can also bind to transcription activators or repressors directly (such as the transcriptional repressor DREAM) controlling the transcription of genes such as prodynorphin and the immediate early gene, c-fos (Carrion et al., 1999; Deisseroth et al., 2003). This translocation is critical for CAMK-mediated CREB activation and the transcription of some genes under the control of CRE-containing promoters.

As membrane and cytosolic proteins respond to localized Ca^{2+} signals, so do nuclear events. For example, CREB activation depends on specific and localized

Ca^{2+} signals. Mice with mutations in the IQ domain of the L-type calcium channel have significantly reduced Ca^{2+}-dependent CREB phosphorylation and transcriptional activity (Dolmetsch et al., 2001). Sustained CREB phosphorylation in the nucleus depends upon the binding of CaM to the IQ domain of L-type channels, and transduction of this signal is mediated through activation of the Ras/Raf/MAPK pathway. Although pharmacological inhibitors of NMDA receptors and N-, P/Q-, and L-type calcium channels are all capable of decreasing intracellular calcium to a similar concentration in wild-type neurons, only L-type channel inhibition blocks depolarization-induced, sustained CREB phosphorylation in neuronal nuclei. Ca^{2+} regulation of CREB activity is complex, because Ca^{2+}-mediated dephosphorylation of this protein also occurs. Ca^{2+} activation of calcineurin causes the dephosphorylation of CREB at serine 133, reducing CREB's transcriptional activity. In fact, inhibition of calcineurin can reduce the threshold of stimulus intensity that is usually required for CREB-mediated transcription in hippocampal neurons (Bito et al., 1996).

4.21.2.4 Calcium Signaling During Memory Processing

In attempts to address the requirement of Ca^{2+} and the specificity of the Ca^{2+} signal required for memory formation and synaptic plasticity, many pharmacological and genetic studies have been conducted. The next sections address a few of the genetic and pharmacological perturbations of Ca^{2+} signaling that have been carried out to more clearly define the role of Ca^{2+} in synaptic plasticity and memory processing.

4.21.2.4.1 Mammalian genetic models of calcium in memory processing

The generation of transgenic or knockout mice with perturbations in calcium signaling has greatly increased our knowledge of calcium's contribution to different forms of plasticity, learning, and memory. Conventional knockout animals have been gene targeted so as to eliminate the function of a specific gene within the genome. Although compensatory mechanisms during development are cause for caution when interpreting data from conventional knockout animals, much can be learned by gene knockout technology and verified by alternative means. Furthermore, conditional knockouts, which involve tissue-specific and, sometimes, inducible gene knockdown properties (accomplished through a site-specific DNA recombination event), have been extremely useful in determining the tissue-specific attributes of certain proteins in synaptic plasticity. Some of the mouse models central to defining a role for Ca^{2+} signaling in synaptic plasticity are listed in **Table 1**.

Collectively, the calcium-sensitive kinases including PKC and the CAMK have been central targets for genetic manipulations. In general, the abolition or reduction of kinase activity has correlated with deficits in LTP and memory, although this is not always the case. For example, there are mouse models in which a reduction in calcium-sensitive kinase activity critically impairs LTP but produces only mild behavioral phenotypes in tasks that are dependent on the same brain region as the observed LTP impairment. Such is the case for the PKCγ knockout animal, for example. Of course, isozyme compensation is always a consideration, especially when perturbation of a kinase isozyme occurs in a family of proteins represented by a large number of isozymes. Proteins of the CAMK family have also been studied using transgenic and gene knockout technologically because of their contribution to synaptic stabilization as well as nuclear events such as CREB-mediated gene transcription. Complete ablation of αCAMKII results in plasticity deficits; specifically, short-term potentiation, and therefore LTP and LTD are completely absent in these animals (Giese et al., 1998). However, animals heterozygous for αCAMKII demonstrate a remarkable phenotype. Interestingly, αCAMKII heterozygotes have impaired cortical LTP and severe remote memory deficits. The remote memory deficits observed in these animals are consistent with the observation that hippocampus-dependent memories undergo systems consolidation (Debiec et al., 2002), a process that provides hippocampal memories with a cortical dependence over time. It is possible that a full dose of CAMKII activity is required to retain synaptic configurations responsible for remote but not hippocampus-dependent memories. Animals in which CAMKIV has been knocked out show severe deficits in both contextual and auditory fear conditioning at 1 and 7 days following training, whereas short-term memory remains intact (Wei et al., 2002). The activation of transcription by CAMKIV is cohesive with the long-term memory phenotype, as long-term but not short-term memory requires *de novo* transcription and translation.

In addition to genetic perturbations of Ca^{2+}-sensitive kinases, receptors and channels that pass Ca^{2+} have been central genetic targets for studying plasticity and memory. For example, mice with mutations in CaV1.2,

the most abundant L-type calcium channel in the hippocampus, show no NMDA-receptor-independent LTP in the hippocampus and also have impairments in spatial memory (Moosmang et al., 2005). As might be expected, genetic manipulations of NMDA receptors demonstrate that the Ca^{2+} signal specific to NMDA-receptor activation plays a key role in both hippocampal LTP and LTD. For example, NR1 subunit ablation in the CA1 area of the hippocampus interferes with both Schaffer collateral LTP and LTD, and these results are mirrored by deficits in the consolidation phase of several hippocampal forms of memory. Conversely, NR2B overexpression reduces the threshold required for CA3-CA1 LTP and also results in behavioral facilitation of auditory and cued fear (see **Table 1**). These and other mammalian genetic models are referenced in **Table 1** and, although an incomplete list, are some of the key models that have resulted in a more concise understanding of how calcium-sensitive proteins (or the lack thereof) affect synaptic plasticity and memory.

4.21.2.4.2 Pharmacological advancements in calcium signaling during memory formation

In general, studies using pharmacological inhibition of intracellular Ca^{2+} strongly corroborate the genetic evidence that Ca^{2+} is central to both synaptic plasticity and memory processing. However, as developmental compensation is to knockout gene technology, so is drug specificity to pharmacological inhibition. For example, inhibition of VGCCs such as the L-type calcium channel has produced confounding results in the synaptic plasticity and memory fields. Although in some cases, inhibition of L-type channels actually enhances contextual fear memories (Cain et al., 2002), inhibition has also been reported to cause deficits in amygdala and hippocampal-dependent memory or LTP (Bauer et al., 2002). Because only low doses of antagonists, such as nifedipine, have resulted in behavioral augmentation, it has been hypothesized that channel modification, rather than complete inhibition, occurs at low doses; memory enhancements are not observed at high doses of antagonists. In spite of these contradictions, within-study comparisons can be made that underscore the specificity of the calcium signal for particular phases of memory processing. For example, systemic administrations of VGCC inhibitors can block extinction of fear memory while having no effect on acquisition or on retrieval-induced reconsolidation. Inhibition of NMDA receptors, on the other hand, results in not only extinction blockade but also blockade of consolidation and reconsolidation phases of memory (Suzuki et al., 2004). The disparate effects of these inhibitors on memory processing underscore the level of specificity required in the calcium signal for these different levels of memory processing. Although less is known about intracellular calcium's contribution to memory-encoding events, pharmacological inhibitors to SER pumps and receptors can and have been used to determine the contribution of SER calcium to events such as vesicle release and the strength of the postsynaptic response. The developments of SERCA pump inhibitors, such as thapsigargin and cyclopiazonic acid (CPA), and the RyR inhibitors (ryanodine red and dentrolene) have assisted in our knowledge of IICR and CICR in synaptic plasticity. CPA application, as well as high doses of ryanodine, for example, can reduce Ca^{2+} transients following synaptic stimulation in hippocampal neurons (Emptage et al., 1999). Inhibition of SERCA pumps by bath application of thapsigargin inhibits LTP at Schaffer collateral-CA1 synapses. Heparin-mediated inhibition of IP3 receptors also interferes with potentiation of CA3 neurons (Bardo et al., 2006).

Whereas inhibitors that block calcium entry and release have proved quite useful in clarifying the temporal and spatial roles for different phases of LTP or memory formation, pharmacological inhibition of calcium-sensitive proteins has also been instrumental in elucidating calcium's plieotropic effects in synaptic plasticity. Pharmacological inhibition of calcineurin, for example, by bilateral ventricular administration of calcineurin antisense oligonucleotides, causes an increase in contextual fear conditioning (Ikegami and Inokuchi, 2000). This study is cohesive with the conditional forebrain calcineurin knockout mouse that showed enhancements in LTP and spatial memory. However, early studies in chicks using intracranial infusions of calcineurin inhibitors cyclosporine A or FK506 resulted in acquisition deficits in a single-trial passive avoidance task (Bennett et al., 1996, 2002). Species differences could account for these discrepancies, or the contribution of calcineurin could be dependent on the type of memory for which the organism was trained and tested. Of course, it is also possible that calcineurin contribution varies according to both the type and location of pharmacological intervention. In short, the role of calcineurin in different memory tasks is complex, and much remains to be clarified about its activity during memory formation. As can be noted in **Table 1**, several calcineurin mouse models

have been made and may lead to more clarification of the role of calcineurin in the different phases of synaptic plasticity and memory processing.

As previously discussed in the section titled 'Calcium's effects in the nucleus,' one of the nuclear effects of synaptic Ca^{2+} is the transcription of BDNF, which is mediated by calcium-sensitive sites in the promoter region of the gene. BDNF strongly potentiates dentate granule cell LTP when synaptic stimuli are coapplied with dendritic BDNF application (Kovalchuk et al., 2002). (There is probably also a presynaptic component to BDNF, as viral rescue of BDNF expression in the hippocampal area CA3 of $BDNF^{-/-}$ mice can recover Schaffer collateral LTP deficits that are induced by 100-Hz stimulus (Zakharenko et al., 2003)). BDNF expression is induced following training for contextual fear conditioning, and BDNF antisense oligonucleotides have been used to study the role of this protein in consolidation and reconsolidation of this task. Infusion of BDNF antisense oligonucleotides into the hippocampus of rats prior to memory training disrupts the consolidation of contextual fear memory, whereas short-term memory is preserved (Lee et al., 2004). As oligonucleotide (such as siRNA and antisense) and viral pharmacological approaches have become more commonly used, the study of Ca^{2+} in synaptic plasticity has been enhanced by the ability to alter protein expression in distinct areas of the brain.

4.21.3 cAMP as a Second Messenger in Plasticity and Memory

The role of cyclic adenosine monophosphate will now be more closely addressed, as it is critical for activation of both cytosolic and nuclear components of synaptic plasticity and memory formation. Adenylyl cyclases convert adenosine triphosphate (ATP) to cyclic adenosine monophosphate. The structure of ATP and its conversion to cAMP are depicted in **Figure 3**.

cAMP functions as a second messenger by coupling extracellular signals to intracellular effectors and integrating specific stimuli into the production of long-lasting neuronal configurations or representations. Two major classes of proteins control cellular concentrations of cAMP: adenylyl cyclases (synthesis) and phosphodiesterases (degradation). Although adenylyl cyclases control the rate of cAMP synthesis, PDE proteins control the rate of its degradation, and these two groups of enzymes tightly regulate intracellular levels of cAMP. Here we focus primarily on the convergence of Ca^{2+} with cAMP signaling at the level of adenylyl cyclases and the effect of this signaling crosstalk on learning and memory.

4.21.3.1 Synthesis and Degradation of cAMP

4.21.3.1.1 Adenylyl cyclases

Ten adenylyl cyclases have been identified to date. Most of these enzymes share several properties, including transmembrane and cytoplasmic domains, the latter being the site of catalysis (Dessauer and Gilman, 1997; Hurley, 1999). With the exception of a soluble adenylyl cyclase, adenylyl cyclases are integral membrane proteins that consist of two groups of six transmembrane domains, a structure that has prompted speculation that these enzymes might have transport activity. The highly conserved cytoplasmic loops form

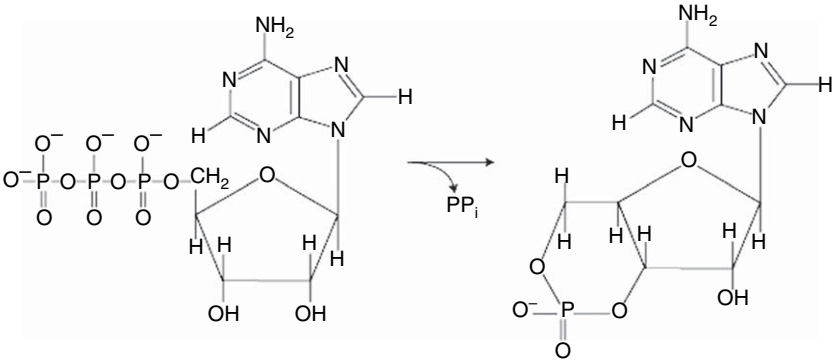


Figure 3 Catalysis of cAMP by adenylyl cyclases. Adenylyl cyclases catalyze the conversion of adenosine triphosphate (ATP) on the left, to cyclic adenosine monophosphate (cAMP) on the right. Adenylyl cyclases catalyze this conversion by attacking the 3′ hydroxyl group on the ribose of the nucleoside triphosphate, ATP.

the catalytic domains of the enzyme and must both be present for adenylyl cyclase activity. Although all isoforms of adenylyl cyclases are expressed in the nervous system, only AC1 is neurospecific. AC2 (a calcium-insensitive cyclase) and the calcium-inhibited AC9 are also highly enriched in the brain (Antoni et al., 1998; Xia et al., 1991). AC8 and AC3, both expressed in the nervous system, are also Ca^{2+} sensitive, with AC8 being Ca^{2+} activated, whereas AC3 is Ca^{2+} inhibited. The presence of distinct classes of adenylyl cyclases (Ca^{2+} sensitive and Ca^{2+} insensitive) was discovered by the application of brain extracts to Ca^{2+}-dependent CaM-Sepharose affinity columns (Westcott et al., 1979). Although most adenylyl cyclases are membrane bound, there is at least one soluble, bicarbonate ion-activated cyclase that appears to be important for several physiological processes, including axon outgrowth (Wu et al., 2006; Zippin et al., 2001).

The structure of adenylyl cyclases and the molecular machinery involved in adenylyl cyclase activation by heterotrimeric G proteins is schematically portrayed in **Figure 4**.

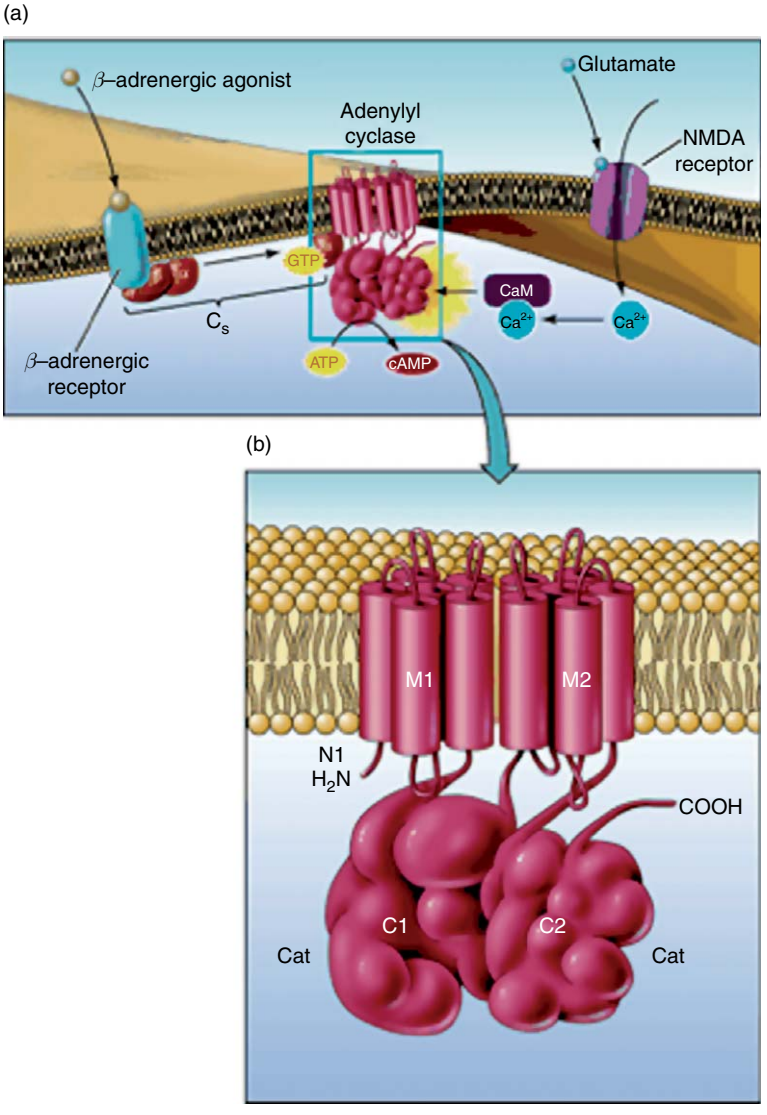


Figure 4 Structure of adenylyl cyclases and activation by G protein–coupled receptors. Activation of G protein–coupled receptors results in a direct association of GTP-bound Gα subunits with adenylyl cyclases in the membrane (a). (b) The catalytic subunits for adenylyl cyclases (cat), composed of the intracellular loops, mediate Ca^{2+}/CaM sensitivity for multiple adenylyl cyclases and are required for enzymatic catalysis of cAMP. Reproduced with permission from Ferguson GD and Storm DR (2004) Why calcium-stimulated adenylyl cyclases? *Physiology* 19: 271–276.

The most common form of adenylyl cyclase regulation is via G protein–coupled receptors, which convey extracellular signals to intracellular signaling pathways. (See Video Clip 1.) Some examples of G protein–coupled receptors include adrenoceptors, metabotropic glutamate, acetylcholine, dopamine, histamine, serotonin, PACAP, somatostatin, vasopressin, melatonin, cannabinoid, opioid, rhodopsin, prokineticin, pheromone, and olfactory receptors. G protein–coupled receptors regulate membrane adenylyl cyclases via direct interaction with the alpha subunits of heterotrimeric G proteins, although in some cases the G$\beta\gamma$ subunits can also activate adenylyl cyclases. Gα subunits are comprised of two domains, a GTPase and an alpha-helical region. These can be subcategorized into the following families: G_s (stimulatory), G_i (inhibitory), G_{olf}, G_q (which couples to phospholipase C), G_t (transducin), and $G_{12/13}$ (motility-activating) proteins. Adenylyl cyclases can be inhibited by binding to the alpha subunit of inhibitory G proteins ($G_{i\alpha}$). There are three $G_{i\alpha}$ isozymes in the hippocampus, and $G_{i\alpha1}$ functions as the primary isozyme for adenylyl cyclase inhibition. $G_{i\alpha1}$ mutant mice demonstrate the importance of this inhibition in normal memory processing and are discussed in the section titled 'Mammalian genetic models of cAMP in memory processing.' Not exempt from affecting cyclase activity, however, are the Gβ,γ protein subunits that can activate adenylyl cyclases II and IV in the presence of $G_{\alpha S}$.

4.21.3.1.2 Phosphodiesterases

Although adenylyl cyclases control the rate of cAMP production in response to external stimuli, PDE proteins are involved in enzymatic catalysis of cAMP. Cyclic nucleotide PDEs comprise a family of ten protein types (from 19 genes) that function to inactivate cAMP (Beavo, 1995; Soderling and Beavo, 2000). PDE proteins were first identified as hydrolyzing enzymes for cAMP by Dr. Earl Sutherland (Butcher and Sutherland, 1962). The PDEs also degrade guanine 3′5′-cyclic monophosphate (cGMP). The family of PDE enzymes provides an important convergence between the Ca^{2+} and cAMP second messenger systems. For example, some PDE proteins are phosphorylated in a Ca^{2+}/CaM-dependent manner and participate in direct interactions with calmodulin. PDE1 is expressed at high levels in the brain and is activated by Ca^{2+}/CaM (Cooper, 2005; Kakkar et al., 1999). To date, there are no known calcium-inhibited PDE proteins.

The *Drosophila* phosphodiesterase *dunce* mutant provided the first evidence that cAMP affects memory formation. The *dunce* mutation caused deficiencies in memory, and since that discovery, the PDEs have become a central object of study in plasticity-related fields. The PDE4 inhibitor rolipram, commonly used as an antidepressant, has been administered to rodents *in vivo*, yielding memory-enhancing tasks in certain forms of memory (Bourtchouladze et al., 2003). This and other studies using pharmacological inhibition of PDE proteins are addressed in the section titled 'Pharmacological advancements in cAMP signaling during memory formation.'

4.21.3.2 cAMP Effector Molecules

Protein kinase A is the primary mediator of cAMP signaling in the cell; however, it is now known to be one of several cAMP effector molecules expressed in the brain. PKA was first discovered by Fischer and Krebs who studied the protein's actions in context of glycogen breakdown (Sutherland and Wosilait, 1955; Walsh et al., 1968). The PKA protein contains two catalytic and two regulatory sites that form what is referred to as the holoenzyme in the absence of cAMP. In the presence of cAMP, the regulatory and catalytic protein subunits disassemble, allowing the catalytic subunits to function as a serine/threonine protein kinase. This kinase activity ultimately affects neurotransmitter release, channel conductance, translocation of proteins to the nucleus, and orchestration of nuclear events such as the activation of gene transcription. The cell maintains tight control over PKA-mediated cAMP signaling; when the catalytic subunits are overexpressed, for example, the regulatory subunit 1 (R1) undergoes posttranscriptional upregulation, producing a more stable protein subunit (Amieux and McKnight, 2002). Animals that aberrantly express some portion of the PKA holoenzyme demonstrate the power of this cAMP-binding protein in transmitting the second messenger signal into lasting, synapse-specific information. For example, animals that express a dominant-negative regulatory subunit R(AB) have deficits in L-LTP and contextual memory (Abel et al., 1997), and animals that do not express one of the catalytic (C1β) or one of the regulatory (RIβ) subunits of PKA have deficits in hippocampal mossy fiber LTP (Brandon et al., 1995). Because PKA is a MAPK activator and appears to mediate its translocation to the nucleus, it is not surprising that deficits in PKA subunit expression result in impairments in synaptic plasticity and memory. Furthermore, PKA is involved in direct binding and activation of several CREB kinases, which will be discussed further.

Although cAMP has traditionally been thought to participate in downstream signaling primarily through the activation of PKA, it can, however, activate MAPK by alternative mechanisms. Specifically, cAMP activation of the small G protein, Rap1, originally believed to be mediated exclusively by PKA phosphorylation (Hata et al., 1991), has now been discovered to occur in a PKA-independent manner. Kawasaki et al. identified two Epac (exchange protein directly activated by cAMP) proteins, both Rap1-activating, cAMP-binding guanine nucleotide exchange factors (GEFs), that activate Rap1 independent of PKA (de Rooij et al., 1998). Considering the high expression of Epacs in areas of the brain known to participate in memory processing, it is likely that these proteins allow coding of information in a way distinct from PKA. Epac proteins generally serve as Ras-activating GEFs, and their effects on Ras and Rap-1 appear to be dependent on both the upstream stimuli presented to the cell and the localization of Ras GTPase-activating protein (GAP) proteins within the cell. Because these exchange proteins have been recently discovered, their role in memory formation and synaptic plasticity remains to be established. Epac proteins have been reported to regulate neurotransmitter release at the crayfish neuromuscular junction, a process that is completely independent of PKA activity (Zhong and Zucker, 2005). New technological advancements in studying Epac proteins are being developed that should allow a delineation of cAMP roles distinct from PKA action.

One of the central targets of cAMP that affects presynaptic plasticity is the family of cyclic nucleotide-gated ion channels (CNGs). CNG channels are nonselective cation channels that bind both cGMP and cAMP. These channels are perhaps most widely studied in the context of photoreceptor cells and olfactory sensory neurons where cGMP and cAMP activate these channels by direct binding. The role of cAMP in activating CNG channels has also been well studied at the crayfish neuromuscular junction, where presynaptic facilitation has a temporal dependence on cAMP modulation of these channels. cAMP activation of CNG channels tags the synapse in such a way that an additional stimulus provides an enhancement of neurotransmission independent of CNG activation. It is unclear exactly how this synaptic tagging occurs, but cAMP activation of CNG channels and Epac proteins appears to increase the number of vesicles ready for release. Epac interaction with the vesicle protein, Rab3a, may be a plausible explanation for this phenomena, but it is unclear why this process requires activation of CNG channels (Zhong and Zucker, 2005).

4.21.3.2.1 Synaptic and Cytoplasmic cAMP Signaling Cascades

Impingement of cAMP on the MAPK pathway has been studied in a variety of cells with cAMP, sometimes activating and sometimes depressing MAPK activation (for review, see Burgering and Bos, 1995). Here we focus on the effects of cAMP signaling in various pathways in the brain, particularly during memory processing. The contribution of MAPK proteins in memory formation is addressed in much greater depth in Chapter 4.25. It is necessary, however, to discuss the Ras/Raf/MAPK pathway here in order to underscore the importance of cAMP-mediated activation of this pathway during memory processing.

Figure 5 illustrates the key interactions important for coupling of cAMP increases to activation of the Ras/MEK/MAPK pathway.

The Raf/MEK/MAPK pathway is critical for the consolidation and reconsolidation of many forms of memory, and inhibition of this pathway interferes with both LTP and long-term memory formation. The small G protein, Ras, is localized by Ras exchange protein, Sos, and the adapter protein, Grb2. Once activated (often by receptor tyrosine kinases) it binds to Raf-1, which mediates its localization and phosphorylation by PKC or other kinases (Kolch et al., 1993). Raf-1 activates the dual-specificity kinase MEK1, which in turn phosphorylates the ERK/MAPK serine/threonine kinases. An alternative mechanism for MAPK pathway activation is through Epac proteins. Rap1, also a small G protein, is activated by Epac proteins and can subsequently activate MEK1/2 through B-raf proteins. Activation of the Raf/MEK/MAPK pathway results in transcriptional and translational activation required for the consolidation of long-term memory. Specifically, inhibition of MAPK kinases in the lateral amygdala will block consolidation of cued fear conditioning while allowing acquisition of the task (Schafe et al., 2000). Activation of the MAPK pathway is also critical in the amygdala for reconsolidation of auditory fear memory (Duvarci et al., 2005). Retrieval of the auditory conditioning results in a molecular cascade of events in the amygdala that includes activation of MAPK during the reconsolidation period. Reconsolidation of the original auditory fear memory is blocked by amygdalar infusion of MAPK inhibitors. Although activation of the Raf/MEK/MAPK

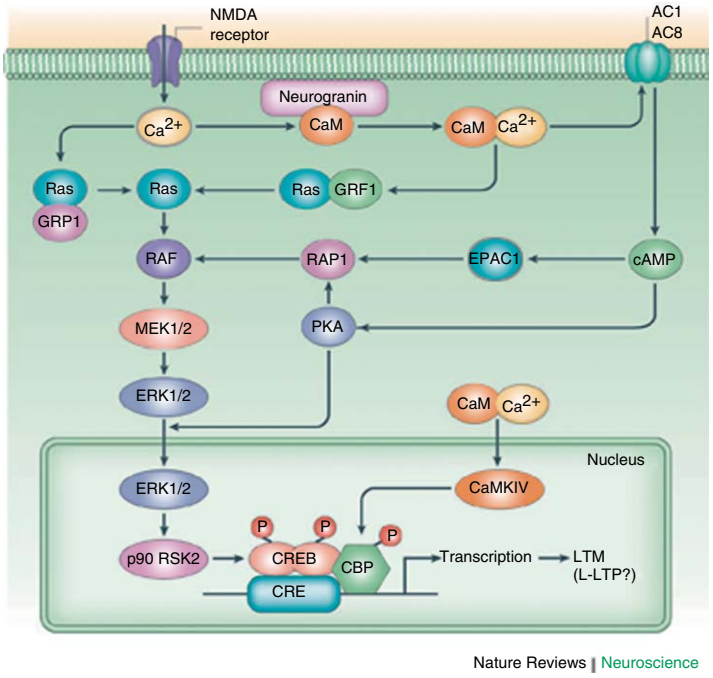


Figure 5 Activation of the ERK/endoplasmic reticulum kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway of calcium and cyclic adenosine monophosphate (cAMP). The postsynaptically expressed protein, neurogranin, controls the level of free calmodulin available for adenylyl cyclase activation. cAMP mediates activation of the MAPK pathway through EPAC proteins, or through protein kinase A (PKA), which can mediate the translocation of ERK/MAPK from the cytosol to the nucleus. Reproduced with permission from Xia Z and Storm DR (2005) The role of calmodulin as a signal integrator for synaptic plasticity. *Nat. Rev. Neurosci.* 6: 267–276.

pathway is critical for auditory fear memories, it is also a requirement for the consolidation of contextual fear memories; training an animal for contextual fear results in an increase in activation of MAPK. Inhibition of MAPK kinases (MEK1/2) with PD98059, UO126, or SL327 inhibits consolidation of contextual fear memories (Atkins et al., 1998).

Phosphatases also play a critical role in cAMP signaling. Two phosphatases that participate in cAMP signaling are the protein tyrosine phosphatase STEP and calcineurin. cAMP impinges on the Raf/MEK/MAPK pathway via the aforementioned Epac proteins, or it can indirectly facilitate persistent MAPK activation by PKA-mediated regulation of STEP (Saxena et al., 1999). Increases in cAMP result in a direct phosphorylation of STEP by PKA, a phosphorylation event that increases MAPK signaling because it interferes with STEP binding to its substrates, such as MAPK (Braithwaite et al., 2006). Whereas STEP is phosphorylated and inactivated by elevations in cAMP, the phosphatase calcineurin functions upstream of cAMP and, through a second mechanism, probably contributes to increases in MAPK activity. It is thought that this phosphatase is responsible for the residual calcium-sensitive cyclase activity in AC1 and AC8 DKO animals. This residual activity can be inhibited by blockade of NMDA receptors; however, general inhibitors of protein serine/threonine kinases do not inhibit depolarization-induced calcineurin activity in hippocampal neurons depleted of AC1 and AC8 (Chan et al., 2005). Pharmacological inhibition of calcineurin indicates that the residual calcium sensitivity in AC1/AC8 double-knockout animals is dependent on this phosphatase. This dependency could arise by a couple of mechanisms: calcineurin might directly dephosphorylate adenylyl cyclases, relieving inhibition of activity, or calcineurin might activate protein phosphatase 1 (PP1) by dephosphorylating inhibitor 1 (I-1), the net result being the dephosphorylation of adenylyl cyclases by PP1.

Calcineurin's participation in cAMP and calcium signaling are not so simple, however. For example, although calcineurin can activate cAMP production through one or more adenylate cyclases, it can reverse PKA phosphorylation of STEP, enabling it to inactivate MAPK and its other targets such as Fyn kinase and the NR2B subunit of NMDA receptors.

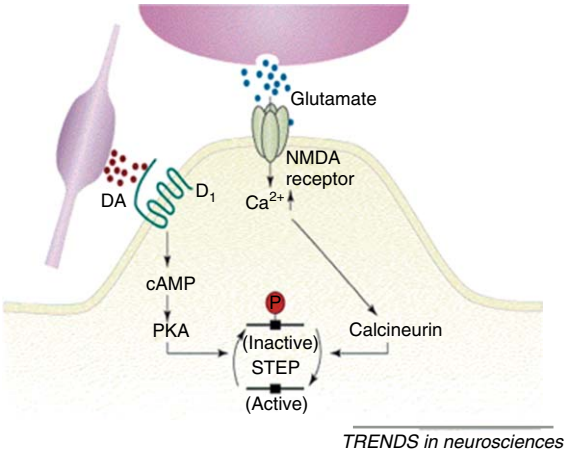


Figure 6 Activity of the phosphatase STEP is controlled by the phosphatase calcineurin. Intracellular increases in cAMP by dopamine (DA) binding to its receptor, D1, induces the phosphorylation of STEP by PKA. Inactive, phosphorylated STEP is incapable of binding to its substrates such as ERK/MAPK. Calcium-activated calcineurin can reverse this phosphorylation, allowing the subsequent activation of STEP and inhibition of MAPK signaling. Reproduced with permission from Braithwaite SP, Paul S, Nairn AC, and Lombroso PJ (2006) Synaptic plasticity: One STEP at a time. *Trends Neurosci.* 29: 452–458.

Therefore, although calcineurin can increase intracellular cAMP levels, it can also serve as a brake for MAPK pathway activation by negatively regulating STEP (see **Figure 6**). Studies are currently in progress looking at the ways in which STEP and calcineurin interact after memory-specific stimuli. One thing is clear, however: cAMP increases are required for activation of the Raf/MEK/MAPK pathway during memory consolidation. Neurons temporally orchestrate the activities of cAMP effectors in such a way that permits the transfer of memory stimuli into long-lasting synaptic configurations that serve as the basis of information storing.

4.21.3.2.2 Functions of cAMP in the nucleus

One of the central nuclear targets (and, perhaps, one of the more thoroughly studied transcription factors) affected by intracellular increases in cAMP is CREB and its family members, cAMP response element modulator (CREM) and activating transcription factor 1 (ATF1). These proteins contain DNA-binding domains in the C-terminal end of the protein that specify their binding to an 8-base pair palindromic region of the DNA, TGACGTCA (Lonze and Ginty, 2002). CREB and CREM both contain a domain known as the KID domain, which contains the serine-133 phosphorylation site that induces binding to the transcriptional coactivator, CREB-binding protein (CBP) (Chrivia et al., 1993). Disruptions in CREB binding to CBP prevent CREB-mediated transcription, and mice expressing CBP that cannot bind to CREB show impairments in long-term memory and LTP (see **Table 2**). This is not surprising, as training induces the transcription of a large number of CRE-regulated genes. Among these are immediate early genes c-fos, zif268, C/EBPbeta, and BDNF. Many of these CREB targets have subsequently been demonstrated to be critical for the consolidation or reconsolidation processes (e.g., see **Table 2** for the effects of genetic disruption of zif268). A later chapter (Chapter 4.27) focuses on transcriptional regulation in plasticity and reviews CREB-mediated gene transcription in detail. A useful tool for analysis of CREB-mediated gene expression during contextual learning is the transgenic CRE-beta-galactosidase mouse, a mouse engineered to express beta-galactosidase under the control of a promoter containing six CRE consensus sites (Impey et al., 1996). Training these animals for hippocampal-dependent memory tasks produces large increases in hippocampal beta-galactosidase gene expression. Contextual memory and passive avoidance memory both stimulate increases in CRE-mediated gene expression in the hippocampus, particularly in area CA1 (Impey et al., 1998). Following auditory fear conditioning, increases in CREB-induced transcription are also observed in the basolateral amygdala (a region that is well established to be required for auditory fear conditioning) but not the hippocampus, indicating that CREB is activated in a stimulus-specific way.

Although cAMP induces the transcription of some genes, cAMP can actually inhibit gene expression in certain cases. For example, the promoter of AC1 has been shown to be inhibited by cAMP, a property that probably explains why AC1 mRNA oscillates in pinealocytes of the pineal gland (Chan et al., 2001). The oscillation of AC1 mRNA and protein in the pineal is important, as it appears to be critical for the activation of enzymes important in the oscillatory release of melatonin.

4.21.3.3 cAMP in Memory Processing (Introduction)

The dependence of memory processing on cAMP signaling extends from *Aplysia* to mammalian systems. Although other chapters focus in detail on the role of

Table 2 Mouse genetic approaches to studying the role of cyclic adenosine monophosphate signaling in memory

Gene modified	*Alteration*	*Affected area of expression*	*Effect on plasticity*	*Effect on memory*
AC1[a]	Knockout	Global	Decreased cerebellar LTP	Deficits in rotarod performance and Morris water maze memory
AC1+[b]	Transgenic, overexpressed	Forebrain	Increased Schaffer collateral LTP, 100-Hz, two-train LTP	Increased recognition memory
AC8[c,18]	Knockout	Global	No LFS-induced LTD	No observable deficits
AC1/AC8[c]	Double knockout	Global	L-LTP deficit, 100 Hz, 4-train LTP	Deficits in passive avoidance and contextual fear memories
Giα1[d]	Knockout	Global	Lower threshold for L-LTP, single 100-Hz stimulus	Impaired passive avoidance, contextual fear, and object recognition memory
PAC1[e,p]	Knockout[e,p] Transgenic[p]	Global or forebrain	Impaired mossy fiber LTPP	Impaired contextual fear memory or context memory persistance but unimpaired in Morris water maze and cued fear
α/ΔCREB[f]	Knockout	Global	Impaired Schaffer collateral LTP, no deficits in STP	Impaired spatial and contextual fear memory but gene dosage and genetic background contribute[m] also; see[n]
αCREB^{1R}[g]	Transgenic, dominant negative	Forebrain	None reported	Deficits in conditioned taste aversion
CBP $^{+/-}$ (RTS model)[h]	Knockout	Global	L-LTP deficit, 100-Hz, 4-train LTP	Deficits in object recognition and fear memory
Zif268$^{-/-,+/-}$[i]	Knockout Heterozygote	Global	L-LTP deficits in dentate gyrus *in vivo*	Deficits in conditioned taste aversion, olfactory discrimination, and spatial memory
R(AB)[j]	Transgenic, dominant negative PKA regulatory subunit	Forebrain	L-LTP deficit in Schaffer collateral-CA1	Spatial and contextual fear memory deficits
PKA (catalytic subunit C1β)[k]	Knockout	Global	Deficits in Schaffer collateral L-LTP	No observable deficits
PKA (regulatory subunit R1β)[k]	Knockout	Global	LTD deficits at Schaffer collateral-CA1, impaired LTP, LTD, and PPF in layers II/III of visual cortex[17]	No observable memory deficits
VP16-CREB[l]	Transgenic, expression of constitutively active CREB	Forebrain	Enhanced Schaffer collateral L-LTP	No behavioral deficits reported

PKA, protein kinase A: LTP, long-term potentiation; LTD, long-term depression.
[a](Storm et al., 1998; Wu et al., 1995); [b](Wang et al., 2004); [c](Wong et al., 1999); [d](Pineda et al., 2004); [e](Sauvage et al., 2000); [f](Bourtchuladze et al., 1994); [g](Josselyn et al., 2004); [h](Bourtchouladze et al., 2003); [i](Jones et al., 2001); [j](Abel et al., 1997); [k](Huang et al., 1995); [l](Barco et al., 2002); [m](Gass et al., 1998); [n](Balschun et al., 2003); [o](Hensch et al., 1998); [p](Otto et al., 2001).

cAMP in synaptic plasticity in specific organisms (*See* Chapters 4.02, 4.03, 4.04, 4.05, 4.06, 4.07, 4.08, 4.09, 4.10), it is worth noting the universality of cAMP signaling in memory. *Drosophila* screens have revealed mutations in cAMP signaling molecules that affect memory (Livingstone et al., 1984). The *Drosophila rutabaga* mutation, for example, has been confirmed to be an X-linked recessive point mutation in an calcium-sensitive

adenylyl cyclase that results in reduced Ca^{2+}/CaM cyclase activity (Levin et al., 1992). Also discovered through *Drosophila* screens is the *dunce* learning mutant (Byers et al., 1981), *dunce* being a mutation that results in loss of phosphodiesterase activity. Similar to the *rutabaga* mutation, the *dunce* mutation results in a net increase in cAMP. Kandel and colleagues pioneered much of the work demonstrating the central role of cAMP signaling in plasticity in the marine snail, *Aplysia californica*. The *Aplysia* sensory to motor synapse has been a central tool in our understanding of cAMP in both pre- and postsynaptic events. In this system, serotonin-induced increases in cAMP lead to CREB-mediated gene expression, a pathway that is necessary for long-term facilitation (Otto et al., 2001) (*See also* Chapters 4.02, 4.03 and 4.10).

4.21.3.3.1 Mammalian genetic models of cAMP in memory processing

In efforts to determine to what extent the cAMP signaling pathways contribute to learning and memory, transgenic and knockout technology has been used to disrupt cAMP signaling. **Table 2** lists some of the animals generated with specific mutations or deletions in cAMP signaling molecules in order to address their function in LTP and memory processing. The neurospecific expression of AC1 makes it an especially appealing drug target for increasing cAMP, specifically in the brain. AC1 knockout animals were created to determine to what extent this enzyme contributes to various forms of LTP or memory formation (Wu et al., 1995). The absence of AC1 results in a 50% reduction in calcium-sensitive adenylyl cyclase activity in both the hippocampus and the cerebellum (Wong et al., 1999). Although spatial memory is impaired in AC1 knockouts (Wu et al., 1995), memory for contextual and passive avoidance training is normal (Wong, 1999). AC8 knockouts also show normal memory for contextual and passive avoidance training. However, AC1/AC8 double knockouts (DKO) exhibit severe deficiencies in spatial, passive avoidance, and contextual long-term memory. Although these animals exhibit impairments in long-term memory, they learn normally and exhibit short-term memory lasting 5–10 min. Interestingly, these memory defects are completely reversed when forskolin, a general activator of adenylyl cyclases, is administered to the hippocampus of DKO mice right before training. Because the memory deficits in DKO mice can be reversed by a general increase in cAMP across the hippocampus, memory deficits exhibited by the mutant mice are attributable to decreased cAMP signaling and not to developmental defects in the CNS. DKO mice also exhibit no long-lasting LTP (L-LTP), whereas the AC1 and AC8 single knockouts do. These data indicate that calcium-stimulated adenylyl cyclases are required for two transcriptionally dependent processes, L-LTP and LTM. The cAMP signal required for these long-lasting processes can be generated by AC1 or AC8. Recently, it was discovered that DKO mice show no training-induced increase in ERK/MAPK activity or the CREB kinase, MSK1, indicating that Ca^{2+}-stimulated adenylyl cyclase activity is required for increases in MAP kinase activity and CREB phosphorylation during memory consolidation.

The contribution of AC1 has also been studied in the context of the anterior cingulated cortex (ACC), a region well known for its contribution to remote fear memory maintenance (Frankland et al., 2004). Unlike AC8, which only contributes to forskolin-induced potentiation, LTP in the ACC requires AC1 activity (Liauw et al., 2005).

To determine whether genetic increases in AC1 activity can improve memory formation, a transgenic mouse strain that overexpresses AC1 in the forebrain ($AC1^{+}$) was used to assess the effect on plasticity and memory. An αCAMKII promoter-driven overexpression of AC1 in the forebrain of mice results in an approximately twofold increase in hippocampal Ca^{2+}-stimulated adenylyl cyclase activity. These mice show an enhancement in novel object memory and also display an enhancement in L-LTP. A 100-Hz, 1-s LTP protocol, normally sufficient for only E-LTP at hippocampal Schaffer-collateral CA1 synapses in wild-type mice, produces L-LTP in $AC1^{+}$ animals (Wang et al., 2004). Contextual fear memory is normal in these animals; however, contextual memory extinction is severely impaired. The decrease in the rate of contextual memory extinction suggests that increased cAMP may impair extinction and is consistent with the observation that $CB1^{-/-}$ mice also show impaired extinction (Marsicano et al., 2002). CB1 receptors couple to inhibition of adenylyl cyclase through G_i proteins, implying that endocannabinoids may mediate extinction through adenylyl cyclase inhibition.

As already mentioned, one of the primary modes for stimulation of adenylyl cyclase is via Gs protein-coupled receptors. One of these receptors, PAC1, is a receptor for pituitary adenylyl cyclase activating peptide (PACAP). PACAP is a circadian-regulated peptide that increases cAMP in target tissues by binding to PAC1, a heterotrimeric G protein-coupled

receptor. The presence of PAC1 in hippocampal presynaptic mossy fiber terminals suggests that it may play a role in LTP or memory processing that uses these synaptic circuits. PAC1 knockout mice show a mild memory phenotype in one-trial contextual fear conditioning as well as in a contextual discrimination task (Sauvage et al., 2000). PAC1 signaling appears to be critical for memory processing in the hippocampus but not the amygdala. Amygdala-dependent memory is unimpaired in PAC1-deficient mice.

4.21.3.3.2 Pharmacological advancements in cAMP signaling during memory formation

Although transgenic technology has proved vital to understanding the importance of adenylyl cyclase activity in long-term memory formation, there are alternative methods to modulate cAMP levels in brain. For example, a direct demonstration of the importance of cAMP signaling for long-term memory formation was a study in which CRE decoy oligonucleotides (nucleotides containing CRE consensus sequences) were directly infused into the CA1 region of the hippocampus after training for contextual fear. These decoys interfered with CRE-mediated gene transcription and memory consolidation, whereas the infusion of scrambled oligonucleotides that did not compete with CREB binding had no effect on behavior (Athos et al., 2002).

Other means of pharmacological interference with cAMP signaling underscore the necessity of cAMP signaling for long term memory formation. For example, the inhibition of PKA by Rp-cAMP (a diastereoisomer of cAMP) infusions into the amygdala accelerates extinction for taste aversion (Koh and Bernstein, 2003). It is worthwhile noting that PKA inhibition, CB1 receptor deficiency, and AC1 overexpression studies all indicate that cAMP may be a molecular restraint on extinction learning.

Forskolin has provided a powerful pharmacological agent by which to stimulate adenylyl cyclases enzymes *in vitro* and *in vivo*. This diterpene activates all of the known adenylyl cyclases except AC9. Forskolin-activated adenylyl cyclase activity can rescue memory deficits in the AC1/AC8 DKO animal. Because PDEs depress cAMP signaling, another way to increase cAMP signaling is through inhibition of PDEs. Phosphodiesterases are strong candidates for memory-enhancing pharmacological targets as interfering with specific PDE proteins can rescue drug-induced memory deficits. For example, the PDE4 antagonist, rolipram, has been used to reverse UO126-induced deficits in radial arm maze retrieval (Zhang et al., 2004). Furthermore, rolipram can also rescue memory deficits in the heterozygous CREB-binding protein (CBP) mice, a mouse with cognitive impairments that have often been compared with those in patients with Rubinstein-Taybi syndrome (Bourtchouladze et al., 2003).

4.21.4 Concluding Remarks

Although it was not possible to describe all of the experiments implicating crosstalk between cAMP and Ca^{2+} in memory formation in this chapter, the examples discussed provide ample evidence for the importance of these second messengers in memory. Crosstalk between cAMP and Ca^{2+} signaling defines the extent to which Hebbian plasticity can occur and, thereby, regulates memory formation.

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4.22 PKMζ, LTP Maintenance, and Long-Term Memory Storage

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4.22.1 Introduction: Protein Kinase Mζ, a Memory Storage Molecule

How long-term memory persists over time is a fundamental question in neuroscience, yet very little is known about the molecular mechanisms that maintain this persistence. In contrast, a vast literature has been written on the molecular mechanisms that trigger learning and the early consolidation of memory. Because this initial phase of memory acquisition is immensely complex, it has been natural to assume that the maintenance of memory storage is similarly complex. In this chapter, however, I would like to present the evidence that a simple molecular mechanism underlies the storage of at least some forms of long-term memory. This core storage mechanism centers around the function of an unusual, persistently active protein kinase C (PKC) isoform, protein kinase Mzeta (PKMζ).

PKMζ's unique mnemonic function was initially characterized through its role in information storage at synapses during long-term potentiation (LTP), the leading candidate for the physiological substrate of memory (Bliss and Collingridge, 1993; Ling et al., 2002; Bliss et al., 2006). LTP can be divided into an early, protein synthesis-independent form and a late, protein synthesis-dependent form (Bliss and Collingridge, 1993). Each of these forms can be further subdivided at the mechanistic level into an induction phase, triggering plasticity, and a maintenance phase, sustaining the plasticity over time (Roberson et al., 1996; Sanes and Lichtman, 1999; Sweatt, 1999). PKMζ's function is specific to one form and phase of LTP: The maintenance phase of

the late, protein synthesis-dependent form of LTP (Serrano et al., 2005).

Late LTP induction is triggered by strong afferent synaptic stimulation that releases glutamate, activating postsynaptic *N*-methyl-D-aspartate receptors (NMDARs) (Bliss and Collingridge, 1993). Postsynaptic Ca^{2+} influx through the receptors then stimulates a complex network of signaling molecules, including many protein kinases, that induces new protein synthesis (Roberson et al., 1996; Sanes and Lichtman, 1999; Sweatt, 1999). Disrupting many of these molecules from the NMDAR to the components of this initial molecular network blocks the formation of LTP. In contrast, inhibiting these molecules after late LTP has been established for approximately 1 h has no effect on the potentiation (Ling et al., 2002). Thus, strictly speaking, these molecules are important for the induction, but not for the maintenance of late LTP.

Because LTP induction initiates the new synthesis of many gene products (Park et al., 2006), one might think that many of these newly expressed molecules would have been shown to be required for maintaining the persistence of LTP, perhaps through the formation of new synapses, and that blocking the effects of these proteins could reverse LTP. But this is not the case. Although it is only one of many proteins synthesized during LTP, PKMζ is the only molecule found to be both necessary and sufficient for the persistence of synaptic potentiation, i.e., introducing PKMζ into neurons causes synaptic potentiation without tetanization, and inhibiting PKMζ reverses established late LTP (Ling et al., 2002). Thus, whereas LTP induction is exceedingly complex, involving a network of dozens, if not hundreds of molecules, LTP maintenance appears to be mediated by a relatively simple molecular mechanism involving the persistent action of PKMζ.

Because the kinase activity of PKMζ is readily inhibited, its role in maintaining the persistence of memory is testable (Pastalkova et al., 2006). Analogous to the ability of PKMζ inhibition to reverse late LTP, PKMζ inhibition eliminates the retention of established long-term memory. Several different forms of memory can be erased, without altering the ability later to establish new long-term memories. Memories up to 1 month after training can be eliminated by the inhibitor, indicating that even remote memories are maintained by PKMζ's persistent action.

PKMζ is thus the first known molecule whose function is to maintain experience-dependent modifications of behavior – a memory storage molecule. In this chapter, we will begin by reviewing the experimental path that led to the discovery of PKMζ, focusing on its unique structure that is key to its ability to store information at synapses. Second, we will examine the enzyme's function in LTP maintenance. Third, we will examine the role of PKMζ in memory persistence and its implications for a new, dynamic view of long-term memory storage.

4.22.2 Protein Kinase Mζ, a Persistently Active Protein Kinase C Isoform Synthesized During Long-Term Potentiation

4.22.2.1 The Discovery of Protein Kinase Mζ, a Constitutively Active Atypical Isoform of Protein Kinase C

The biochemical property of PKMζ that allows it to maintain long-term memory is its persistent kinase activity (Sacktor et al., 1993). Most of the approximately 500 protein kinases in a typical mammalian genome are activated only transiently by second messengers and thus transduce information, but not store it. This is because most kinases are autoinhibited in their basal states in cells, often by a pseudosubstrate sequence within the kinase itself. Second messengers, produced in response to ligand–receptor interactions, bind to the kinases and activate them by producing conformational changes that relieve their autoinhibition. However, because second messengers are usually then rapidly eliminated, the kinases quickly return to their inactive basal states, awaiting the next round of extracellular signaling. Thus most kinases are activated by posttranslational modifications and their effects are transient.

PKMζ, in contrast, is activated through *de novo* synthesis and its effects are persistent (Hernandez et al., 2003). These unusual properties of PKMζ are the result of its unique structure as an independent PKC catalytic domain gene product that completely lacks autoinhibition (Sacktor et al., 1993; Kelly et al., 2007), allowing it to be a constitutively active form of PKC.

PKC is a gene family of closely related serine-threonine kinases, each consisting of a single polypeptide (Dekker et al., 1995; Nishizuka, 1995; Newton, 2001). All full-length PKCs consist of an N-terminal regulatory domain and a C-terminal catalytic domain. The regulatory domain of PKC contains both second messenger binding sites and a pseudosubstrate, which

binds to and inhibits the catalytic domain. Second messengers stimulate PKC by binding to the regulatory domain and causing a conformational change that relieves the autoinhibition. Because lipid membrane is an obligatory cofactor for the binding of these second messengers to the PKC regulatory domain, activation of full-length PKCs is associated with translocation from cytosolic to membranous compartments within a cell. The three classes of PKC isoforms are distinguished by the different second messengers that activate the isoforms. Conventional PKCs (cPKCs: α, βI, βII, γ) are activated by Ca^{2+} and/or diacylglycerol (DAG); novel PKCs (nPKCs: δ, ε, η, θ) by DAG, but not Ca^{2+}; and atypical PKCs (aPKCs: ζ, ι/λ) are stimulated not by DAG or Ca^{2+}, but by other lipid second messengers, including arachidonic acid, phosphatidic acid, and ceramide, and by specific proteins that bind to its regulatory domain (Zhou et al., 1994; Hirai and Chida, 2003).

The translocation of PKC to membrane by second messengers is generally short-lived, because increases in lipid second messengers or intracellular Ca^{2+} are usually transient (Nishizuka, 1995). However, Nishizuka, who discovered PKC, in his very first papers on the enzyme, described an *in vitro* mechanism for permanently activating the kinase (Inoue et al., 1977; Takai et al., 1977). He and his colleagues found that proteases, in particular the Ca^{2+}-dependent calpains, could cleave PKC at its hinge, separating the regulatory from the catalytic domain, thus permanently removing the enzyme's autoinhibitory constraint to form a constitutively active kinase (Inoue et al., 1977; Kishimoto et al., 1983). The resulting independent catalytic domain was called PKM (M standing for the Mg^{2+} in Mg^{2+}-adenosine triphosphate (ATP), the sole requirement for the enzyme's activity) (Inoue et al., 1977). Although an attractive mechanism for sustaining long-term cellular responses, particularly as a potential biochemical basis for long-term memory, PKM formation had never been observed under physiological conditions.

The notion of a persistent kinase as a possible mechanism of memory storage came largely from the work of James Schwartz (1933–2006), who pioneered the biochemical analysis of memory in a model system, the mollusk *Aplysia* (Schwartz, 1993; Schwartz and Greenberg, 1987). Following speculation by Francis Crick (Crick, 1984), Schwartz had formulated the idea that protein kinases critical for short-term memory might be transformed into persistently active forms for storing long-term memory. Specifically, Schwartz proposed that certain kinases, which normally depend upon second messengers for activation, could, through extensive or repetitive stimulation during behavioral training, become partially or fully independent of second messenger stimulation, and thus persistently active to maintain memories. In support of this notion, Schwartz found that two kinases in *Aplysia* neurons, the cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA), which, together with Eric Kandel and colleagues had been shown to be critical for short-term memory (Kandel and Schwartz, 1982), and the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), became less dependent on second messengers after stimulation by the facilitory neurotransmitter serotonin. PKA became autonomously active through a protein synthesis-dependent loss of its autoinhibitory regulatory subunit (Greenberg et al., 1987; Bergold et al., 1990); CaMKII was converted into an autonomously active form through posttranslational mechanisms – autophosphorylation and translocation from cytosol to a membranous compartment of neurons (Saitoh and Schwartz, 1985).

Schwartz also examined PKC, and, together with the author, found the kinase was activated by translocation to membrane, its standard mechanism of activation, during short-term facilitation and behavioral sensitization in *Aplysia* (Sacktor et al., 1993). This work was continued by Wayne Sossin, who later showed that the Ca^{2+}-independent, novel *Aplysia* PKC was important specifically for activity-independent short-term facilitation, particularly for facilitation from a previously depressed state, whereas the Ca^{2+}-dependent, conventional PKC was important for activity-dependent short-term facilitation (Zhao et al., 2006). Sossin also showed that novel *Aplysia* PKC could be persistently activated, perhaps through a sustained phosphorylation (Lim and Sossin, 2006), which, he proposed, might be important for an intermediate form of facilitation (Sossin et al., 1994).

Around the time of the initial work of PKC in *Aplysia*, there was also growing interest in PKC in LTP. Malenka, Madison, and Nicoll found that a PKC activator, phorbol esters, increased synaptic transmission (Malenka et al., 1986), and Malinow et al. (1988) and Routtenberg and colleagues (Colley et al., 1990) showed that H7, a PKC catalytic domain inhibitor, both prevented LTP induction and reversed LTP maintenance. Furthermore, Routtenberg had also detected a persistent translocation of PKC during *in vivo* LTP (Akers et al., 1986), and, in 1991, Klann and Sweatt showed that the constitutive activity of

PKC persistently increased in LTP maintenance in hippocampal slices (Klann et al., 1991, 1993). Subsequently, phosphorylation (Klann et al., 1993; Sweatt et al., 1998) and oxidative activation (Klann et al., 1998) were found to contribute to these persistent effects of PKC. The functional role of PKC in maintaining LTP, however, was soon questioned, both because the effects of H7 were found to be relatively nonspecific (Muller et al., 1990), and because other PKC inhibitors that blocked the enzyme's regulatory domain (e.g., sphingosine (Malinow et al., 1988) and calphostin C (Lopez-Molina et al., 1993) and a second inhibitor of the PKC catalytic domain (staurosporine)), while preventing LTP induction, did not reverse established LTP maintenance (Denny et al., 1990; Muller et al., 1992). Thus, interest in persistently active PKC as a mechanism for sustaining LTP began to decline.

However, around the time that these doubts were raised, PKC was found to be a family of kinases, derived from nine separate genes (Dekker et al., 1995; Nishizuka, 1995). Therefore, the apparent discrepancies in the initial inhibitor studies could have been due to isoform-specific effects. (Indeed, both regulatory domain inhibitors and staurosporine (McGlynn et al., 1992; Kochs et al., 1993; Ling et al., 2002) do not effectively inhibit PKMζ.) The possibility that specific isoforms might be important in LTP was explored in detail by our laboratory.

When we began our investigations into PKC in LTP, our initial hypothesis was that some PKC isoforms might be important for induction, and others for maintenance, either through persistent translocation of PKC or formation of a PKM. Because we did not know which isoforms might be important for which phase, we generated antisera specific to all known isoforms of PKC (Sacktor et al., 1993). The antisera were made to epitopes in each isoform's C-terminal. This site was chosen for two reasons: first, the C-terminals were specific for each PKC known at that time, and second, because the PKC catalytic domain is C-terminal, we could detect the formation of the smaller PKM fragment by Western blot.

We found that, although hippocampus expressed nearly all the PKCs, only the single PKCζ isozyme (~72 kDa) was expressed as a PKM form at approximately 55 kDa (Sacktor et al., 1993; Naik et al., 2000). Immunoprecipitation of PKMζ from hippocampal extracts, followed by autophosphorylation, demonstrated the autonomous activity of the kinase (Sacktor et al., 1993).

By comparing PKC levels from tetanized CA1 regions to control CA1 regions from adjacent hippocampal slices that had received only test stimulation, we found that most of the isoforms were activated by translocation to membrane (Sacktor et al., 1993). However, this translocation was observed 15 s after the tetanus and rapidly reversed by 1 min. The only form of PKC to increase at a later time point, 40 min after the tetanization, was PKMζ (Sacktor et al., 1993; Osten et al., 1996b).

The increase in PKMζ correlated well with the persistence of LTP. A time course showed that the increase occurs 10 min after the tetanization and persists for at least 2 h (Osten et al., 1996b). The level of the persistent increase in PKMζ correlated significantly with the level of the persistent increase in synaptic transmission (Osten et al., 1996b). The increase in PKMζ was blocked by NMDAR antagonists (Sacktor et al., 1993; Osten et al., 1996b) and was specific for LTP, because we observed that PKMζ did not change with test stimulation alone, short-term potentiation (Osten et al., 1996b), or supramaximal tetanic stimulation that produced spreading depression (Osten et al., 1996a). Conversely, PKMζ decreased after a 3-Hz stimulation that produced long-term depression (LTD) (Hrabetova and Sacktor, 1996, 2001).

Because the only mechanism known at the time to form PKM was through proteolysis of PKC, we assumed in these early studies that PKMζ was formed from PKCζ by calpains (Sacktor et al., 1993), Ca^{2+}-dependent proteases that become active during LTP (Vanderklish et al., 1995) and could generate PKM *in vitro* (Kishimoto et al., 1983). Curiously, however, while we observed that PKMζ increased during LTP, we did not detect a concomitant decrease in what we believed to be PKCζ, i.e., we did not observe a precursor–product relationship (Osten et al., 1996b). We therefore guessed that there might be a newly synthesized pool of PKCζ that replaces the cleaved PKCζ. To test this hypothesis, we applied protein synthesis inhibitors, anisomycin or cycloheximide, during the LTP to reveal the precursor–product relationship (Osten et al., 1996b). As expected, the inhibitors blocked the persistent increase in synaptic potentiation. Unexpectedly, however, the inhibitors also prevented the persistent increase of PKMζ (Osten et al., 1996b). This experiment was the first indication that the formation of PKMζ required new protein synthesis and, therefore, was generated by a novel, nonproteolytic mechanism.

4.22.2.2 Protein Kinase Mζ Synthesis from an Internal Promoter Within the Protein Kinase Cζ Gene

The formation of PKMζ during LTP requires new protein synthesis because PKMζ in brain is produced not by proteolysis, but by the direct synthesis of the independent catalytic domain of PKCζ from the ζ gene (Hernandez et al., 2003). Because PKC isoforms are defined as distinct PKC gene products (Dekker et al., 1995; Nishizuka, 1995; Newton, 2001), PKMζ is now classified as an atypical PKC isoform (Hernandez et al., 2003).

The ζ gene produces two sets of RNAs from two distinct promoters (Marshall et al., 2000): a full-length PKCζ mRNA (Ono et al., 1989) and a PKMζ mRNA (Ono et al., 1988) (**Figure 1**). The 5′ end of the PKMζ mRNA is a unique sequence not present in the PKCζ mRNA, whereas its 3′ end is identical to that of PKCζ mRNA. The open reading frame (ORF) for the kinase domain of PKMζ mRNA begins in the hinge and extends to the end of the catalytic domain, thus encoding the PKMζ protein (Hernandez et al., 2003). By screening a panel of tissues, we found that both PKMζ mRNA and protein are expressed specifically in brain (except for a low abundant PKMζ mRNA in kidney that does not express detectable levels of protein) (Hernandez et al., 2003). (The only other brain-specific PKC isozymes are PKCγ (Kikkawa et al., 1987) and a form of PKCη (Sublette et al., 1993).) PKMζ is expressed in most, if not all regions of the brain, with twice as much of the protein present in forebrain structures, such as neocortex, striatum, and hippocampus, relative to hindbrain structures, cerebellum, and tegmentum (Naik et al., 2000).

Translational regulation is believed to be the primary mechanism for the initial increase of PKMζ in LTP. The PKMζ mRNA has a long 5′ untranslated region (UTR) and multiple short ORFs prior to

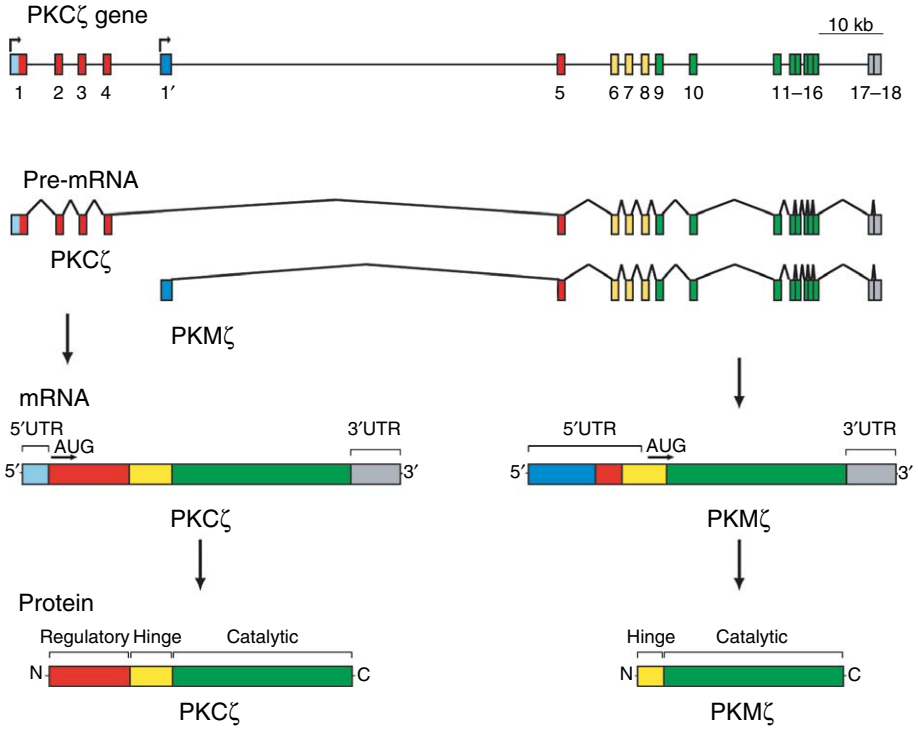


Figure 1 PKMζ mRNA formation from an internal promoter within the PKCζ gene. (Top) The intron–exon structure of the human PKCζ gene shows two exon clusters separated by a large intron: exons 1–4, encoding the PKCζ 5′UTR (light blue) and regulatory domain (red), and exons 5–18, encoding the remaining regulatory domain, hinge (yellow), catalytic domain (green), and 3′UTR (grey). The unique 5′PKMζ mRNA sequence is in a single exon (exon 1′, dark blue) within the large intron. PKCζ mRNA transcription begins at exon 1, resulting in full-length PKCζ (bottom left). Transcription from exon 1′ and splicing to exon 5 generates PKMζ mRNA, translation of which begins in the hinge to generate PKMζ (bottom right). From Hernandez AI, Blace N, Crary JF, et al. (2003) Protein kinase Mζ synthesis from a brain mRNA encoding an independent protein kinase Cζ catalytic domain. Implications for the molecular mechanism of memory. *J. Biol. Chem.* 278: 40305–40316; used with permission.

the kinase ORF, consistent with an inhibitory constraint of the 5′UTR on the translation of the message (Hernandez et al., 2003). This inhibitory mechanism was confirmed by sequential truncation of the 5′UTR, which greatly increased PKMζ translation *in vitro* (Hernandez et al., 2003). Furthermore, metabolic labeling of hippocampal slices with ^{3}H-methionine/cysteine followed by immunoprecipitation of PKMζ showed a large increase in the rate of PKMζ synthesis between 10 and 45 min after tetanization (Hernandez et al., 2003). Since the rate of transcription is 1–2 kb/min (Shilatifard, 1998) and the length of DNA encoding the PKMζ mRNA is approximately 100 kb (Hernandez et al., 2003), new transcription of PKMζ mRNA would begin only as early as roughly 1 h after tetanization.

Because the PKMζ message is dendritic (Muslimov et al., 2004), local translational regulation may provide a mechanism for the synapse specificity that is a characteristic feature of LTP, which would be important for its ability to store memory (Bliss and Collingridge, 1993). *In situ* hybridization with a specific probe to the 5′UTR of PKMζ mRNA showed PKMζ mRNA both in the pyramidal cell body layer and in dendritic layers of the hippocampus (Muslimov et al., 2004). The dendritic location of PKMζ mRNA was confirmed by observations of primary cultures of hippocampal neurons where individual neuritic processes could be clearly identified. Furthermore, injection of truncated versions of the PKMζ mRNA revealed two dendritic localization signals, one in the kinase ORF and a second in the 3′UTR. In addition to local dendritic synthesis, other mechanisms may also contribute to the synapse-specific effects of PKMζ in LTP, including synaptic tagging and capture (Sajikumar et al., 2005) (see the section titled 'Protein kinase Mζ maintains potentiation after synaptic tagging').

Although translational regulation is important for the initial increase of PKMζ in LTP, transcriptional upregulation may also contribute to more persistent increases in PKMζ. Indeed, an *in vivo* LTP study suggested a trend upward in the level of ζ mRNA at 2 and 24 h after tetanization (Thomas et al., 1994). The promoter region of the PKMζ mRNA in the mouse, rat, and human PKCζ genes contains a canonical cAMP response element (CRE), as well as putative sites for nuclear factor-κB (NF-κB) and CCATT/enhancer binding protein (C/EBP) (Hernandez et al., 2003). Each of these transcription factors has been implicated in memory formation (Dash et al., 1990; Alberini et al., 1994; Yin and Tully, 1996; Silva et al., 1998; Merlo et al., 2002; Yeh et al., 2002; Meffert et al., 2003). Interestingly, both the human and chimpanzee PKMζ promoters contain an additional partial CRE duplication immediately adjacent to the canonical mammalian CRE (Hernandez et al., 2003). Whether this duplication enhances CREB binding and aids the transcription of PKMζ during memory formation in primates is speculative, but the strong conservation of putative activity-dependent transcription factor binding regions in the PKMζ mRNA promoter strongly suggests that PKMζ mRNA transcription may be functionally important.

4.22.2.3 Regulation of Protein Kinase Mζ Synthesis by Multiple Protein Kinases and Actin Filaments in Long-Term Potentiation Induction

Following NMDAR activation, several multifunctional protein kinases are critical for inducing LTP, in particular CaMKII, mitogen-activated protein kinase (MAPK), and PKA (Roberson et al., 1996; Sweatt, 1999). Other kinases important for translational regulation, the lipid kinase, phosphatidylinositol 3-kinase (PI3-kinase) (Kelly and Lynch, 2000; Sanna et al., 2002; Opazo et al., 2003), and mammalian target of rapamycin (mTOR) (Tang et al., 2002; Cammalleri et al., 2003; Cracco et al., 2005) are also important for LTP induction. We found that selective inhibitors of each of these kinases block the synthesis of PKMζ in LTP (Kelly et al., 2007). Thus the actions of many disparate signaling molecules converge to regulate PKMζ synthesis.

Another important effector mechanism for LTP is the actin-based cytoskeleton. Although usually thought of as part of structural changes assumed to contribute to the expression of potentiation (Fukazawa et al., 2003; Lang et al., 2004; Okamoto et al., 2004; Huang et al., 2005; Ouyang et al., 2005; Kramar et al., 2006), the function of actin in LTP is unknown. Inhibition of actin filament formation by latrunculin B (which binds to monomeric G-actin and prevents its formation into filaments) blocks LTP induction (Kim and Lisman, 1999; Krucker et al., 2000; Fukazawa et al., 2003). Inhibition of actin filament formation with latrunculin also blocked PKMζ synthesis (Kelly et al., 2006). This is consistent with the notion that actin filaments serve as a scaffolding for assembling proteins important for translation (Stapulionis et al., 1997), particularly elongation factor 1A (Kandl et al., 2002), or perhaps as a local trafficking mechanism for targeting dendritic mRNAs into spines. In contrast to

the agent's effect when applied around the time of tetanization, when latrunculin was applied 1 h after tetanization, the drug did not reverse LTP maintenance (Kelly et al., 2006). Furthermore, latrunculin had no effect on PKMζ-mediated potentiation when placed together with the kinase in a whole-cell recording pipette (Kelly et al., 2006). Thus, actin filament formation regulates the synthesis of PKMζ and therefore is part of the signaling network of induction, rather than maintenance.

Although the induction period of LTP lasts only roughly 30–60 min after a tetanus, actin filament formation can be long-lasting, even weeks in the case of *in vivo* LTP at perforant pathway-dentate gyrus granule cell synapses (Fukazawa et al., 2003). This suggests the possibility that actin filament formation may persistently upregulate local PKMζ synthesis for maintaining increased levels of PKMζ in the face of its turnover.

Interestingly, PKMζ activity itself is also necessary for the *de novo* synthesis of PKMζ in LTP, because both chelerythrine, a PKC inhibitor that appears selective for PKMζ at low doses, and the PKMζ-selective inhibitor ζ-inhibitory peptide (ZIP) block PKMζ synthesis after tetanization and prevent LTP (Kelly et al., 2006). These results suggest the possibility of a positive feedback loop at the level of translation that might maintain the persistent increased PKMζ. Further characterization of this and other potential positive feedback mechanisms will be critical topics for future studies of long-term and remote memory persistence.

4.22.2.4 Phosphatidylinositol-Dependent Protein Kinase 1 Forms a Complex with Protein Kinase Mζ to Maintain Autonomous Kinase Activity in Long-Term Potentiation

Because PKMζ is an independent PKC catalytic domain without an autoinhibitory regulatory domain, it does not require stimulation by second messengers that are necessary for the activation of full-length PKCs, but instead is regulated by activity-dependent protein synthesis (Hernandez et al., 2003). In order for this increase in the amount of PKMζ to translate into an increase in phosphotransferase activity, the kinase must be enzymatically active. Many protein kinases, including PKCs, however, are inactive (or with relatively little activity) immediately after synthesis. This is because their kinase domains are initially in an inactive conformation that inefficiently binds protein substrates. These kinases require an additional kinase, phosphatidylinositol-dependent protein kinase 1 (PDK1), to phosphorylate the activation loop of their catalytic domain to obtain a maximally active conformation (Newton, 2003; Biondi, 2004).

The specific cellular role of PDK1 phosphorylation is different for the various classes of PKC. For cPKCs, PDK1 phosphorylation is constitutive, rendering the kinases competent for subsequent regulation by second messengers (Newton, 2003). In contrast, for full-length aPKCs, like PKCζ, PDK1 phosphorylation is regulated and is an important part of aPKC activation by extracellular signals (Chou et al., 1998; Le Good et al., 1998). Growth factors or hormones, most notably insulin, stimulate PI3-kinase, producing phosphatidylinositol-(3,4,5)-triphosphate (PIP3) that can bind to both the regulatory domain of PKCζ and PDK1. A PDK1-binding site in the catalytic domain of PKCζ can also serve to direct interactions between the two kinases (Balendran et al., 2000). PDK1 then phosphorylates the activation loop of PKCζ, increasing its enzymatic activity.

We found that the function of PDK1 phosphorylation of PKMζ is different from that of the other PKCs and may be essential for PKMζ's role in memory persistence (Kelly et al., 2007). In brain, basal PKMζ directly binds to PDK1, which maximally phosphorylates the PKC on its activation loop. Within 1 h of LTP, the newly synthesized PKMζ is also maximally phosphorylated. Thus, in contrast to full-length PKCζ, in which activation loop phosphorylation is regulated, PKMζ is constitutively phosphorylated, ensuring maximal autonomous activity of the kinase for sustaining responses in neurons.

To summarize this section, PKMζ is a unique, autonomously active isoform of PKC derived from an internal promoter within the PKCζ gene. The PKMζ mRNA is transported to dendrites, and strong afferent tetanization that produces LTP increases the *de novo* synthesis of PKMζ. This initial step is regulated by NMDAR activation, as well as by many signaling molecules and pathways critical for LTP induction, including CaMKII, MAP, PKA, mTOR, and actin filament formation. The newly synthesized PKMζ rapidly binds to PDK1 and is phosphorylated on its activation loop to obtain maximal autonomous activity. In hippocampal slices, the increase in PKMζ protein persists for hours after tetanization and correlates with the degree of synaptic potentiation. The next section examines the function of this persistent increased activity in maintaining LTP.

4.22.3 Protein Kinase Mζ Is Both Necessary and Sufficient for Long-Term Potentiation Maintenance

4.22.3.1 Protein Kinase Mζ Potentiates Synaptic Transmission by Persistently Upregulating Postsynaptic AMPAR Trafficking

Does the persistent kinase activity of PKMζ maintain LTP? Is it necessary and sufficient for the persistent enhancement of synaptic transmission? To examine the question of sufficiency, PKMζ was recombinantly expressed and purified from the baculovirus/*Spodoptera frugiperda* (*Sf*9) cell overexpression system, in which endogenous PDK1 of the *Sf*9 insect cells phosphorylates the activation loop of the overexpressed PKMζ (Ling et al., 2002). We then patched CA1 pyramidal cells in hippocampal slices with low concentrations (1–3 nmol l^{-1}) of the purified kinase in the whole-cell recording solution (**Figure 2(a)**), thus bypassing the synthesis of the kinase that occurs during LTP induction (Ling et al., 2002). Diffusion of PKMζ into cells rapidly doubled the amplitude of the evoked alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor (AMPAR)-mediated excitatory postsynaptic current (EPSC), which then stabilized for the length of the recordings. Bath application of the PKC catalytic domain inhibitor chelerythrine (Herbert et al., 1990) reversed the potentiation to baseline, indicating that PKMζ activity was continually required for the synaptic potentiation. Bath applications of the PKMζ-selective inhibitor ZIP also prevented the potentiation (Serrano et al., 2005) (**Figure 2(a)**).

PKMζ is by far the most potent potentiating substance known. In comparison, postsynaptic perfusion of 100–1000 times more CaMKII produces approximately half the potentiation of AMPAR responses (Lledo et al., 1995; Shirke and Malinow, 1997). PKMζ-mediated increases in EPSCs completely occluded LTP induced by tetanization paired with postsynaptic depolarization, indicating that PKMζ and LTP enhance transmission through the same mechanism (Ling et al., 2002).

We next determined the site of synaptic transmission enhanced by PKMζ (Ling et al., 2006). LTP affects several sites of synaptic transmission, both pre- and postsynaptic (Bliss and Collingridge, 1993; Malenka and Bear, 2004). It is important to note, however, that almost all the studies of LTP expression have focused on its early phase, not its late phase. As we will see in the section titled 'Protein kinase Mζ, maintains late long-term potentiation,' however, PKMζ functions specifically in the late phase. As all possibilities were

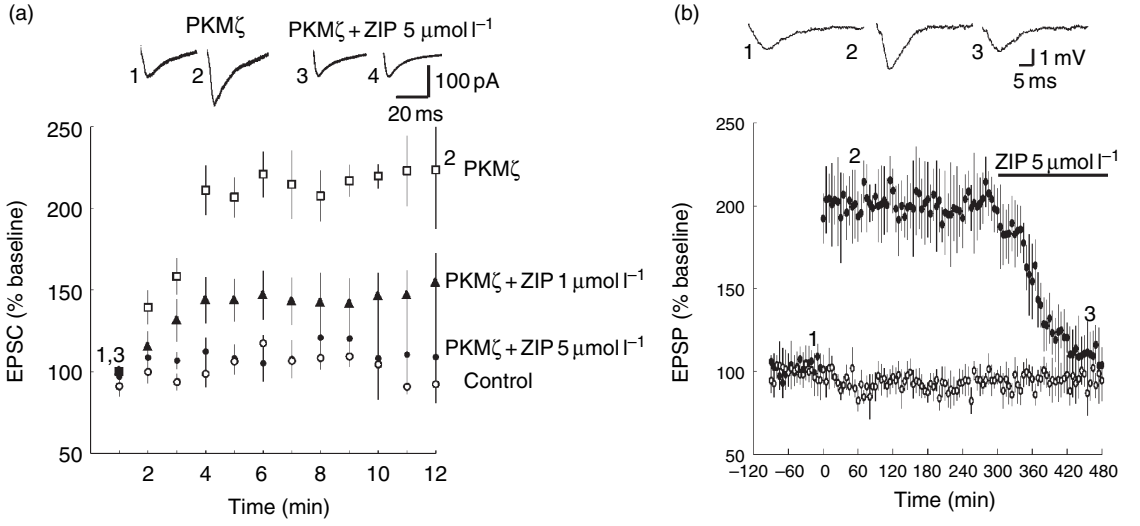


Figure 2 PKMζ is necessary and sufficient for LTP maintenance. (a) Whole-cell perfusion of PKMζ into a CA1 pyramidal cell potentiates AMPAR-mediated synaptic transmission. A total of 1 μmol l^{-1} ZIP applied to the bath partially blocks and 5 μmol l^{-1} ZIP fully blocks the PKMζ-mediated potentiation of AMPAR responses. (b) A total of 5 μmol l^{-1} ZIP applied to the bath 5 h after tetanization reverses LTP maintenance, but does not affect synaptic transmission of a control pathway simultaneously recorded within the hippocampal slice. EPSC, excitatory postsynaptic current; EPSP, excitatory postsynaptic potential. From Serrano P, Yao Y, and Sacktor TC (2005) Persistent phosphorylation by protein kinase Mζ maintains late-phase long-term potentiation. *J. Neurosci.* 25: 1979–1984; used with permission.

open, we examined multiple potential sites for the action of PKMζ on AMPAR-mediated synaptic transmission (Ling et al., 2006). Analysis of the coefficient of variance of evoked responses recorded from CA1 pyramidal cells before and after PKMζ-mediated potentiation showed no evidence of retrograde presynaptic enhancement. Consistent with this result, postsynaptic PKMζ perfusion doubled the amplitude of AMPAR-mediated, miniature excitatory postsynaptic currents (mEPSCs), without affecting their frequency, confirming that PKMζ enhanced synaptic transmission at a postsynaptic site. These results also indicate that PKMζ enhances synaptic transmission without unsilencing NMDAR-only, silent synapses, which would also have led to changes in the coefficient of variance of evoked responses and an increase in mEPSC frequency. The kinetics of the mEPSCs during PKMζ-mediated potentiation, dendritic filtering, and input resistance of the cell also did not change with PKMζ-mediated synaptic enhancement.

This left two possible sites for PKMζ-mediated potentiation of AMPAR responses: channel unit conductance and the number of active channels (defined as the product of the number of physical channels and their open probability). To distinguish between these two sites of action, we used a statistical technique: peaked-scale, nonstationary fluctuation analysis (Traynelis et al., 1993) (NSFA), also referred to as noise analysis, to examine the effect of PKMζ on mEPSCs. NSFA showed that PKMζ did not affect unit conductance, but doubled the number of active AMPAR channels. The doubling was rapidly reversed by applying chelerythrine to PKMζ-potentiated mEPSCs (Ling et al., 2006).

These results indicated that PKMζ enhances synaptic transmission exclusively by increasing the number of functional postsynaptic AMPARs. As mentioned, however, peaked-scale NSFA does not distinguish between an increase in the number of physical channels and an increase in open probability. The open probability of AMPAR channels on CA1 pyramidal cells, however, has been suggested to be already high in its basal state (~0.9) and not to increase with LTP (Andrasfalvy and Magee, 2004), suggesting that a change in the number of receptors was the more likely possibility. Therefore, we explored further the mechanisms of AMPAR trafficking that PKMζ might regulate to increase the numbers of postsynaptic receptors.

AMPARs rapidly move into and out of the synapse through several distinct pathways. One of these pathways involves the trafficking protein *N*-ethylmaleimide-sensitive factor (NSF) that interacts directly with the AMPAR subunit glutamate receptor 2 (GluR2) in order to maintain the receptors at postsynaptic sites (Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998). Inhibiting NSF–GluR2 interactions by a peptide, called pep2m (Nishimune et al., 1998), that mimics the NSF binding site on GluR2 and prevents their interaction, blocked PKMζ-mediated potentiation of AMPARs (Yao and Sacktor, 2006). Furthermore, bath applications of a cell-permeable myristoylated version of the peptide both blocked PKMζ-mediated potentiation and reversed the late phase of LTP (as observed for the PKMζ kinase inhibitor ZIP; see the section titled 'Protein kinase Mζ maintains late long-term potentiation'). In contrast, inhibiting a second AMPAR trafficking pathway that involves vesicle-associated membrane protein (VAMP)/synaptobrevin-mediated, constitutive exocytosis of receptors from internal stores to the plasma membrane (Luscher et al., 1999) had no effect on PKMζ-potentiation of AMPARs. The NSF/GluR2-dependent pathway may involve lateral movement of plasma membrane receptors into the synapse (Gardner et al., 2005). Consistent with this mechanism in LTP, the AMPAR subunits GluR2 and 3 were found to increase in synaptosomes through the action of PKMζ and NSF/GluR2-dependent trafficking, whereas the total level of surface GluR2 (and its total level overall) did not change (Kelly and Sacktor, 2006). Thus PKMζ enhances synaptic transmission through a specific molecular mechanism – persistently increasing NSF/GluR2-mediated trafficking of AMPARs from extrasynaptic to postsynaptic sites.

4.22.3.2 Atypical Protein Kinase M Increases Synaptic Size

Although persistent enhancement of AMPAR trafficking to the synapse is the mechanism of synaptic potentiation by PKMζ, it may not be the only PKMζ-mediated mechanism important for the maintenance of LTP and long-term memory. One indication that PKMζ also affects the structure of synapses comes from the study of the *Drosophila* atypical PKM. Unlike vertebrates that have two aPKC genes, PKCζ and PKCι/λ, *Drosophila* has a single gene, DaPKC (Drier et al., 2002). DaPKC also comes in a PKM form, which, as we will see in the section titled 'Protein kinase Mζ enhances the persistence of associative memory in *Drosophila*,' has a critical role in persistence of memory in the fly (Drier et al., 2002).

A well-characterized model system for synapse development in *Drosophila* is the neuromuscular junction, a glutamatergic synapse for which there are techniques for overexpression of genes at either the pre- or postsynaptic side (Ruiz-Canada et al., 2004). On the presynaptic side, overexpression of DaPKC regulates the stability of microtubules by promoting their association with the microtubule-associated protein 1B (MAP1B)-related protein, Futsch (Ruiz-Canada et al., 2004). On the postsynaptic side, which corresponds to the site of the increase of PKMζ in LTP, overexpression of DaPKM increases the size of the synapse by regulating the synaptic cytoskeleton through control of the extent of actin-rich and microtubule-rich areas (Ruiz-Canada et al., 2004). Interestingly, both the post- and presynaptic sides appeared to increase in size after postsynaptic overexpression, indicating a retrograde effect across the synapse. Conversely, postsynaptic overexpression of a dominant negative form of DaPKM decreased postsynaptic as well as presynaptic size.

Curiously, overexpression of postsynaptic DaPKM decreased the density of glutamate receptors and attenuated synaptic transmission at the neuromuscular junction, opposite to the effect of postsynaptically perfused PKMζ at hippocampal synapses. One possible explanation for this difference is that the overexpression of the kinase in the fly was relatively slow and in a developmental context, whereas the perfusion of PKMζ was rapid into a mature synapse. Indeed, rapid overexpression of PKMζ in a mature fly had no effect on motor responses to shock (Drier et al., 2002). Another interesting possibility is that the NSF/GluR2-mediated trafficking mechanism for driving AMPARs into a hippocampal synapse is absent in the fly neuromuscular junction.

Recent work suggests that the size of hippocampal synapses in culture may also be increased by overexpression of PKMζ and be decreased through inhibition of endogenous PKMζ (Sondhi et al., 2006). This regulation appears to be mediated through actions on the state of postsynaptic density 95 (PSD-95) aggregation.

Thus there may be two arms to PKMζ's effects at synapses: an AMPAR trafficking pathway that increases synaptic strength and a structural modification pathway that increases the size and, perhaps, the longevity of synapses, which correlates with synaptic size (Holtmaat et al., 2006). In the next section we will examine when these PKMζ actions on synapses are critical for maintaining LTP.

4.22.3.3 Protein Kinase Mζ Maintains Late Long-Term Potentiation

If PKMζ is sufficient for synaptic potentiation, is it necessary for the maintenance of LTP? As mentioned in the introduction, although many inhibitors block LTP induction when applied around the time of tetanization, none had reversed established late-phase LTP prior to our work on PKMζ (Ling et al., 2002). In our initial investigation, we examined LTP maintenance 1 h after tetanization by comparing the effects of applications of two PKMζ inhibitors: chelerythrine, an inhibitor of the catalytic domain of PKC, which selectively inhibits PKMζ at low concentrations relative to conventional/novel PKCs (c/nPKCs) and CaMKII (Ling et al., 2002); and the myristoylated ζ-pseudosubstrate peptide (ZIP), a cell-permeable selective PKMζ inhibitor (Bandyopadhyay et al., 1997; Ling et al., 2002; Braun and Mochly-Rosen, 2003). As a control, we examined staurosporine, a general kinase inhibitor that blocks c/nPKCs, CaMKII, and PKA, but not atypical PKCs such as PKMζ (McGlynn et al., 1992; Kochs et al., 1993; Ling et al., 2002). Consistent with several earlier studies (Denny et al., 1990; Muller et al., 1992), staurosporine, while blocking LTP induction when applied prior to tetanization, had no effect on maintenance when bath-applied 1–5 h after tetanization (Ling et al., 2002). Therefore, CaMKII, c/nPKCs, PKA, and the many other protein kinases that are blocked by staurosporine are not required for LTP maintenance. In contrast, both chelerythrine and ZIP reversed maintenance when applied 1 h after tetanization. The reversal was specific to potentiated synapses because a nontetanized pathway, simultaneously recorded in each experiment, remained stable.

Because the ability to reverse established LTP is very unusual, we explored the effect of PKMζ inhibition by ZIP on LTP maintenance in more detail (Serrano et al., 2005). In general, in order to avoid nonspecific effects of a drug, it is important to use the lowest concentration that effectively blocks the relevant function of the target in the cellular context of interest. We therefore determined the lowest concentration of ZIP that, when applied to the bath of hippocampal slices, would block the ability of PKMζ to enhance synaptic transmission by intracellular perfusion of the kinase. We found that 1 μmol l^{-1} attenuated the enhancement, whereas 5 μmol l^{-1} ZIP completely blocked PKMζ-mediated potentiation of synaptic transmission (**Figure 2(a)**).

We then bath-applied 5 μmol l^{-1} ZIP continuously throughout an LTP experiment, and found that there was a residual potentiation in the presence of the inhibitor that was indistinguishable from the early, protein synthesis-independent phase of LTP (Serrano et al., 2005). Therefore, PKMζ did not contribute to early LTP.

We then examined the ability of 5 μmol l^{-1} ZIP to reverse established LTP when applied 1, 3, and 5 h after tetanization (**Figure 2(b)**) (Serrano et al., 2005). The agent reversed LTP at all time points, but had no effects on control untetanized pathways, simultaneously recorded within each slice. Furthermore, the IC50 of ZIP for reversal of established late LTP at 3 h was nearly identical to its IC50 for the ability to block PKMζ-mediated potentiation of AMPAR responses. These results indicated that the persistent activity of PKMζ is required for maintaining late LTP.

4.22.3.4 Protein Kinase Mζ Maintains Potentiation after Synaptic Tagging

PKMζ is synthesized by strong afferent stimulation of synapses that produces late LTP, but not weak afferent stimulation that produces only transient potentiation (Osten et al., 1996b; Hernandez et al., 2003) and maintains late but not early LTP (Serrano et al., 2005). How does newly synthesized PKMζ affect only specific, activated synapses? One possibility is that the kinase might be involved in the process of synaptic tagging and capture (Frey and Morris, 1997; Sajikumar et al., 2005). Synaptic tagging is the hypothesis, first proposed by Frey and Morris, to explain how newly synthesized plasticity-related proteins (PRPs), which are presumably synthesized away from synapses in the soma or dendrites, can maintain potentiation specifically at recently activated synapses but not at neighboring synapses that had not been activated. They proposed that a tag could be set at activated synapses to which the PRPs might bind or be captured. To test their hypothesis, they showed that a weak tetanization, which normally would produce only early, decremental LTP, could nonetheless result in persistent late LTP, if late LTP had been produced elsewhere in the neuron by an independent synaptic pathway (Frey and Morris, 1997). In their model, the weak tetanization, although not strong enough to stimulate protein synthesis, could still set synaptic tags that could capture the PRPs that had been synthesized by the strongly tetanized input.

Sajikumar and Frey (Leibniz Institute for Neurobiology, Magdeburg, Germany) tested the hypothesis that PKMζ was a PRP that could be captured to maintain potentiation at tagged synapses (Sajikumar et al., 2005). They first showed that ZIP reversed late LTP, as expected, but did not affect the maintenance of the transient early LTP. Then during a tagging experiment, when the early LTP pathway would normally have been converted to late LTP, they found that ZIP reversed potentiation at both the strongly and the weakly tetanized synapses (Sajikumar et al., 2005). These results demonstrated that persistent PKMζ activity maintains potentiation not only at strongly tetanized synapses, but also is critical for the conversion of early into late LTP at tagged synapses.

Sajikumar and Frey had also discovered another type of tagging phenomenon known as cross-tagging, which revealed a surprising relationship between LTP and LTD. They found that strong LTD, just like strong LTP, could allow a subsequent weak tetanus to produce late LTP. Conversely, strong LTP could allow a subsequent weak LTD stimulus to produce a late, long-lasting depression. Is PKMζ's role to produce the persistence of any change in synaptic strength, regardless of its direction, or is it specific to the persistence of potentiation? Sajikumar and Frey first found that ZIP had no effect on reversing LTD maintenance (although it did block LTD induction). They then showed that ZIP could nonetheless reverse the persistent synaptic potentiation produced by cross-tagging after strong LTD. On the other hand, ZIP did not affect the persistent depression produced by cross-tagging after strong LTP, even though the agent reversed the persistent potentiation produced by the strong tetanization. These results demonstrated that PKMζ specifically maintains synaptic potentiation, not depression, and were consistent with our results showing that the postsynaptic perfusion of PKMζ caused potentiation of synapses.

How is PKMζ's role in synaptic tagging commensurate with the possibility that the kinase is produced from a dendritic mRNA during LTP? One notion is that activity-dependent synthesis of PKMζ is local in dendrites, but that tagging is required for finer compartmentalization of the synthesized PKMζ to only activated synapses. Alternatively, the phenomenon of tagging may be caused by metaplastic changes in the efficiency of dendritic local synthesis (Tsokas et al., 2006). Regardless of which is correct, the results of this section showed that the inhibition of PKMζ

rapidly reverses late LTP maintenance. This meant that, for the first time, there was a tool to test the hypothesis that the molecular mechanism of LTP maintenance, the persistent activity of PKMζ, stores information in behaving animals.

4.22.4 Protein Kinase Mζ and Memory Storage

4.22.4.1 Protein Kinase Mζ Enhances the Persistence of Associative Memory in *Drosophila*

PKMζ's role in behavior was first examined by Jerry Yin, Eric Drier, and colleagues at the University of Wisconsin in *Drosophila*, a model system with several advantages for the study of the molecular pathways of learning and memory. One advantage is the ability to rapidly express transgenes through the heat shock promoter, allowing expression of proteins in less than 1 h. A second advantage is that a relatively simple behavior, associative olfactory conditioning (Quinn et al., 1974; Aceves-Pina et al., 1983), has been well characterized, with various stimulation protocols producing different forms of memory: A single training session produces short-term memory lasting a few hours, mass training (i.e., multiple training sessions with short between training intervals) produces memory lasting around a day, and spaced training produces long-term memory lasting several days.

Either DaPKM or mouse PKMζ overexpressed by the heat shock promoter shortly after short-term memory training converted the behavioral response into 24-h memory (Drier et al., 2002). Similarly, overexpression of either transgene could convert a mass-trained memory lasting 1 day into a memory lasting 4 days, the phenotype of long-term memory (Drier et al., 2002). Conversely, overexpression of a dominant negative version of the mouse PKMζ (Lallena et al., 1999; Romanelli et al., 1999; Drier et al., 2002) (which also blocked LTP (Ling et al., 2002)), or feeding flies the PKC inhibitor chelerythrine, prevented the persistence of memory after mass training, but did not affect initial learning (Drier et al., 2002). The ability of mouse PKMζ to substitute for fly DaPKM demonstrates strong evolutionary conservation of PKMζ function.

Surprisingly, the overexpression of PKMζ has to occur in a narrow time window of less than 1 h after the training. Overexpression prior to training or 2 h after had no effect on memory enhancement. This suggests that exogenous PKMζ has to be overexpressed at the time when endogenous DaPKM would have been synthesized in response to synaptic activity, if flies generated DaPKM during memory formation as hippocampal neurons do in LTP. Perhaps just as remarkable, the enhancement of memory by the transgenic expression of PKMζ by the heat shock promoter occurs in all tissues of the fly. Therefore being at the right time appears essential for the ability of PKMζ to prolong memory, but being at the right place is not. As noted by Yin and colleagues (Drier et al., 2002), these results are consistent with the notion of synaptic tagging, in which PKMζ is a newly synthesized PRP that is captured by a synaptic tag, set in the fly by behavioral short-term memory, rather than in hippocampal slices by early LTP.

PKM forms of PKC may also be important in other forms of memory in invertebrates. A constitutively active form of PKC, i.e., PKC activity not requiring regulatory domain activators such as Ca^{2+} or DAG, was observed to increase after long-term olfactory conditioning in the antennal lobes of the honeybee (Grunbaum and Muller, 1998). A transient increase required proteolysis, whereas a persistent increase lasting for days required new protein synthesis, like PKMζ formation in LTP. In *Aplysia*, a PKM form of PKC may be generated by proteolysis, rather than protein synthesis, to mediate an intermediate form of activity-dependent facilitation (Sutton and Carew, 2000).

This sampling of various species of different phyla suggests that PKM might be formed from PKC by either proteolysis or synthesis to mediate memory, but that mammals (at least as assayed by hippocampal LTP) had selected the synthetic pathway. This suggested that the persistent activity of PKMζ might maintain a form of memory in mammals.

4.22.4.2 Protein Kinase Mζ Maintains Long-Term Memories in Multiple Regions of the Brain

In order to translate our findings from hippocampal slices to behavior, we began by focusing on the role of PKMζ in spatial memory that is initially encoded and stored in the hippocampus (Pastalkova et al., 2006). Because the synapses that are thought to encode spatial information are widely distributed in the hippocampus (Moser and Moser, 1998; Kubik and Fenton, 2005), to test the hypothesis that PKMζ maintained this information it was important to inhibit PKMζ widely in the hippocampus. We

therefore examined *in vivo* LTP at perforant path-dentate gyrus synapses, using an array of recording electrodes spaced up to 2 mm away from the injection site in CA3. We determined that 10 nmol ZIP (in a 1-μl bolus) rapidly reversed LTP when injected 22 h after LTP induction. Labeling the ZIP peptide with biotin showed that the agent diffused approximately 3–4 mm within the hippocampus but not to other brain regions.

We then were in a position to examine the role of PKMζ in maintaining hippocampus-dependent spatial memory. We chose active place avoidance (Cimadevilla et al., 2000a,b, 2001; Wesierska et al., 2005), a behavior with several experimental advantages for the initial study of PKMζ function in spatial memory. Hippocampus inactivation by tetrodotoxin blocks both initial learning and short-term memory, as well as long-term memory retention of active place avoidance (Cimadevilla et al., 2000a, 2001). This would allow us to distinguish between an effect of PKMζ inhibition on short-term and long-term retention of the conditioned responses. Learning is rapid (optimal performance observed in roughly 2.5 h of training) and remains hippocampus-dependent for at least 1 month, allowing us also to examine the role of PKMζ in remote spatial memory.

The apparatus for active place avoidance consists of a slowly rotating platform, open to the room environment, within which a nonrotating 60-degree sector is a shock zone (Cimadevilla et al., 2000b). The rotation brings the animal into the shock zone, and the animal rapidly learns to avoid the shock by actively moving to the nonshock areas of the platform. After an initial 10-min exposure to the apparatus without shock, rats were trained in eight 10-min sessions with the shock on, separated by 10-min rest intervals in their home cages.

Twenty-two hours after the last training session, we injected either ZIP or saline into both hippocampi. Two hours later, retention testing on the apparatus without shock (i.e., extinction testing) showed that the saline-injected animals exhibited long-term memory by avoiding entry into the shock zone. In contrast, the ZIP-injected animals failed to avoid the shock zone and actively explored the entire apparatus as if naive. Whereas the ZIP-injected animals did not recall the long-term memory, spending time in the shock zone at the level of chance, immediate reconditioning and then retesting without the shock showed that the animals could nonetheless recall the short-term memory of the conditioned response. These results indicate that ZIP produces retrograde amnesia specifically for long-term memory, not short-term memory of place avoidance. Staurosporine, the nonspecific kinase inhibitor that blocks conventional and novel PKCs, CaMKII, and PKA, and which blocks LTP induction but not maintenance (Ling et al., 2002), had no effect on long-term memory retention at a dose that completely prevented learning when injected prior to training.

A critical question concerning the effect of PKMζ inhibition of memory retention is whether ZIP disrupts information retrieval or information storage. One way to distinguish between these possibilities is to inject ZIP after long-term memory has been established, wait for the drug to be eliminated, and then test whether the memory returns. If the conditioned behavior returns, this would indicate an effect on information retrieval; if the memory were persistently eliminated, this would indicate an effect on storage. We therefore trained animals and then 1 day later injected ZIP without testing. One week later (when the ZIP had been eliminated), the previously ZIP-injected animals had no recall of the long-term memory, whereas the saline-injected animals avoided the shock zone. Then to test whether ZIP caused permanent damage to memory function, we immediately retrained the previously ZIP-injected animals and found that they learned and could remember place avoidance as a new long-term memory. Thus ZIP erases previously acquired memory, but does not affect the ability to subsequently store new long-term memory.

We also examined whether memories older than 1 day were also sustained by PKMζ and found that 1-month-old place avoidance memory could be eliminated by ZIP.

Subsequent to our initial study, other forms of long-term memory have also been shown to be maintained by PKMζ. Spatial accuracy in the water maze and in the appetitively conditioned eight-arm radial maze are strongly affected by intrahippocampal injections of ZIP after training (Serrano et al., 2007). In the latter, the deficit resulted from increases of reference memory errors and not working memory errors, indicating a specific effect on spatial information.

Other areas of the brain in which memories are thought to be stored have also been examined. ZIP injection in the basolateral amygdala complex inhibited memory retention for auditory fear conditioning (Friedman et al., 2007). Injection in the insular neocortex erased memory for conditioned taste preference (Shema et al., 2007) either days or weeks after training, yet ZIP had no anterograde effect on long-term

memory formation when injected immediately prior to training. As in place avoidance, the memory for conditioned taste aversion did not return even 1 month after the agent's injection. Because the neocortex is thought to be the ultimate repository of many long-term memories, these results indicate that the persistent activity of PKMζ is a general brain mechanism for long-term memory storage.

4.22.4.3 Protein Kinase Mζ and Alzheimer's Disease

Because of PKMζ's critical function in memory storage, we examined the role of the kinase in the most common disorder of memory, Alzheimer's disease (AD) (Crary et al., 2006). The characteristic features of the pathology of AD are extracellular amyloid plaques and intracellular neurofibrillary tangles (Braak and Braak, 1991). Using antisera specific to each of the PKC isoforms, we found that only PKMζ and the other atypical PKCι/λ were present in a subpopulation of tangles. Aggregates of AMPARs GluR1 and GluR2 were also associated with the aggregates of PKMζ in perisomatic granules, another neuropathological feature of AD (Aronica et al., 1998; Probst et al., 2001). Several other neurodegenerative diseases also are characterized by tangles, frontotemporal dementia (Pick's disease), corticobasal degeneration, and progressive supernuclear palsy, collectively known as tauopathies. These tangles contain PKCι/λ, but not PKMζ (Shao et al., 2006).

PKMζ has two unique features compared to all other known components of the tangles in AD. Tangles are primarily composed of hyperphosphorylated tau, as well as smaller amounts of other molecules, including ubiquitin and several protein kinases, notably glycogen synthase kinase-3β. Tangles are found widely in the brains of patients with AD, and most of its molecular components are present in the tangles from all of these regions (Papasozomenos, 1989). PKMζ, in contrast, is found in tangles only in the limbic system and medial temporal lobe, i.e., hippocampus, entorhinal cortex, and amygdala. PKMζ is not found in tangles in regions of the neocortex, despite the widespread expression of PKMζ in human neocortex. PKMζ is also not in the tangles present in nonlimbic subcortical regions, including nucleus basalis of Meynert and locus ceruleus. This distribution of aggregated PKMζ only to limbic structures suggests the possibility that high levels of PKMζ may be generated as a compensatory mechanism in some remaining healthy neurons in memory-encoding regions, as synaptic plasticity generally fails in affected neurons because of the burden of amyloid and neurofibrillary degeneration.

The second unusual feature of PKMζ is that it is found only in the tangles of patients with dementia. Some tangles in the limbic areas are a common finding in the elderly without dementia. These tangles had previously been found to be indistinguishable at the molecular level from the tangles in the brains of patients with AD. PKMζ, however, was not present in the tangles of the elderly individuals without known dementia. This suggests that the aggregation of PKMζ may signal the exhaustion of the compensatory increased plasticity, mentioned earlier, thus leading to memory loss, the characteristic symptom of AD.

4.22.5 Conclusions and Future Prospects

The unique structure and function of the PKMζ gene, mRNA, and protein provides a simple molecular mechanism of memory storage through synthesis of a persistently active protein kinase (Sacktor et al., 1993; Hernandez et al., 2003). This mechanism is radically different from the standard notion of memory storage as a passive change in the large-scale structure of synaptic connections between neurons. Although the standard model may yet prove to be important for memory storage in invertebrates and in some forms of memory in the mammalian brain, the persistent activity of PKMζ maintains spatial memory in hippocampus, fear memory in the amygdala, and associative memory in the neocortex.

Thus PKMζ is a fundamental biological molecule for information storage in the brain. How did this mechanism of storage evolve? A clue to its origin may be in the general function of atypical PKC in other cellular contexts. In many different cell types, from *Caenorhabditis elegans* (Izumi et al., 1998; Tabuse et al., 1998; Wu et al., 1998) to *Drosophila* (Wodarz et al., 2000) to mammalian species (Lin et al., 2000; Qiu et al., 2000), atypical PKC is critical for the establishment and maintenance of cell polarity within cells and junctional complexes between cells (Ohno, 2001), including neurons early in development (Shi et al., 2003). At many of these polarized regions of the cell and junctions, the activity of atypical PKC regulates the assembly of various receptors and structural

proteins. Because of PKCζ's role in the regulation of protein trafficking at junctions in general, the kinase may have been easy to adapt to a similar role in the synaptic junctions between neurons. The key development for the formation of long-term memory, the formation of the internal promoter within the PKCζ gene that generates the persistent activity of PKMζ, may have been an evolutionary accident. At synapses, the formation of PKMζ may lead to persistently enhanced transmission, but outside neurons, the constitutive atypical PKC activity of PKMζ would most likely have been developmentally lethal. Therefore, only a promoter capable of neuron-specific regulation might benefit the organism. How the expression of PKMζ then came to be under the control of strong but not weak synaptic activity is a mystery, but as Francis Jacob famously said, "Evolution is a tinkerer" (Jacob, 1977). One may surmise, however, that any animal with even a rudimentary capacity for long-term memory as a mechanism to adapt to its environment would have considerable evolutionary advantage. In this context, the recently evolved, tandem CREB-binding site in the PKMζ promoter of chimpanzees and humans is of particular interest (Hernandez et al., 2003).

Several important avenues are now open for future investigation. First, what are the substrates of PKMζ that mediate its function at synapses? Although both AMPAR trafficking through NSF/GluR2-mediated mechanisms and structural changes by PSD-95 may be downstream of PKMζ, the specific substrates mediating these processes are unknown. Unfortunately, identifying functionally important substrates of any kinase is a hit-or-miss affair, and there is no straightforward path other than informed guesswork.

Second, because the effects of PKMζ are essential for long-term memory maintenance yet are rapidly reversible, memory storage can now be viewed as a much more dynamic process than conceived of in the purely structural growth model, and one that is more amenable to experimental, even therapeutic, manipulation. Injections of ZIP eliminate previously established LTP maintenance but spare short-term synaptic plasticity and basal synaptic transmission. Thus for the first time, the information storage function of a brain region can be distinguished from its information processing function, including its role in transferring information to another brain region. Can other dynamic features of long-term memory, such as reconsolidation, now be explained by the degradation and resynthesis of PKMζ? Can we visualize the engram by monitoring changes in PKMζ after conditioned behavior? Can human disorders thought to be mediated by pathophysiologically enhanced LTP, such as neuropathic pain, posttraumatic stress, even addictive behavior, now be reversed by local injections of ZIP?

Third, what do the basic properties of PKMζ tell us about memory? What is the half-life of PKMζ? Presuming it is shorter than 1 month, a time when memories are still maintained by the persistent activity of the kinase, how then could PKMζ sustain memory beyond the half-life of the PKMζ protein? Are there multiple transcriptional, translational, and posttranslational positive feedback mechanisms that maintain persistently increased PKMζ at specific synapses? Perhaps there will be a network of molecules maintaining PKMζ, just as there is a network of molecules initiating its formation in LTP induction. Whether such a putative maintenance network is large, involving persistent transcriptional or translational upregulation, or small, involving perhaps only a single synaptic substrate of PKMζ with which it interacts, we are only at the beginning of our understanding of PKMζ, LTP maintenance, and memory storage.

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4.23 CaMKII: Mechanisms of a Prototypical Memory Model

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4.23.1 Introduction

Elucidating the molecular basis for complex behaviors like learning and memory is one of the largest challenges currently facing neuroscientists. It is generally accepted that such complex behaviors reflect changes in connectivity in neuronal circuits, which are believed to arise due to the intrinsic ability of individual synapses to adapt their properties in the face of changes in the ongoing activity. Consequently, synaptic plasticity in several brain regions has been studied as a more tractable surrogate for learning and memory, and many molecules implicated in synaptic plasticity have been linked to behavioral consequences.

The rodent hippocampus is the most widely studied model system for synaptic plasticity, particularly the glutamatergic synapses formed by Schaffer collateral projections of CA3 neurons to dendrites of CA1 pyramidal neurons. In general, strong stimulation (e.g., brief high-frequency stimulation) results in long-term potentiation (LTP), whereas more prolonged weaker stimulation paradigms (e.g., low-frequency stimulation) result in long-term depression (LTD). Both LTP and LTD at these synapses require Ca^{+2} influx via postsynaptic *N*-methyl-D-aspartate (NMDA)-type glutamate receptors (NMDARs) and generally are restricted to the same synapses that receive modified input, rather than spilling over to modulate nearby unstimulated synapses. These fundamental properties imply that postsynaptic Ca^{2+} signaling responds in frequency-dependent and spatially restricted manners to effect opposing modifications of synaptic properties. Dendritic spine morphology undoubtedly provides a spatial constraint on postsynaptic Ca^{2+} signals, but compartmentalization of signaling proteins is also likely to be important for precise synaptic modulation.

Central among known Ca^{2+}-sensitive signaling molecules is Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). Overwhelming amounts of biochemical, electrophysiological, and genetic data suggest that CaMKIIα is essential for normal hippocampal-based synaptic plasticity, learning, and memory, and that CaMKIIα autophosphorylation at both Thr^{286} and Thr^{305}/Thr^{306} is critical (see Lisman et al., 2002; Colbran and Brown, 2004; Elgersma et al., 2004). However, the molecular basis of CaMKII actions to regulate excitatory synaptic transmission

during induction or maintenance of hippocampal LTP are less clear. The complexity of these underlying mechanisms is demonstrated by recent findings that autophosphorylation of CaMKIIα at Thr^{305}/Thr^{306} is essential for hippocampal metaplasticity (Zhang et al., 2005) and that CaMKIIα is required for LTD, and not LTP, in the cerebellum (Hansel et al., 2006). Moreover, mutation of Thr^{305} and Thr^{306} to prevent autophosphorylation rescued both behavioral and electrophysiological deficits in a mouse model of Angelman Syndrome (van Woerden et al., 2007), a mental retardation disorder resulting from mutation in the Ube3a ubiquitin ligase. These studies underscore the importance of understanding the molecular basis of CaMKII regulation.

This chapter will discuss the intrinsic regulatory properties of CaMKII that are ideally suited to integrate postsynaptic Ca^{2+} signals and recent studies describing CaMKII interactions with other synaptic proteins, providing insight into the compartmentalization of CaMKII actions in neuronal dendrites. These findings suggest new ways in which CaMKII may fulfill its pleiotropic roles in modulating synaptic transmission.

4.23.2 The Molecular Nature of CaMKII

CaMKII was originally identified as an abundant neuronal protein kinase that is highly enriched in subcellular fractions containing postsynaptic densities (Kennedy et al., 1983; Goldenring et al., 1984; Kelly et al., 1984). Mammalian genomes encode four CaMKII isoforms, and each mRNA is subject to alternative splicing to yield multiple transcripts. Consequently, at least 25 distinct CaMKII proteins may be differentially expressed in cells and during development. In neurons, CaMKIIα and CaMKIIβ predominate. CaMKIIβ is expressed in most brain regions throughout development, but with a changing representation of multiple splice variants (Brocke et al., 1995). In contrast, CaMKIIα is expressed predominantly in the forebrain beginning at about postnatal day 5 in rodents, and expression levels increase in parallel with ongoing postnatal synaptogenesis (Brocke et al., 1995; Bayer et al., 1999). CaMKIIα transcripts, but not those of CaMKIIβ, contain an extended 3′ untranslated region that directs the mRNA to dendritic protein translation sites (Mayford et al., 1996). Indeed, NMDAR activation stimulates the local dendritic translation of CaMKIIα, possibly via a CaMKII-mediated pathway (Atkins et al., 2004, 2005), and dendritic translation of CaMKIIα is essential for the stabilization of synaptic plasticity and memory consolidation (Miller et al., 2002).

Almost all CaMKII isoforms contain highly similar amino-terminal catalytic and regulatory domains, which interact to suppress kinase activity in the basal state. The carboxyl-terminal association domain assembles subunits into the dodecameric holoenzyme structure (see **Figure 1**). Recent studies provide structural insight into the nature of the CaMKII holoenzyme. (Kolodziej et al., 2000; Morris and Torok, 2001; Hoelz et al., 2003; Gaertner et al., 2004; Rosenberg et al., 2005, 2006). Unresolved discrepancies in the interpretation of data from different laboratories may represent different naturally occurring conformations. The holoenzyme structure is essential for normal regulation by autophosphorylation and CaMKII association with other synaptic proteins, as will be described.

4.23.3 Regulation of CaMKII: Formation of a Molecular Memory

Residues 281–302 within the regulatory domain interact with the catalytic domain to interfere with the binding of both ATP and protein substrates (Colbran et al., 1989). Recent x-ray crystal structures of an inactive mutant of *Caenorhabditis elegans* CaMKII suggest that the regulatory domain interacts with the substrate binding lobe of the catalytic domain to displace helix D from the active conformation seen in many other kinases, distorting the nucleotide binding site (Rosenberg et al., 2005). Ca^{2+}/CaM interacts with residues 294–302 of the regulatory domain (Meador et al., 1993), disrupting the autoinhibitory interactions. This presumably allows helix D to relax into an active conformation that allows nucleotide and protein substrate binding (Rosenberg et al., 2005).

Ca^{2+}/CaM randomly binds to and activates individual CaMKII subunits within the holoenzyme. Simultaneous activation of adjacent subunits allows for trans-autophosphorylation at Thr^{286} within the regulatory domain (Hanson et al., 1994; Mukherji and Soderling, 1994). Thr^{286} autophosphorylation slows Ca^{2+}/CaM dissociation by over 100-fold and interferes with the autoinhibitory interaction to generate CaMKII with Ca^{2+}-independent (autonomous) activity (Colbran et al., 1989; Meyer et al., 1992). These dual effects are generally thought to prolong

CaMKII-mediated signaling initiated by transient changes in intracellular Ca^{2+}. Thus, the Thr^{286} autophosphorylated CaMKII serves as a molecular memory of a transient Ca^{2+} signal and must be dephosphorylated to return CaMKII to the basal state (reviewed in Hudmon and Schulman, 2002).

Binding of Ca^{2+}/CaM also blocks autophosphorylation at Thr^{306} and Thr^{305}. In the absence of Ca^{2+}/CaM, inactive CaMKII is slowly autophosphorylated, predominantly at Thr^{306}, blocking Ca^{2+}/CaM binding and preventing CaMKII activation (Colbran, 1993). Prior Thr^{286} autophosphorylation greatly enhances Ca^{2+}/CaM-independent autophosphorylation at Thr^{305} or Thr^{306}; although this dually phosphorylated CaMKII cannot bind Ca^{2+}/CaM, it remains active due to the prior autophosphorylation at Thr^{286} (Hashimoto et al., 1987; Hanson and Schulman, 1992). Thus, CaMKII that is autophosphorylated at Thr^{305} or Thr^{306} is Ca^{2+}/CaM-insensitive, but may exist in inactive or partially active forms depending on the level of Thr^{286} autophosphorylation.

In addition to the well-established effects of autophosphorylation at Thr^{286} and $Thr^{305/306}$ on CaMKII activity, several additional *in vitro* autophosphorylated sites have been identified (Miller et al., 1988; Dosemeci et al., 1994; Patton et al., 1990). Most of these additional sites are not known to regulate CaMKII activity directly, but recent studies found that Thr^{253} autophosphorylation in intact neurons (Migues et al., 2006) may play a role in controlling CaMKII subcellular localization.

Since autophosphorylation profoundly regulates CaMKII, it follows that protein phosphatases (PPs) affect CaMKII signaling. PP1, PP2A, and PP2C can dephosphorylate CaMKII *in vitro* (reviewed in Colbran, 2004a). In forebrain extracts, soluble Thr^{286}-phosphorylated CaMKII is primarily dephosphorylated by PP2A, but Thr^{286}-phosphorylated CaMKII associated with postsynaptic densities (PSDs) is predominantly dephosphorylated by PP1 (Strack et al., 1997). However, PP2C was suggested to play a significant role in CaMKII dephosphorylation in cerebellar granule cells (Fukunaga et al., 1989, 1993). Thus, the identity of specific enzymes responsible for CaMKII dephosphorylation likely depends on the specific cell type and the subcellular localization of CaMKII. Moreover, regulation of the expression and/or activity of these phosphatases during physiological stimulation, in diseased states, or during aging may critically modulate CaMKII activation and downstream signaling.

4.23.4 Integration of Dynamic Ca^{2+} Signals by CaMKII

The *in vitro* regulatory properties of CaMKII are ideally suited to respond to dynamic Ca^{2+} signals according to the amplitude, duration, and frequency of intracellular Ca^{2+} transients. In an idealized *in vitro* model, CaMKII is not significantly autophosphorylated following stimulation with short duration pulses (80 ms) delivered at <3 Hz, but Thr^{286} autophosphorylation acutely increases as the frequency of Ca^{2+} transients increases to 10 Hz (De Koninck and Schulman, 1998). Modification of the duration of the individual Ca^{2+} transients dramatically modifies the frequency-response relationship. Interestingly, the 80-ms Ca^{2+} pulses approximate the duration of postsynaptic Ca^{2+} transients during normal synaptic transmission, and 3–10 Hz is similar to the natural hippocampal theta frequency. Thus, this hypersensitivity to stimulation frequency may provide the fundamental basis for the frequency-dependent activation of CaMKII to induce LTP. More recent work showed that CaMKIIβ is similarly regulated, but that the relationship between stimulation frequency and autophosphorylation is different than for CaMKIIα and is modulated by alternative splicing (Bayer et al., 2002). In part, this may reflect the fact CaMKIIβ has a higher affinity for Ca^{2+}/CaM than does CaMKIIα (Brocke et al., 1999). Thus, it is likely that cells expressing different ratios of CaMKII isoforms and splice variants exhibit different stimulation-response properties in CaMKII signaling.

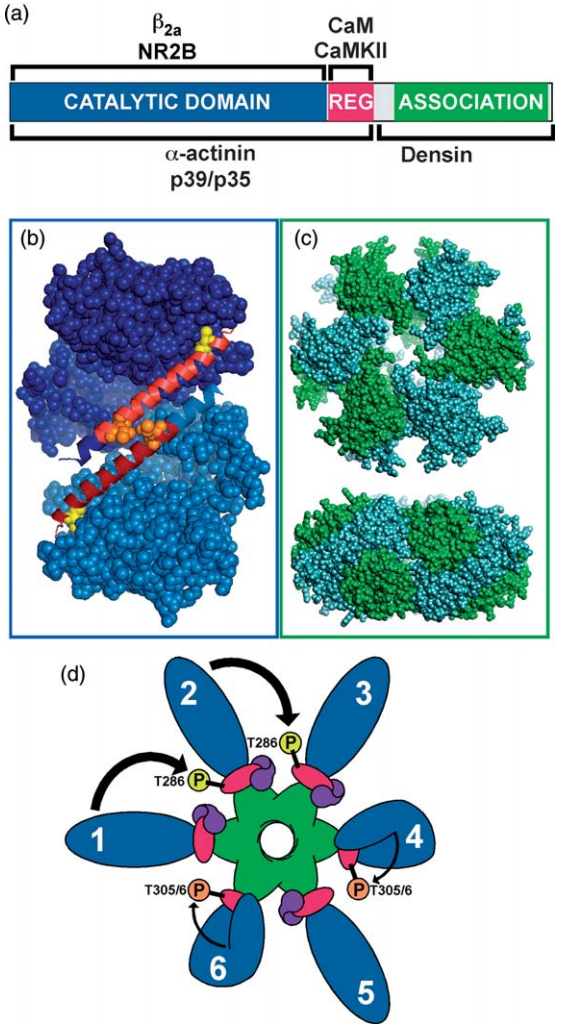
It also should be noted that the experiments described were conducted in the absence of opposing phosphatase activities. PP1 and PP2A appear to be the major activities responsible for CaMKII dephosphorylation, depending on the localization. *In vitro* studies showed that PP1 enhances the cooperativity for CaMKII activation by Ca^{2+}/CaM, providing ultrasensitive responsiveness to increasing Ca^{2+} concentrations (Bradshaw et al., 2003). PP1 has been hypothesized to couple with CaMKII to form a bistable molecular switch controlling synaptic plasticity (Zhabotinsky, 2000; Lisman and Zhabotinsky, 2001). The inhibition of hippocampal PP1 is necessary for increased Thr^{286} autophosphorylation of CaMKII, LTP induction, and memory, at least in some paradigms (Blitzer et al., 1998; Genoux et al., 2002). In addition, PP2A is inhibited following LTP induction (Fukunaga et al., 2000), possibly contributing to prolonged CaMKII autophosphorylation following LTP. However, it is not yet clear whether phosphatases

modify the frequency responsiveness to repeated Ca^{2+} transients.

Aside from Ca^{2+} transient frequency, Ca^{2+} transient dynamics are discretely regulated in the spines and shafts of neuronal dendrites (Sabatini et al., 2001), likely playing an important role in modulating CaMKII activation. In addition, small microdomains close to individual Ca^{2+} channels may contain substantially higher concentrations of Ca^{2+}, allowing associated pools of CaMKII to be preferentially activated at submaximal stimulation intensities (see below). Thus, CaMKII localization to discrete cellular domains may have a profound impact on the dynamics of CaMKII activation by Ca^{2+}.

Several studies have shown that CaMKII activity/autophosphorylation is stimulated by Ca^{2+} entry via NMDARs or voltage-gated Ca^{2+} channels (Fukunaga et al., 1989, 1992; Molloy and Kennedy, 1991; Ocorr and Schulman, 1991) and during LTP induction in hippocampus (Fukunaga et al., 1993; Ouyang et al., 1997, 1999). In cultured hippocampal neurons, brief depolarization was shown to induce compartmentalized CaMKII activation (Menegon et al., 2002). In immature neurons, CaMKIIα was activated in the somatodendritic compartment, but not in the axonal compartment. However, inclusion of PP1/PP2A inhibitors revealed axonal CaMKII activation. CaMKII activation remained somatodendritic in more mature neurons, but the activated kinase is localized to dendritic spines rather than dendritic shafts in the more distal dendrites (Menegon et al., 2002). This may reflect the translocation of activated kinase into spines.

CaMKII autophosphorylation is coupled to extracellular Ca^{2+} concentrations in hippocampal slices (Molloy and Kennedy, 1991). A recent study in



dorsal root ganglion neurons may provide additional insight into this coupling (Cohen and Fields, 2006). Under standard culture conditions the level of Thr^{286} autophosphorylation was substantial, but removal of extracellular Ca^{2+} resulted in almost complete Thr^{286} dephosphorylation within 20 min. Interestingly, removal of extracellular Ca^{2+} had no detectable effect on bulk cytoplasmic Ca^{2+}, but it depleted intracellular Ca^{2+} stores. Depletion of intracellular stores using caffeine similarly reduced CaMKII autophosphorylation. Thus, CaMKII autophosphorylation at Thr^{286} is tightly coupled to Ca^{2+} entry via intracellular Ca^{2+} stores, at least in dorsal root ganglion neurons (Cohen and Fields, 2006).

In combination, these observations suggest that Ca^{2+} changes in small subcellular microdomains due to specific mechanisms of Ca^{2+} mobilization are tightly coupled to CaMKII activation and Thr^{286} autophosphorylation. Uncovering these mechanisms will require the ability to resolve changes in activation in distinct subcellular compartments, as promised by recent development of fluorescence resonance energy transfer (FRET)-based reporters of CaMKII activation (Takao et al., 2005).

4.23.5 Synaptic Targeting of CaMKII

CaMKII is highly abundant in neurons, particularly in the forebrain, where it represents 1–2% of the total protein (Erondu and Kennedy, 1985), which calculates to an average concentration of 10–20 $\mu mol\ l^{-1}$ CaMKII subunit. The size of dendritic spines is highly variable, ranging from 0.004 to 0.6 μm^3 (Harris and Kater, 1994), but there would be ≈50 holoenzymes per average dendritic spine (volume of 0.05 μm^3) if CaMKII were randomly distributed. However, early studies showed that CaMKII is tightly associated with a variety of cytoskeletal components, preparations of plasma, synaptic and vesicular membranes, and especially with PSDs (Kennedy et al., 1983; Goldenring et al., 1984; Kelly et al., 1984; Ouimet et al., 1984; Fukunaga et al., 1988). Recent analyses suggest that there are ≈80 CaMKII holoenzymes per average isolated PSD, representing approximately 6% of the total PSD protein (Chen et al., 2005). This estimate is in broad agreement with quantitative mass spectrometry analyses (Cheng et al., 2006) and represents a substantial enrichment over the number expected based on random distribution (3 CaMKII holoenzymes per average

Figure 1 Structure-function of CaMKII holoenzymes. (a) Domain structure of a CaMKII subunit. Each CaMKII subunit contains an N-terminal catalytic domain, a central regulatory domain (REG), and a C-terminal association domain. The approximate domains needed for interaction with CaMKAPs and other proteins are indicated above and below the bar. (b) Structure of CaMKII catalytic/regulatory domain. The figure was generated using MacPyMol from the protein database file 2BDW. This is an x-ray crystal structure of the *Caenorhabditis elegans* CaMKII catalytic/regulatory domain (residues 1–340) containing an inactivating mutation. The protein appears monomeric in solution but forms a dimer in the crystal. In this orientation, the approximate dimensions of this structure are 90 Å by 50 Å. Each catalytic domain in the dimeric unit is shown as a space-filling model in different shades of blue. A critical component of dimer formation appears to be interactions between the adjacent regulatory domains, which formed long α-helices (shown as ribbon structures in different shades of red). In this inactive structure, the Thr^{286} autophosphorylation sites (yellow) are buried in pockets on the same catalytic domain, whereas Thr^{305}/Thr^{306} autophosphorylation sites in the Ca^{2+}/CaM-binding domain (orange) are at the dimerization interface. Generated from protein database from Rosenberg OS, Deindl S, Sung RJ, Nairn AC, and Kuriyan J (2005) Structure of the autoinhibited kinase domain of CaMKII and SAXS analysis of the holoenzyme. *Cell* 123: 849–860. (c) Structure of the dodecameric CaMKII holoenzyme hub. The figure was generated using MacPyMol from a structural model of the dodecameric association domain (residues 336–478). The subunits are arranged as a stacked pair of hexameric rings, and alternating subunits are shown in different shades of green when viewed from above (top panel) and from the side (bottom panel). The dimensions of the holoenzyme hub are approximately 145 Å in diameter by 60 Å high. Structural model of the dodecameric association domain is available online as supplementary material to Rosenberg OS, Deindl S, Comolli LR, et al. (2006) Oligomerization states of the association domain and the holoenyzme of Ca^{2+}/CaM kinase II. *FEBS J.* 273: 682–694. (d) Model for regulation of CaMKII by autophosphorylation. The diagram illustrates a top view of a single hexameric ring from the dodecameric holoenzyme. In the absence of Ca^{2+}/CaM, the regulatory domain (red) interacts with the catalytic domain (blue) to suppress activity (subunits 4 and 6) (see panel (b)). Ca^{2+}/CaM-binding (purple) activates individual CaMKII subunits by disrupting this interaction (e.g., subunits 1, 2, 3, 5). Activation of adjacent subunits in the holoenzyme results in the rapid trans-autophosphorylation at Thr^{286} (yellow circles) (subunits 2, 3). It is unknown whether trans-autophosphorylation occurs within (as shown) or between hexameric rings in the dodecameric holoenzyme. The Thr^{286}-autophosphorylated kinase has increased affinity for Ca^{2+}/CaM and retains autonomous kinase activity after dissociation of Ca^{2+}/CaM because the interaction of catalytic and regulatory domains is disrupted. 'Inactive' subunits (4 and 6) undergo slow intra-subunit autophosphorylation at Thr^{305} or Thr^{306} (orange circles) to block binding of Ca^{2+}/CaM, inhibiting CaMKII activation. Adapted from Colbran RJ (2004b) Targeting of calcium/calmodulin-dependent protein kinase II. *Biochem. J.* 378: 1–16; used with permission from The Biochemical Society.

PSD with 0.36 μm diameter, 30 nm depth). However, it should be emphasized that there is considerable variability between individual PSDs (Chen et al., 2005), as estimated in isolated PSDs and by immunoelectron microscopy in brain slices (Ouimet et al., 1984; Fukunaga et al., 1988).

Variability of CaMKII abundance in PSDs suggests that this interaction may be dynamically regulated. Initial studies showed that CaMKII interactions with PSD-enriched proteins are dependent on Thr^{286} autophosphorylation (or CaMKII activation) (McNeill and Colbran, 1995). Moreover, isolated PSDs containing substantial amounts of endogenous CaMKII were shown to have spare binding capacity for activated CaMKII (Strack et al., 1997; Yamauchi and Yoshimura, 1998). Consistent with these *in vitro* studies, CaMKII activation in hippocampal slices was shown to result in increased CaMKII association with PSD-enriched fractions (Strack et al., 1997). Subsequent studies examined the localization of green fluorescent protein (GFP)-tagged CaMKII isoforms expressed in cultured neurons. GFP-CaMKIIα initially adopts a freely diffusible cytosolic localization in the dendrites and soma, but translocates to dendritic spines following Ca^{2+} mobilization via NMDARs or voltage-dependent Ca^{2+} channels, where it colocalizes with PSD95, the classical PSD marker. Translocation of CaMKIIα can occur independent of the kinase activity and is dependent on binding of Ca^{2+}/CaM (Shen and Meyer, 1999; Shen et al., 2000). However, it seems likely that under physiological conditions activated cytosolic kinase would bind ATP and undergo Thr^{286} autophosphorylation before it could diffuse and bind to the PSD. Mutagenesis studies suggest that Thr^{286} autophosphorylation stabilizes CaMKII localization to spines, increasing the time constant for dissociation from spines from ≈10 s to ≈50 s. Similarly, inhibition of PP1 also results in more sustained synaptic translocation of CaMKIIα (Shen et al., 2000). In contrast, autophosphorylation at Thr^{305} and Thr^{306} appears to destabilize the punctate localization of CaMKII in spines, because a mutated kinase lacking these sites had an increased time constant for spine dissociation of ≈300 s (Shen et al., 2000).

Certain splice variants of GFP-CaMKIIβ associate with F-actin filaments under basal conditions when expressed in neurons and other cells (Shen et al., 1998; Fink et al., 2003). Binding of Ca^{2+}/CaM to GFP-CaMKIIβ following Ca^{2+} mobilization displaces the kinase from F-actin, allowing translocation to synapses. However, GFP-CaMKIIβ accumulates at synapses more slowly than does GFP-CaMKIIα, presumably because of the requirement for dissociation from F-actin. Thus, the relative expression of these two isoforms dictates the kinetics of synaptic translocation (Shen and Meyer, 1999).

In recent studies using more mature neuronal cultures, intrinsic neuronal activity resulted in synaptic accumulation of GFP-CaMKIIα, which could be dispersed by blocking NMDARs, but not by removal of extracellular Ca^{2+} (Bayer et al., 2006). Similarly, transient translocation of endogenous CaMKII to spines following Ca^{2+} mobilization induced by glutamate-, NMDA-, or KCl-induced depolarization was demonstrated using immunocytochemical methods (Dosemeci et al., 2001; Merrill et al., 2005). This endogenous synaptic CaMKII is highly autophosphorylated at Thr^{286}, and inhibition of PPs results in a more sustained synaptic localization (Dosemeci et al., 2002), as seen with GFP-CaMKIIα.

The localization of GFP-CaMKIIα also has been investigated in hippocampal slice cultures. In this system, chemical LTP induction resulted in persistent accumulation of GFP-CaMKIIα in dendritic spines in parallel with increases in PSD-associated CaMKII, as determined by immuno-gold labeling (Otmakhov et al., 2004). Moreover, sensory stimulation of zebrafish induces the sustained translocation of GFP-CaMKII to PSDs *in vivo* (Gleason et al., 2003). Presumably, induction of bona fide LTP recruits additional signaling processes that stabilize CaMKII binding to PSDs, perhaps by inhibiting phosphatases that are active toward Thr^{286}. Investigation of these apparently physiological mechanisms for CaMKII translocation is complicated by observations that a variety of pathological conditions also induce CaMKII translocation to spines. Some of these synaptic punctae may result from activity-dependent clustering of CaMKII into higher-order assemblies of the holoenzyme, as will be discussed below. It will be important to examine the localization of CaMKII *in vivo* using mammalian models in order to investigate the relationship between learning, memory, synaptic activity, and CaMKII translocation.

The idea that targeting of CaMKII to the PSD might potentiate phosphorylation of nearby substrates was supported by the initial observation that binding of exogenous CaMKII to PSDs potentiates phosphorylation of many proteins *in vitro,* including alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor glutamate receptor 1 (GluR1) subunits (Strack et al., 1997). However, recent studies suggest considerable nuance to the role of PSD-associated CaMKII. A fragment of

vimentin containing a specific CaMKII phosphorylation site was fused to four different proteins that were themselves phosphorylated by CaMKII *in vitro* and in human embryonic kidney 293 (HEK293) cells and that were targeted to synapses by different mechanisms (Tsui et al., 2005). When expressed in neurons, all of these proteins were targeted to synapses. However, phosphorylation of the vimentin model site could be detected with a phospho-vimentin antibody only when this domain was fused to stargazin or PSD95, but not when it was fused to NMDAR 2B (NR2B) or GluR1. Moreover, phosphorylation of the PSD95 and stargazin model substrates appeared to be coupled to Ca^{2+} entry via the NMDAR, but not to Ca^{2+} entering via voltage-gated Ca^{2+} channels. These data are surprising because CaMKII is only known to bind directly to NR2B, and this interaction potentiates phosphorylation of the vimentin model site in HEK293 cells. In combination, these studies suggest that CaMKII is targeted to specific microdomains within the PSD where it can access only a subset of the total CaMKII substrates in the PSD (Tsui and Malenka, 2006). Unfortunately, co-localization of CaMKII with the different substrates was not monitored in these experiments, and the authors were unable to detect CaMKII-dependent phosphorylation of the endogenous sites in GluR1 or NR2B in these experiments (Tsui and Malenka, 2006). Nevertheless, taken at face value, the data suggest that multiple mechanisms (perhaps involving several other proteins) may be required to direct CaMKII to specific PSD microdomains that are coupled to different substrates and distinct routes of Ca^{2+} entry.

4.23.6 Interactions of CaMKII with CaMKAPS

It has been increasingly recognized over the last several years that intracellular signaling pathways are tightly compartmentalized, often by assembly of large multi-protein signaling complexes that are sometimes referred to as signalosomes. Perhaps the best characterized system is the interaction of protein kinase A (PKA) with a family of A-kinase anchoring proteins (AKAPs), which nucleate complexes containing PKA and a diverse array of additional signaling proteins (Smith et al., 2006). Based on this paradigm and emerging evidence that CaMKII localization is dynamically regulated, several laboratories have investigated the association of CaMKII with other cellular proteins, which we have termed CaMKAPs (CaMKII-associated proteins). Here, I will focus on CaMKAPs that may play a role in postsynaptic targeting of CaMKII and/or modulate functions likely to be relevant for synaptic plasticity, learning, and memory. **Figure 2** shows domain structures and interacting proteins for these CaMKAPs.

4.23.6.1 NMDA-Type Glutamate Receptor Subunits

As discussed above, CaMKII is partially localized to excitatory synapses that contain NMDARs. The NR2B subunit was initially identified as a CaMKAP that binds only to the activated Thr^{286}-autophosphorylated kinase (Strack and Colbran, 1998). Recent studies have shown that CaMKII activation by Ca^{2+}/CaM binding coupled with nucleotide binding to the active site is sufficient for formation of a stable complex with NR2B (Robison et al., 2005a). CaMKII interacts with two distinct domains in the C-terminal tail of NR2B: a central domain (residues 1290–1309) appears to have higher affinity for CaMKII than a less-well defined membrane-proximal domain (Strack and Colbran, 1998; Leonard et al., 1999, 2002; Strack et al., 2000a; Bayer et al., 2001) (**Figure 2**). NR2A contains an amino acid sequence with similarity to the high-affinity binding site in NR2B, but insertion of two additional amino acids disrupts CaMKII binding (Strack et al., 2000a; Barria and Malinow, 2005) (**Figure 3**). CaMKII co-localizes only with NMDARs that contain NR2B in heterologous cells, and mutations within the central high-affinity CaMKII-binding domain that disrupt *in vitro* interactions of CaMKII with NR2B also disrupt co-localization (Strack et al., 2000a).

Recently, NR2B was shown to interact close to the CaMKII active site, possibly in two different binding modes that mediate a transition from transient to more sustained interaction (Bayer et al., 2006). These data are interesting in light of studies showing that NR2B can have two distinct effects on CaMKII activity. On one hand, binding of NR2B has been shown to prop open the regulatory domain, trapping an autonomously active form of CaMKII in the absence of Thr^{286} autophosphorylation (Bayer et al., 2001). In contrast, under different conditions NR2B inhibits CaMKII activity (Robison et al., 2005a), perhaps not surprisingly because the CaMKII binding domain of NR2B shares considerable amino acid sequence similarity with the CaMKII regulatory domain (Strack et al., 2000a) (**Figure 3**). The impact of these two modes of binding in intact cells remains unclear.

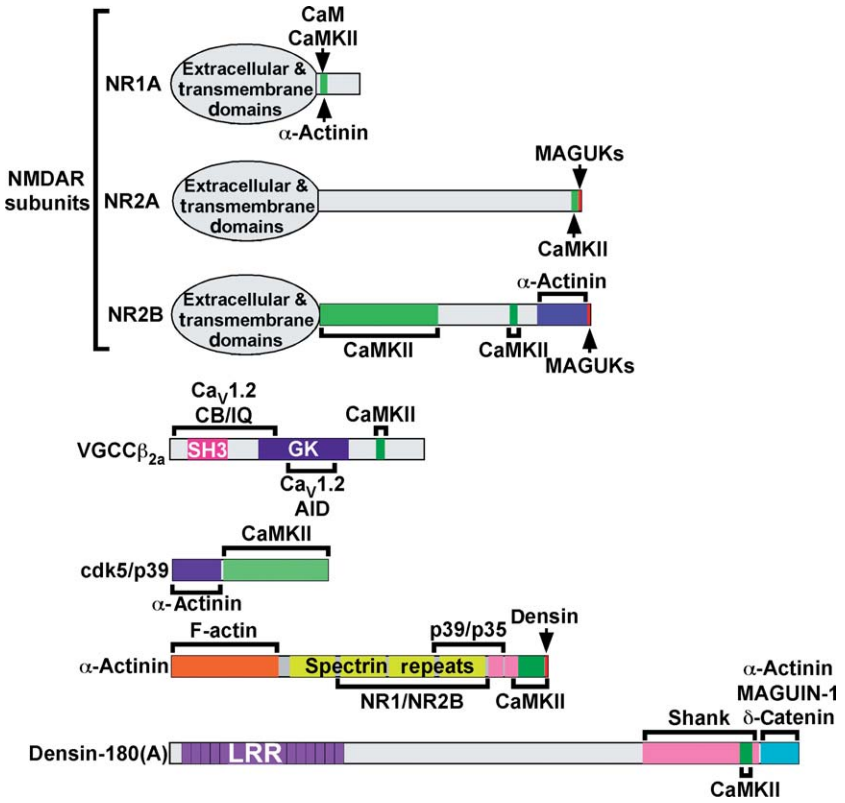


Figure 2 CaMKII-associated proteins (CaMKAPs) domain structures and known interacting proteins. Linear domain maps of CaMKAPs showing conserved protein interactions domains. Domains known to interact with CaMKII and selected additional proteins are indicated above and below each protein. SH3, Src-homology 3 domain; GK, guanylyl kinase-like domain; LRR, leucine-rich repeats; cdk5; cyclin-dependent kinase 5; NR, NMDA receptor; CB/IQ, a domain in the C-terminal tail of $Ca_V1.2$ containing multiple Ca^{2+}/CaM-binding motifs; AID, alpha-interacting domain in the I-II domain linker of $Ca_V1.2$; MAGUKs, membrane-associated guanylate kinases, a family of proteins. See text for details.

Activated CaMKII has also been shown to interact with the NR2A and NR1 subunits of the NMDAR (see **Figure 2**). An alternatively spliced region of NR1 binds CaMKII, and both calmodulin and α-actinin compete for this interaction (Leonard et al., 1999, 2002). CaMKII also competes with PSD95 for binding near the extreme C-terminus of NR2A (Gardoni et al., 1998, 1999, 2001b). However, interactions of CaMKII with NR1 and NR2A are not detected in all laboratories (e.g., see Strack and Colbran, 1998; Leonard et al., 1999), and CaMKII does not appear to significantly colocalize with NR1/NR2A NMDARs in heterologous cells (Strack and Colbran, 1998). Thus, interactions with NR1 and NR2A subunits may be too weak to mediate CaMKII targeting to NMDARs. Perhaps targeting of activated CaMKII to the NMDAR complex via NR2B facilitates these interactions.

Several studies have shown that CaMKII is dynamically associated with neuronal NMDARs using coimmunoprecipitation approaches. Interpretation of these data is complicated by the assembly of NMDAR subunits into functional receptor ion channel complexes with many other proteins unless they are fully solubilized using harsh ionic detergents, and also depends on the specificity of the antibodies for the NMDAR subunits. Initial studies in hippocampal slices found that activation of NMDARs promotes CaMKII association with the entire NMDAR complex (Leonard et al., 1999). Association of CaMKII with the C-terminus of NR2A appears to be inhibited by protein kinase C (PKC) phosphorylation at Ser^{1416} (Gardoni et al., 2001a), allowing for increased binding of PSD95 that may be critical for LTP induction (Gardoni et al., 2001b). However, more recent experiments suggested that CaMKII is preferentially associated with the NR2B subunit and not with NR2A (Kim et al., 2005). Taken together, these findings are consistent with biochemical data demonstrating binding of activated CaMKII to NMDAR



Figure 3 Alignment of amino acid sequences from CaMKII-binding domains in NMDA receptor 2B (NR2B) and β_{2a} with the CaMKIIα regulatory domain. Residues in green boxes are identical, and residues in grey boxes are conserved. The yellow circle indicates CaMKII phosphorylation sites in NR2B and β_{2a} and the Thr^{286} autophosphorylation site in CaMKII. These sites are highly efficient CaMKII substrates, and the domains surrounding these sites all form stable interactions with the CaMKII catalytic domain. Despite strong sequence similarity, the regulatory domain of CaMKII competes with ATP for binding to and inhibiting CaMKII (Colbran RJ, Smith MK, Schworer CM, Fong YL, and Soderling TR (1989) Regulatory domain of calcium/calmodulin-dependent protein kinase II and synaptic plasticity. *Curr. Opin. Neurobiol.* 14: 318–327), whereas Ca^{2+}/CaM-dependent binding of CaMKII to NR2B and β_{2a} requires that ATP first bind to the kinase (Robison AJ, Bartlett RK, Bass MA, and Colbran RJ (2005a) Differential modulation of Ca^{2+}/calmodulin-dependent protein kinase II activity by regulated interactions with NMDA receptor NR2B subunits and alpha-actinin. *J. Biol. Chem.* 280: 39316–39323). The arrow above the NR2B sequence indicates the location of an isoleucine-asparagine insertion in the corresponding domain of NR2A that appears to disrupt CaMKII binding (Barria A and Malinow R (2005) NMDA subunit composition controls synaptic plasticity by regulating binding to CaMKII. *Neuron* 48: 289–301).

subunits, but more studies are needed to establish the precise mechanisms and role for this interaction in neurons.

The original CaMKAP hypothesis suggested that CaMKAPs target CaMKII to particular subcellular compartments to facilitate signaling to specific downstream substrates. This raises the question of whether interactions with NMDARs can account for the known enrichment of CaMKII in PSDs. A CaMKII-binding glutathione-S-transferase-NR2B fusion protein blocked ≈75% of CaMKII binding to PSDs *in vitro* (Strack et al., 2000a), suggesting that NR2B makes a significant contribution to binding, although a role for other CaMKII-binding proteins sharing a similar interaction mechanism cannot be excluded. Excitatory synapses may contain about 50 NMDARs (reviewed in Kennedy, 2000), in the same range as estimated numbers of PSD-associated CaMKII holoenzymes (Chen et al., 2005), suggesting that NMDARs may be quantitatively important for PSD targeting if each receptor complex targets one CaMKII holoenzyme. In contrast, a recent mass spectrometry study found that PSDs contain a substantial (>20-fold) excess of CaMKII holoenzymes over NMDAR channels (Cheng et al., 2006). These data suggest that bulk CaMKII targeting to PSDs must involve interactions with additional proteins or the formation of higher-order clusters of CaMKII holoenzymes. More studies are needed to understand the quantitative contribution of NMDARs to PSD targeting of CaMKII.

High local concentrations of Ca^{2+} near cytoplasmic domains of the NMDAR would be predicted to potentiate activation of associated CaMKII to enhance the phosphorylation of nearby substrates. Consistent with this hypothesis, phosphorylation of a membrane-bound model CaMKII substrate (a vimentin fragment) in HEK293 cells was shown to be facilitated by binding of CaMKII to NR2B localized to the same membrane compartment (Tsui et al., 2005). However, this effect is independent of Ca^{2+} influx, because the NR2B was not present in a functional NMDAR, and is perhaps more consistent with the possibility that NR2B is trapping an autonomously active form of CaMKII. More studies are needed to determine the effect of disrupting the CaMKII-NR2B interaction on phosphorylation of PSD proteins, LTP induction, learning, and memory.

An additional function for CaMKII binding to NMDARs may be to provide feedback modulation of NMDAR activity. Activation of CaMKII in isolated PSDs enhanced the binding of MK801, an open channel blocker of the NMDAR, suggesting that CaMKII increases channel opening (Kitamura et al., 1993). Consistent with these findings, CaMKII increased NMDAR currents in spinal dorsal horn neurons (Kolaj et al., 1994). However, these studies did not address contributions of NMDARs with different subunit combinations to the CaMKII response. CaMKII potently phosphorylates NR2B at Ser^{1303} within the high-affinity CaMKII-binding domain, and this residue is phosphorylated *in situ* (Omkumar et al., 1996). In heterologous cells, CaMKII enhanced the desensitization of NMDARs formed from NR2B and one of multiple NR1 subunit slice variants but modestly reduced the desensitization of NMDARs containing NR2A in place of NR2B (Sessoms-Sikes et al., 2005). Effects of CaMKII on NMDARs containing both NR2A and NR2B were not investigated. Thus, CaMKII may function as a negative feedback regulator of Ca^{2+} entry via NMDARs containing NR2B, but a positive feedback regulator of those containing NR2A. Interactions of CaMKII with NMDAR subunits may facilitate specific CaMKII actions on NMDARs with different subunit compositions. It will be important to elucidate the roles of

these NR2 subunit–specific effects of CaMKII in modulating synaptic and extrasynaptic NMDARs and synaptic plasticity.

Initial insights into the impact of CaMKII binding to NR2B on synaptic plasticity have been provided recently. Insertion of two amino acids into the CaMKII-binding domain of NR2B significantly reduced Ser^{1303} phosphorylation *in vitro* (Mayadevi et al., 2002). This mutation disrupts CaMKII binding to NR2B, and overexpression of the mutant protein interfered with LTP induction in organotypic hippocampal slice cultures (Barria and Malinow, 2005). Although the effects of this mutation on NR2B phosphorylation *in situ* were not investigated, these data suggest that CaMKII binding to NR2B is important for LTP induction. These findings appear at odds with pharmacological studies suggesting that synaptic NR2A-containing NMDARs are critical for LTP, whereas extra- or perisynaptic NR2B NMDARs are critical for normal LTD (Liu et al., 2004; Massey et al., 2004). However, the specificity of the pharmacological reagents has been questioned (Weitlauf et al., 2005), and a recent study suggests that the subunit dependence of synaptic plasticity varies with the developmental stage (Bartlett et al., 2007). The precise relationships between CaMKII interactions with, and phosphorylation of, specific NMDAR subunits and different forms of synaptic plasticity clearly warrant further study.

4.23.6.2 The Densin-180/α-Actinin/cdk5 Complex

Prompted by the realization that NMDARs may not fully account for PSD targeting of CaMKII (see above), and by the development of yeast two-hybrid strategies for identifying novel protein–protein interactions in unbiased screens, CaMKII was shown to interact directly with densin-180, α-actinin, and the p35/39 subunit of cyclin-dependent kinase 5 (cdk5) (Strack et al., 2000a; Walikonis et al., 2001; Dhavan et al., 2002). In addition, α-actinin also binds to densin-180 and to p35/39 independently of CaMKII. Characterization of these interactions revealed a diversity of new mechanisms by which CaMKII interacts with other cellular proteins to regulate synaptic function.

4.23.6.2.1 Densin-180

Densin-180 was originally identified as a relatively abundant 180-kDa PSD protein containing a leucine-rich repeat (LRR) domain at the N-terminus and a single PDZ domain at the C-terminus (Apperson et al., 1996) (see **Figure 2**). It became a founding member of the LAP protein family, which contain **L**eucine-rich repeats (LRRs) **A**nd (1 or more) **P**DZ domains (Bilder et al., 2000; but see Wilson et al., 2001). Initial studies suggested that densin-180 had a single transmembrane-spanning domain with a large extracellular N-terminal domain containing the LRRs and some glycosylation sites and only a short cytosolic C-terminal domain. However, other LAP protein family members are exclusively intracellular, and cell surface biotinylation of dissociated hippocampal neurons failed to modify densin-180 (Izawa et al., 2002). Thus, the entire densin-180 protein may be cytosolic. Additional studies are needed to clarify this issue.

In part because of the putative transmembrane structure of densin-180, most studies focused on protein interactions mediated by the C-terminal domain of densin-180. Biochemical and yeast two-hybrid studies showed that CaMKII holoenzymes bound to a 29-amino-acid domain adjacent to the PDZ domain (Strack et al., 2000a; Walikonis et al., 2001; Robison et al., 2005b). This CaMKII-binding domain exhibits no amino acid sequence similarity to CaMKII-binding domains in the NMDAR subunits. Interestingly, multiple mRNA splice variants are differentially expressed during embryonic and postnatal development, and one variant lacks the CaMKII-binding domain (Strack et al., 2000b). The major adult densin-180 splice variant (densin-A) interacts with nonphosphorylated inactive CaMKIIα (Walikonis et al., 2001; Robison et al., 2005b), and this interaction is insensitive to Ca^{2+}/calmodulin or to $Thr^{305/306}$ autophosphorylation (Robison et al., 2005a). However, autophosphorylation at Thr^{286} potentiates the interaction to a varying extent depending on assay conditions (Strack et al., 2000b; Walikonis et al., 2001; Robison et al., 2005a). Densin-A is unable to bind CaMKIIα monomers generated by C-terminal truncation (Robison et al., 2005b), and the oligomeric C-terminal association domain of CaMKIIα binds densin-A in the yeast two-hybrid assay (Walikonis et al., 2001). Thus, it seems that densin-A interacts with the C-terminal association domain of CaMKIIα and/or requires the holoenzyme structure for interaction. In contrast, an embryonic densin splice variant (densin-D) binds to monomeric, C-terminal truncated forms of CaMKIIα and also to CaMKIIβ, CaMKIIγ, and CaMKIIδ isoforms (Robison and Colbran, unpublished observations). Thus, this embryonic form of densin-180 can interact with CaMKII isoforms that are expressed at similar developmental stages. In combination, these data suggest that densin-180 splice variants can have different roles during development, some independent

of CaMKII. Additional cellular studies are required to further explore the role of alternative splicing of densin-180.

In addition to CaMKII, the C-terminal domain of densin-180 has also been shown to interact with α-actinin (Walikonis et al., 2001), δ-catenin (Izawa et al., 2002), Shank (Quitsch et al., 2005), and MAGUIN-1 (Ohtakara et al., 2002), providing links to many other PSD proteins. Densin-180 overexpression induces branching of neuronal dendrites, and this effect is antagonized by coexpression of Shank, which competes with δ-catenin, but not CaMKII, for binding to densin-180 (Quitsch et al., 2005). In combination, these data suggest that densin-180 serves as a scaffolding protein, assembling postsynaptic signaling complexes to modulate dendritic morphology. However, although the LRR domain appears sufficient for membrane targeting (Quitsch et al., 2005), it remains unclear whether the N-terminal domain is intracellular or extracellular, and its specific role remains largely unknown. It will be important to identify additional binding partners for densin-180, determine the effects of CaMKII on these interactions, and elucidate their biological role.

4.23.6.2.2 α-Actinin

Dendritic spines are highly enriched in F-actin and F-actin-binding proteins, such as α-actinin. All four mammalian α-actinin isoforms contain an N-terminal F-actin-binding domain, a central spectrin-repeat domain, and a C-terminus containing an EF-hand divalent metal ion–binding domain that may not be functional in all isoforms (see **Figure 2**). The spectrin repeat domains form antiparallel dimers in a long, rodlike structure, with single F-actin-binding and C-terminal domains at each end (reviewed in Broderick and Winder, 2005). Yeast two-hybrid screens identified interactions between the extreme C-terminus of α-actinin-4 and the densin-180 PDZ domain (Walikonis et al., 2001), the C-terminal domain of α-actinin-1 and the N-terminal domain of the cdk5 regulatory subunits p35 and p39 (Dhavan et al., 2002), and the central rod domain of α-actinin-2 and NMDAR NR1 and NR2B subunits (Wyszynski et al., 1997).

CaMKII was shown to bind to residues 713–892 of α-actinin-1 and residues 638–879 of α-actinin-4 using the yeast two-hybrid assay (Walikonis et al., 2001; Dhavan et al., 2002). Biochemical experiments found that residues 819–894 constitute the smallest CaMKII-binding fragment of α-actinin-2, which is thought to be the major isoform present in dendritic spines (Robison et al., 2005b). This domain is highly conserved in all the α-actinin isoforms, but is considerably larger than, and shares no homology with, CaMKII-binding domains in NMDARs or densin-180. All CaMKII isoforms, as well as monomeric catalytic/regulatory domain fragments, can bind α-actinin. Interestingly, Ca^{2+}/calmodulin competes with α-actinin for binding to CaMKII. Moreover, $Thr^{305/306}$ autophosphorylation of CaMKII appears to block α-actinin binding, but Thr^{286} autophosphorylation has little effect (Robison et al., 2005a). Coimmunoprecipitation and colocalization experiments are consistent with the interaction of CaMKII with the α-actinins at synapses *in situ* (Walikonis et al., 2001; Dhavan et al., 2002; Robison et al., 2005b). Together, these data suggest the intriguing hypothesis that α-actinin localizes inactive CaMKII under basal conditions, and that localized elevations of Ca^{2+} would displace CaMKII and activate the kinase.

4.23.6.2.3 p35/p39 regulatory subunits of cyclin-dependent kinase 5

CaMKII was identified as a direct binding partner for p35/p39 in a yeast two-hybrid screen (Dhavan et al., 2002). A monomeric catalytic/regulatory domain fragment of CaMKII interacts with the C-terminal domain of p35/p39, separate from the α-actinin-binding site in the N-terminal domain of p35/p39 (**Figure 2**). Notably, CaMKII colocalizes with α-actinin, cdk5, and p35 in cultured neurons, especially in dendritic spines. Coimmunoprecipitation experiments isolated complexes containing CaMKII, α-actinin, and p35 from brain extracts, and pretreatment of cultured neurons or hippocampal slices with NMDAR agonists increased the amount of coimmunoprecipitation in a manner that required CaMKII activity (Dhavan et al., 2002). These data suggest an intriguing regulatory relationship between CaMKII and cdk5. Given recent progress in elucidating critical roles for cdk5 in regulating synaptic morphology and transmission (Cheung et al., 2006), it will be important to determine the role of α-actinin as a scaffolding protein to facilitate this potential cross talk.

4.23.6.3 Voltage-Gated Calcium Channels

Voltage-gated calcium channels are essential both presynaptically for release of neurotransmitter and postsynaptically in synaptic plasticity. They are formed from a pore-forming α subunit, encoded by multiple genes in three distinct families (Ca_V1, Ca_V2, and Ca_V3), which associate with β subunits (four genes, β_1, β_2, β_3, and β_4, that encode many mRNA splice variants) and other regulatory subunits/

proteins, yielding a tremendous diversity of neuronal calcium channels. There are multiple mechanisms for the feedback regulation of voltage-gated calcium channels, including binding of heterotrimeric G protein subunits and phosphorylation by multiple kinases. Ca^{2+}-dependent feedback is also an important mechanism for regulation of voltage-gated Ca^{2+} channels. For example, direct binding of Ca^{2+}/CaM to α subunit C-terminal domains controls Ca^{2+}-dependent inactivation of several calcium channel subtypes (reviewed in Catterall, 2000). More complex Ca^{2+}-dependent mechanisms are also important for integrating the actions of calcium channels, such as the activation of dendritic L-type calcium channels, which activates a CaMKII-dependent pathway to inhibit Ca^{2+} entry via R-type channels, interfering with LTP induction under some conditions (Yasuda et al., 2003). In addition, contributions of T-type and L-type channels to Ca^{2+} transients in spines and dendritic shafts of striatal medium spiny neurons are dynamically modulated by up/down-state transitions between two resting membrane potentials (Carter and Sabatini, 2004). Association of CaMKII with the calcium channels may be important for modulation of these activities.

4.23.6.3.1 L-type calcium channels

Neuronal L-type calcium channels (LTCCs) contain $Ca_V1.2$ or $Ca_V1.3$ α subunits associated with diverse β subunits, which exert differential effects on the trafficking and biophysical properties of the channels (reviewed in Dolphin, 2003). LTCCs were initially localized to the soma and proximal dendrites (Hell et al., 1993), but they are also enriched at more distal synapses (Obermair et al., 2004). LTCCs play a key role in modulating neuronal gene transcription and synaptic plasticity (Deisseroth et al., 2003), with $Ca_V1.2$ and $Ca_V1.3$ subunits making distinct contributions to transcriptional responses in the hippocampus (Zhang et al., 2006). Recent studies found that the loss of dendritic spines on striatal medium spiny neurons following dopamine depletion *in vivo* requires LTCC activity that is specifically mediated by $Ca_V1.3$ (Day et al., 2006).

CaMKII can facilitate LTCC current via actions on α subunits. The cytosolic N- and C-terminal domains, as well as the intracellular linker domains, of $Ca_V1.2$ can be phosphorylated by CaMKII, and CaMKII also binds to the N- and C-terminal domains (Hudmon et al., 2005). Interaction of CaMKII with the C-terminal domain was suggested to be important for facilitation of a mutated $Ca_V1.2$ channel in oocytes (Hudmon et al., 2005). In addition, a genetic mutation associated with Timothy syndrome creates a CaMKII phosphorylation site (Ser^{439} in the I-II linker domain) that mediates an increase in channel open probability (Erxleben et al., 2006). CaMKII may also act to increase channel activity by phosphorylating the membrane-proximal region of the C-terminal domain (Erxleben et al., 2006; Lee et al., 2006). However, the physiological relevance of these forms of LTCC regulation by CaMKII is unclear.

LTCCs are also facilitated in cardiomyocytes during trains of action potential stimulation, in part due to an increase in channel open probability mediated by CaMKII (Dzhura et al., 2000). CaMKII is specifically targeted to the cardiac LTCC complex because the open probability of channels presented in isolated membrane patches is increased by an associated CaMKII activity (Dzhura et al., 2002). The regulation of channel open probability by CaMKII can be reconstituted in heterologous cells coexpressing $Ca_V1.2$ with the β_{2a} subunit, but not in cells lacking β_{2a} (Grueter et al., 2006). CaMKII binds to β_{2a} and efficiently phosphorylates Thr^{498}. Interestingly, the amino acid sequence surrounding Thr^{498} is very similar to those surrounding Ser^{1303} in the CaMKII-binding domain of NR2B and Thr^{286} in the regulatory domain of CaMKII itself (**Figure 3**). Mutation of Thr^{498} to alanine essentially prevents CaMKII-dependent increases in both single LTCC open probability and whole-cell LTCC current in cardiomyocytes. These studies define the β subunit as a key locus for physiological modulation of LTCCs by CaMKII-mediated protein phosphorylation (Grueter et al., 2006), in contrast to the direct actions of Ca^{2+}/calmodulin and PKA to modulate channel activity via the α subunit (see Catterall, 2000).

The actions of CaMKII on the α and β subunits to facilitate LTCCs are not necessarily mutually exclusive. Thus, it will be interesting to investigate whether specific β subunits are required for CaMKII actions via the α subunits and to determine their role in modulating neuronal LTCCs. The four β subunit genes exhibit distinct but overlapping expression patterns in the brain (reviewed in Dolphin, 2003). Whereas the β_1 and β_3 subunits contain amino acid sequences similar to those surrounding Thr^{498} in β_{2a}, similar sequences cannot be identified in β_4. Thus, one prediction is that CaMKII will differentially modulate LTCCs in neurons, depending on the identity of the β subunit present

in the channel complex. Moreover, β subunits associate with non-L-type calcium channel α subunits, and evidence is beginning to emerge that CaMKII can also regulate Ca_V2 channels (Jiang et al., 2004). Thus, specific β subunits may be involved in CaMKII modulation of N-, P/Q, and/or R-type channels.

4.23.6.3.2 T-type calcium channels

T-type channels are somewhat distinct from other calcium channels in that they are activated at lower voltages and are thought to operate independent from β subunits. Three genes encoding T-type channel variants ($Ca_V3.1$, $Ca_V3.2$, and $Ca_V3.3$) are differentially expressed in different brain regions. Single-nucleotide polymorphisms in the $Ca_V3.2$ gene have been linked to a form of epilepsy (reviewed in Perez-Reyes, 2006). Neuronal T-type channels are dynamically regulated by diverse mechanisms, including feedback modulation by intracellular Ca^{2+} (reviewed in Chemin et al., 2006). Intracellular Ca^{2+} facilitates T-type calcium channels by shifting the voltage dependence for channel activation to more negative potentials via the activation of CaMKII (Lu et al., 1994; Barrett et al., 2000; Welsby et al., 2003). CaMKII directly regulates recombinant $Ca_V3.2$ T-type channels by phosphorylating Ser^{1198} in the II-III loop (Welsby et al., 2003; Yao et al., 2006). Recent studies have shown that CaMKII binds to the II-III loop of $Ca_V3.2$, but there is little amino acid sequence similarity with CaMKII-binding domains in other proteins. Notably, CaMKII does not regulate $Ca_V3.1$ channels and does not bind to or phosphorylate the II-III loop of $Ca_V3.1$. Phosphorylation of the adrenal T-type channels at Ser^{1198} is dynamically regulated by angiotensin II *in vivo* (Yao et al., 2006). It will be important to investigate Ser^{1198} phosphorylation in neurons and determine the contribution of this phosphorylation to modulation of dendritic Ca^{2+} signaling.

4.23.6.4 SynGAP/MUPP1

Guanosine triphosphate (GTP)ase activating proteins (GAPs) inhibit G proteins by stimulating GTP hydrolysis. SynGAP binds to PSD95 and is enriched at synapses where it regulates ras, thereby controlling glutamatergic signaling via extracellular signal-regulated protein kinase (ERK) and p38 MAP kinase pathways. Reductions in synGAP expression activate ERK pathways and inhibit the p38 MAP kinase pathway (Rumbaugh et al., 2006). Recent studies specifically linked NMDARs containing NR2B to the modulation of synGAP and synaptic MAP kinases (Krapivinsky et al., 2003; Kim et al., 2005). Selective interactions of CaMKII with NR2B (see Kim et al., 2005) may be important for this selectivity, because CaMKII phosphorylates and activates synGAP, thereby downregulating the ERK kinase pathway following Ca^{2+} influx (Oh et al., 2004). Modulation of synGAP is an important control point for AMPA-type glutamate receptor (AMPAR) trafficking, contributing to synaptic plasticity (Krapivinsky et al., 2004; Rumbaugh et al., 2006).

In other studies, synGAP was shown to be significantly phosphorylated by CaMKII under basal conditions (Krapivinsky et al., 2004). Binding of CaMKII and synGAP to distinct PDZ domains in **mu**ltiple-**P**DZ **p**rotein-1 (MUPP-1) appeared to promote this basal phosphorylation. In these studies, Ca^{2+} influx via the NMDAR disrupted the interaction of CaMKII with MUPP-1, reducing synGAP phosphorylation at the same time that the calcineurin phosphatase was activated to dephosphorylate and inhibit synGAP (Krapivinsky et al., 2004). These data suggest that MUPP-1 mediates assembly of synGAP and CaMKII and is an important mechanism for modulating synGAPα isoforms. However, a more minor synGAPβ isoform appears to bind CaMKII directly (Li et al., 2001). The existence of multiple mechanisms for assembly of CaMKII-synGAP complexes may allow for diversity in the regulatory interactions and contribute to some of the discrepancies in this literature.

4.23.7 Roles for CaMKII in Synaptic Plasticity

Abundant evidence suggests that CaMKII activation is important for LTP induction, and many investigators have studied relevant molecular mechanisms. The most prominent substrate for CaMKII is the Thr^{286} autophosphorylation site in CaMKIIα, which is essential for normal synaptic plasticity, learning, and memory (reviewed in Elgersma et al., 2004). Several interesting postsynaptic CaMKII substrates have been identified. Most attention has focused on the regulation of the activity and subcellular localization of subunits of the AMPAR. CaMKII and PKC phosphorylate the GluR1 subunit of AMPARs at Ser^{831} to increase unitary conductance of GluR1 homomeric channels (Barria et al., 1997a,b). Indeed, Ser^{831} and a nearby PKA phosphorylation

site (Ser^{845}) in GluR1 are essential for normal synaptic plasticity (Lee et al., 2003). However, it was recently shown that Ser^{831} phosphorylation does not affect the conductance of heteromeric GluR1/GluR2 channels that are physiologically relevant (Oh and Derkach, 2005), so the significance of Ser^{831} phosphorylation to LTP induction is unclear. In addition, CaMKII promotes GluR1 trafficking to synapses by a mechanism that is independent of GluR1 phosphorylation and requires binding of the C-terminus of GluR1 to a PDZ domain in an unidentified protein (Hayashi et al., 2000). Thus, with the exception of Thr^{286} autophosphorylation, the CaMKII substrates relevant to LTP induction are unclear.

There is even greater uncertainty about the role of CaMKII activity during maintenance of LTP. Early studies focused on the hypothesis that LTP induction would increase Thr^{286} autophosphorylation of CaMKII, resulting in a sustained increase of autonomous CaMKII activity that played a critical role in maintenance. However, this hypothesis was confounded by many studies showing that CaMKII inhibitors cannot reverse preexisting LTP, even though they effectively block LTP induction (discussed in Chen et al., 2001). A potential explanation for this conundrum was suggested in a recent study that found that LTP induction rapidly increased both Thr^{286} autophosphorylation and autonomous CaMKII activity, but that the activity returned to basal levels within a few minutes even though Thr^{286} autophosphorylation was maintained for at least 1 h (Lengyel et al., 2004). Possibly the autonomous kinase activity of the Thr^{286} autophosphorylated kinase is suppressed by interactions with other proteins, such as NR2B. Thus, CaMKII and sustained Thr^{286} autophosphorylation may be important for maintenance of LTP without an essential role for ongoing kinase activity. The temporal distinction between requirements for Thr^{286} autophosphorylation and autonomous kinase activity may extend to certain learning/memory paradigms, because experiments using a chemical-genetic approach to inhibit CaMKII activity at various times after a fear conditioning paradigm showed that kinase activity is not required indefinitely (Wang et al., 2003).

Investigators have long questioned why a highly efficient enzyme like CaMKIIα is expressed in forebrain at levels that are more comparable with cytoskeletal proteins such as actin or tubulin (Erondu and Kennedy, 1985). The high expression levels were suggested to imply some sort of structural role. Intriguing insight into this possibility was provided by recent analyses of isolated PSDs by electron microscopy (Petersen et al., 2003). Variable amounts of CaMKII were found on the cytosolic face of PSDs, as expected based on the dynamic regulation of CaMKII binding to PSDs. However, rather than being randomly distributed, the CaMKII appeared to be present in an orderly array, forming 'towers' projecting from the PSD into the cytosol. The CaMKII towers may be related to hypothesized 'slots' for synaptic insertion of AMPARs (Lisman and Zhabotinsky, 2001), and their dimensions were reasonably consistent with the possibility that they are formed from stacks of CaMKII holoenzymes (Petersen et al., 2003). Whatever the nature and function of the CaMKII towers, the orderly distribution of the kinase on the cytosolic face hints at a structural role.

If CaMKII indeed plays a structural role, one might then ask why a structural protein needs to be an enzyme, especially one with such intricate regulatory mechanisms. In this regard, two possible interrelated mechanisms might be involved. First, CaMKII holoenzymes may assemble into higher-order complexes in response to Ca^{2+}-mobilizing stimuli in intact cells. Second, CaMKII may coordinate the assembly of multiple CaMKAPs in a complex. Ca^{2+}/calmodulin-binding and autophosphorylation modulates both of these processes, suggesting that CaMKII may function as an autoregulated scaffolding protein. Data supporting these two mechanisms are reviewed in turn.

4.23.7.1 Higher-Order Assemblies of CaMKII Holoenzymes

Early *in vitro* studies showed that autophosphorylation of CaMKII at pH 6.5 using low concentrations of ATP resulted in clustering of CaMKII holoenzymes into loosely packed, large branched complexes which then associated to form larger complexes (Hudmon et al., 1996, 2001). Electron microscopy subsequently revealed that spherical clusters of CaMKII contaminated isolated PSD preparations (Dosemeci et al., 2000). The reversible formation of these spherical clusters could be induced in intact cultured neurons and hippocampal slices by glutamate and calcium mobilizing stimuli (Tao-Cheng et al., 2001, 2002). CaMKII clustering may be a neuroprotective mechanism to sequester the kinase away from critical substrates when abnormally activated, perhaps under excitotoxic conditions that result in reduced ATP

levels and cytosolic pH. Recent studies found that in intact cells CaMKII holoenzymes reversibly oligomerized by a mechanism involving interaction of regulatory and catalytic domains in different holoenzymes (Hudmon et al., 2005). Formation of CaMKII clusters under more pathological conditions may be related in some way to the perhaps more physiologically relevant CaMKII 'towers' observed in PSDs (Petersen et al., 2003) (discussed earlier). An understanding of the structural basis for formation of the towers will likely provide insight into their function.

4.23.7.2 Binding to CaMKAPs

CaMKII interactions with the CaMKAPs are differentially regulated by Ca^{2+}/calmodulin-binding and autophosphorylation. Moreover, different domains in CaMKII appear to be involved in binding to CaMKAPs (**Figure 1(a)**), as reflected by the lack of competition between CaMKAPs for binding to CaMKII and by the fact that C-terminal truncation of CaMKII has different effects on each interaction (Strack et al., 2000a; Robison et al., 2005b). Consistent with these observations, Thr^{286} autophosphorylated CaMKII can bind simultaneously to fragments of NR2B, densin-180, and α-actinin *in vitro,* suggesting that under appropriate conditions CaMKII can nucleate complexes containing these three proteins in intact cells (Robison et al., 2005b). Despite the distinct regulation of their interactions with CaMKII and their different effects on CaMKII activity, NR2B, densin-180, and α-actinin are at least partially colocalized in dendritic spines. Moreover, multiprotein complexes containing these proteins can be immunoprecipitated from brain extracts (Wyszynski et al., 1997; Dunah et al., 2000; Walikonis et al., 2001; Robison et al., 2005b). However, the role of CaMKII in formation of these multiprotein complexes in cells is unclear, and it is not clear how these protein assemblies relate to the regularly spaced arrays of CaMKII towers observed in PSDs. However, one can speculate that regulated interactions of CaMKII with different CaMKAPs might induce structural rearrangements of these complexes that may be important for ultrastructural or functional changes at the synapse.

4.23.8 Concluding Comments

Studies over the last 20 years have revealed tremendous complexity in the biochemical properties of CaMKII. Thr^{286} autophosphorylation provides an intuitively appealing mechanism for a molecular memory, and there is abundant evidence that this process is critical for synaptic plasticity and learning. However, the exact molecular basis for modulation of synaptic transmission by CaMKII remains unclear. Ongoing studies are revealing new ways in which CaMKII signaling can be modulated by its interactions with a plethora of additional synaptic proteins. These interactions are differentially regulated and can have diverse effects on CaMKII activity. These findings are revealing considerable nuance to CaMKII regulation that suggest new ways in which CaMKII may modulate synaptic transmission. The challenge will be to link these biochemical mechanisms to specific aspects within the continuum of physiological responses that are collectively described as synaptic plasticity. The reward for meeting this challenge will likely be new strategies for therapeutic intervention that might target disrupted excitatory synaptic transmission that is associated with numerous neurological diseases and disorders.

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4.24 Angelman Syndrome

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4.24.1 Introduction

The ability to perceive our environment, store information about our experiences, and retrieve that information when needed is a fundamental necessity for human cognition. Neuroscience research has only recently begun to unravel the mysteries of learning and memory by identifying the elemental molecules, important proteins, and signal transduction pathways that underlie the formation of long-lasting memories. The identification of specific genes and their products involved in disorders associated with human mental retardation has greatly facilitated our overall understanding of the mechanisms underlying human cognition. Recent research of Angelman Syndrome (AS) represents a quintessential example of this process at work.

Angelman syndrome is a devastating human mental retardation condition that affects specific areas of the central nervous system, including the hippocampus. The discovery of this brain region–specific disorder and subsequent production of a mouse model has allowed researchers the unique opportunity to explore the molecular mechanisms at work in the adult hippocampus that directly underlie human memory processes. This chapter recounts the important discoveries surrounding this disorder and the implications of these discoveries for our current knowledge of the molecular players present in the hippocampus that are thought to facilitate learning and memory.

4.24.2 Understanding the Genetics of AS

4.24.2.1 The Prevalence of AS

The prevalence of AS among children and young adults is between 1/10,000 and 1/20,000 and is commonly accompanied by severe mental retardation, epilepsy, a puppet-like gait, dysmorphic facial features, a happy disposition with bouts of inappropriate laughter, hyperactivity, sleep disorders, and lack of speech. The etiology of AS arises from an absence of genetic contribution from a localized region on chromosome 15. Nearly 70% of AS cases involve cytogenetic deletion of q11-q13 (~4 Mb) on the maternally inherited chromosome 15 (15_{mat}).

Interestingly, 15q11 deletions also occur in Prader-Willi syndrome (PWI); however, the deletion is found on the paternally inherited chromosome 15 (15_{pat}), and the two disorders are characterized by distinct phenotypic differences (Cassidy et al., 2000). This suggests that there is differential expression of the genes in the homologous chromosome 15 and that the etiology of AS is specifically the consequence of cytogenetic disruption of the maternal chromosome (first to postulate this were Magenis in 1990 and Williams in 1990).

Understanding the genetic anomaly that defines AS begins in understanding how genes can be inherited in an active state from one parent and, alternatively, in an inactive state from the other parent. This phenomenon, referred to as genomic imprinting, is a poorly understood epigenetic mechanism affecting a relatively few known autosomal mammalian genes (Redei et al., 2006). Usually, autosomal genes are present in duplicate (i.e., two alleles), with one inherited from the father and one from the mother. For the majority of genes, both alleles are transcribed (or expressed) equally. However, for a small subset of genes, known as imprinted genes, only one allele is expressed in a parent-of-origin-dependent manner. Note that the 'imprint' here refers to the epigenetic mechanism through which one allele is silenced and is completely unrelated to the classical 'filial imprinting' manifest at the behavioral level.

4.24.2.2 Maternal Imprinting and AS

The mechanism of imprinting involves the biochemical marking of DNA by methylation of CpG-rich domains in association with particular chromatin conformations. This epigenetic mark allows the molecular machinery of each cell in the progeny to recognize and appropriately express only one allele at a particular locus. For example, during maternal expression, paternally allelic DNA methylation silences the paternal transcript, and the remaining functional genes in the locus are exclusively expressed maternally. Chromosome 15 contains a cluster of imprinted genes in the q11-q13 region, many of which are involved in brain development and function and normally undergo exclusively maternal expression. Therefore, if cytogenetic deletion of q11-q13 occurs on 15_{mat}, none of the imprinted gene products are expressed. There are additional genetic mutations that can occur to produce null expression of these imprinted genes. For example, 7–9% of AS cases are the result of imprinting mutations in which both the maternal and paternal copies undergo paternal-like methylation, and 2–3% show paternal unpaired disomy (UPD), whereby two copies of the paternal chromosome region are inherited with no maternal copies present (**Figure 1**). Finally, there is a subpopulation of patients with clinically diagnosable AS who do not fall into the aforementioned three groups but, rather, represent familial-derived mutations of one or more genes in the maternal 15q11-q13 critical region.

The discovery that 15q11-q13 represents the AS critical region narrowed the search for candidate genes that give rise to AS. In 1997, a gene within the AS critical region named *Ube3a* was found to be mutated in approximately 5% of AS individuals. These mutations can be as small as 1 base pair. The product of the *Ube3a* gene was initially identified following viral studies of p53 tumor suppressor protein degradation by the oncogenic human papilloma virus (HPV). Researchers interested in HPV *E6* gene expression following infection found that the HPV E6 protein alone had no effect on p53; however, in the presence of an associated protein p53, degradation was observed (Huibregtse et al., 1991). This associated protein, referred to as E6-associated protein, or E6-AP, was found to be the 100-kDa protein encoded by the *Ube3a* gene. The *Ube3a* gene has a spatially restricted imprinted expression pattern.

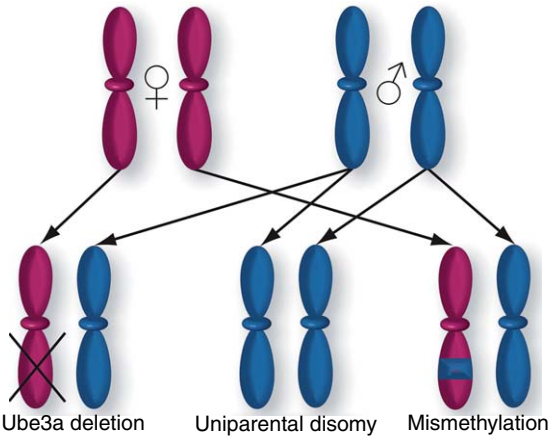


Figure 1 Maternal imprinting disorders resulting in Angelman Syndrome (AS). Genomic imprinting results in parent-specific epigenetic differentiation and monoallelic gene expression. Parental imprints are established during gametogenesis, where alleles of imprinted genes are maintained as either paternal or maternal origin. AS can arise from maternal deletion, uniparental disomy, or mismethylation of the AS critical region.

Ube3a shows imprinted expression in the brain but biallelic expression in other tissues. Each of the four genetic mechanisms described above that cause AS – large deletion, imprinting mutation, UPD, and point mutation – also cause inactivation or absence of the *Ube3a* gene.

4.24.2.3 The Ubiquitin Ligase Pathway

E6-AP is an enzymatic component of a complex protein degradation system termed the ubiquitin-proteosome pathway. This pathway is located in the cytoplasm of all cells. The pathway involves a small molecule, ubiquitin, which can be attached to proteins causing them to be degraded. Multiple steps are involved in the ubiquitination process (**Figure 2**). In the initial enzymatic step, the ubiquitin-activating enzyme E1 activates ubiquitin in an ATP-dependent reaction that links the E1 with the C terminus of a ubiquitin molecule. The ubiquitin-conjugating E2 enzymes then accept and transfer the activated ubiquitin moiety from E1 either to an E3 ligase or directly to a target substrate. The E3 ligase confers specificity to substrate recognition for ubiquitination. E6-AP ubiquitin ligase falls into the E3 category of ubiquitin lipases; therefore, it was believed that identifying the protein target(s) of E6-AP would unlock the mystery surrounding the AS disorder. To date, only four proteins have been identified as targets of E6-AP-dependent ubiquitination. These include the p53 tumor suppressor protein; the HHR23A protein, a human homolog to the yeast DNA repair protein Rad23 (Kumar et al., 1999); the multicopy maintenance protein (Mcm) 7 subunit involved in the initiation of DNA replication (Kuhne and Banks, 1998); and E6-AP, which is a target for itself (Nuber et al., 1998). Unfortunately, no obvious connection can be made between these identified targets and the etiology of AS.

4.24.3 Modeling AS in a Mouse

4.24.3.1 Production of the AS Mouse Model

The lack of a clear connection between E6-AP function and AS, coupled with the scarce availability of

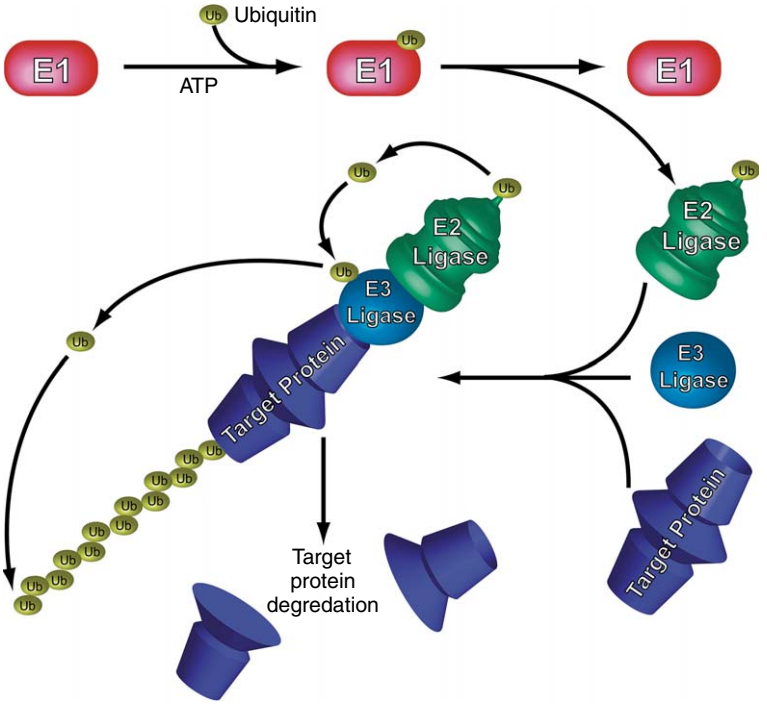


Figure 2 Ubiquitin ligase pathway. Association of an E1 ubiquitin ligase with a single ubiquitin (Ub) molecule results in an ATP-dependent manner. Ub is subsequently transferred to an E2 ligase. The E2 then associates with the E3, in this case Ube3a, and the target protein specified by the E3 ligase. The E3 ligase transfers successive Ub molecules to the target protein forming poly-Ub chains. When a critical length of the Ub chain is reached, the targeted protein associates with the proteosome and undergoes degradation.

AS postmortem brain tissue, necessitated the production of a mouse model for AS. Two mouse models were developed, each with a different strategy to disrupt the murine homolog of *Ube3a*. The mouse chromosome 7 contains a region exhibiting a similar gene compliment to the human AS critical region. The first mouse line, created by Gabriel and colleagues, utilized an Epstein-Barr virus latent membrane protein 2A (LMP2A) transgenic insertion that resulted in the deletion of the entire murine equivalent AS critical region. The rational for this method lies in mimicking what is seen in humans that exhibit the most common genetic defect leading to AS: an approximately 4-Mb deletion in the 15_{mat}q11-q13 region. Alternatively, the mouse model developed by Jiang and colleagues utilized a null mutation in *Ube3a* via a single gene knockout (Jiang et al., 1998). While both of these mouse models effectively disrupt *Ube3a*, there exists some controversy over whether *Ube3a* deficiency in and of itself is responsible for the manifestation of AS, or if the full phenotype is due to the combined affect of disrupting several genes in the human chromosome 15_{mat}q11-q13 critical region (Lee and Wevrick, 2000).

A phenotypic assessment in patients with the cytogenetic deletion (class I) exhibit a profile more true to what is commonly considered to be clinically classical AS. These patients show the full realm of AS characteristics including microcephaly and hypopigmentation. In contrast, patients for whom AS arises from specific *Ube3a* mutation (class II) or *Ube3a* mismethylation (class III) result in patients with fewer instances of hypopigmentation, microcephaly, and seizure. This indicates that AS caused by *Ube3a* disruption is exacerbated by the disruption of additional genes located at the same locus. Among these genes is the $GABA_A$ receptor $\beta 3$ subunit (*GABRB3*). There is evidence that the inhibitory neurotransmitter GABA is involved in the suppression of seizures, which may explain why class II and III AS patients experience fewer occurrences of seizure. In support of this hypothesis, a lower instance of seizure activity in the Jiang et al. mouse model compared to the other mouse model was observed. Because nearly all available evidence points to *Ube3a* as the locus for AS, and given the inherent difficulties in interpreting the effects of multiple gene deletions in mice, the consensus was that the single *Ube3a*-null mutation mouse would provide a better instrument to investigate the molecular mechanisms underlying the AS etiology. The following section discusses experiments conducted using the Jiang et al. AS mouse model and relates the results to the human condition.

4.24.3.2 Characterization of the AS Mouse Model

The initial characterization of the maternal deficient *Ube3a*-null mouse mutant (*Ube3a* m^-/p^+) revealed striking similarities to the human phenotype (Jiang et al., 1998). These phenotypic similarities are more easily discernible when they are subdivided into categories of physical and physiologic characteristics.

4.24.3.2.1 Physical Similarities of AS and the Maternal Deficient Ube3a-Null Mouse

The brains of *Ube3a*-m^-/p^+ mice showed an overall normal morphology, with no obvious abnormalities in any particular brain regions, suggesting that maternal E6-AP deficiency does not effect neuronal development or organization. However, by 18 days of age *Ube3a*-m^-/p^+ mice were observed to have smaller bodies and brains than wild-type mice. This size difference was seen in both the cortex and cerebellum of the brain. Imprinted expression analysis revealed that, similar to the human *Ube3a* gene, the murine *Ube3a* gene also exhibited a spatially restricted imprinted expression pattern in which exclusively maternal expression was observed in the brain. In adult wild-type mice, the detection of *Ube3a* mRNA revealed high expression levels in areas CA1 and CA3 of the hippocampus, basal ganglia, cerebellum, and cerebral cortex and the periglomerular, granual and mitral cells of the olfactory bulb. This was compared to maternal deficient mice that showed no detectable hippocampal expression, reduced cerebellar expression with no detectable expression in the Purkinje cell layer, and an overall reduced expression in the olfactory region associated with a lack of detectable expression in the mitral cell layer.

Motor function and coordination was assessed using hind-paw footprint analysis, the bar crossing test and the accelerating Rotorod test. Each of these tests confirmed a distinct and significant motor deficit in the *Ube3a*-m^-/p^+ mice, which mimics the tremor, ataxia and motor coordination defect described in human AS patients. In addition, seizure in AS patients is very prominent and found to affect greater than 90% of diagnosed AS patients. Likewise, seizures in *Ube3a*-m^-/p^+ mice could easily be induced through audiogenic means by simply running an object vigorously over the metal grate lid of

the mouse's home cage. Physical changes are more easily quantifiable and tend to be a more convincing argument for the recapitulation of a human mouse model. However, there are several ways to measure the behavioral correlates to human cognitive ability in the mouse by assessing its learning ability through behavioral testing and its function at a synaptic level through electrophysiologic techniques.

4.24.3.2.2 Cognitive Similarities of AS and the Maternal Deficient Ube3a-Null Mouse

The hippocampus is intimately involved in spatial memory processes and is linked to numerous cortical regions. Acquisition of explicit memory involves the hippocampus as part of a polymodal sensory integration scheme that processes visual, auditory, and somatosensory inputs. A great deal of evidence now implicates the hippocampus as an important brain structure involved in cognitive processing in humans. Although determining cognition is less straightforward in mice than it is in humans, we can call upon Pavlovian conditioning paradigms to asses the associative learning ability of *Ube3a*-m^-/p^+ mice.

The cognitive ability of the *Ube3a*-m^-/p^+ mice was assessed with the fear conditioning learning paradigm. The training paradigm consisted of an auditory conditioned stimulus (CS) paired twice with an unconditioned aversive stimulus (US: a mild foot shock) (*See* Chapter 4.11). The mice demonstrated their ability to associate the CS as a cue for the US or the training chamber context and the US by exhibiting a freezing behavior when re-presented with the CS or the training chamber. Although both contextual and cued fear-conditioned learning is dependent on the proper function of the amygdala, only the contextual learning is hippocampus dependent. The *Ube3a*-m^-/p^+ mutant mice exhibited robust freezing when re-presented with the CS 24 h after training, but significantly less freezing when reintroduced to the training chamber. These results indicate that hippocampus-dependent associative learning is disrupted in the *Ube3a*-m^-/p^+ mutant mice and is concordant with the imprinted expression pattern of *Ube3a*.

4.24.3.2.3 Physiologic Similarities of AS and the Maternal Deficient Ube3a-Null Mouse

The lack of hippocampal *Ube3a* expression coupled with the severe hippocampus-dependent learning deficit made the characterization of hippocampal synaptic function essential. The hippocampus is separated into three distinct synaptic pathways: the perforant path, the mossy fiber synapse, and the Schaffer collateral synapses. Of these three major synaptic connections, the Schaffer collaterals in area CA1 of the hippocampus are by far the most well characterized. It is in this region that synaptic transmission and plasticity were characterized in the *Ube3a*-m^-/p^+ mice. Jiang and colleagues found that basal synaptic transmission was unaltered in the AS mouse. This indicated that overall connectivity and single-stimulus synaptic function were essentially normal. Next, synaptic plasticity induced with high-frequency stimulation (HFS) was tested. It was shown that *Ube3a*-m^-/p^+ mice exhibited a severe deficit in long-term potentiation (LTP) induction. Although controversial, parallels can be drawn between the mechanisms of synaptic strengthening that occur during hippocampal LTP and those postulated to occur during hippocampus-dependent learning and memory events. Thus, a deficit in hippocampal LTP is consistent with a deficit in learning and memory. Perhaps most importantly, these studies were the first to implicate a deficit in hippocampal LTP in a human learning disability.

4.24.3.3 AS Mouse Hippocampal Physiology

It took nearly 3 years to identify a specific biochemical alteration in the AS mouse. Initially, the strategy employed identification of proteins that exhibited (1) a lack of ubiquitination in the AS mouse compared to normal mouse brain protein complement and (2) increased levels due to lack of degradation through the ubiquitin proteosome pathway. Although sound in design, identifying short-lived ubiquitinated proteins is problematic, and no gross changes in protein levels were observed in the AS mouse imprinted brain regions. It was not until a more in-depth analysis of hippocampal synaptic plasticity was performed that new and interesting implications of maternal Ube3a deficiency in the hippocampus were revealed.

Multiple stimulation patterns will elicit LTP in area CA1 of the hippocampus. To better understand the basis of the LTP deficit in the AS mouse, it is prudent to consider the LTP response to different patterns of stimulation (*See* Chapter 4.16). A standard LTP-inducing stimulation consisting of a 1-s, 100-Hz

stimulation elicits a long-lasting increase in synaptic plasticity. Alternatively, increasing the number of high-frequency trains of stimulation can produce a potentiation that is both substantially higher in magnitude and longer lasting. Manipulating these variables in the LTP induction protocol is often used to determine the efficacy or potency of an applied drug or, as in this case, to evaluate the severity or penetrance of the Ube3a deficiency. In order to test the possibility that the LTP deficits in *Ube3a*-m^-/p^+ mutants were due to an increase in the threshold of LTP induction, LTP was induced with a saturating amount of HFS designed to give the maximum potentiation in a given hippocampal slice. It was found that the saturating HFS could rescue the LTP deficit in the AS mouse.

What was the mechanism by which the LTP deficit could be overcome? The induction of LTP is highly dependent upon an influx of postsynaptic Ca^{2+}. Increasing the number of stimulations would increase the amount of Ca^{2+} influx significantly and could explain the LTP rescue. The question then would be whether the derangement of LTP induction was upstream (receptor activation) or downstream of calcium influx. The major neurotransmitter-activated calcium channel at the CA1 hippocampal synapse is the *N*-methyl-D-aspartate receptor (NMDAR) (*See* Chapter 4.20). Slices stimulated with repeated very high frequency stimulation in the presence of the NMDA receptor antagonist DL-2-Amino-5-phosphonopentanoic acid (AP5) would determine whether the increase in LTP threshold observed in *Ube3a* m^-/p^+ mutants was due to insufficient postsynaptic NMDAR-dependent Ca^{2+} influx. Under these conditions, the LTP deficit in the *Ube3a*-m^-/p^+ mice remained. The inability of *Ube3a*-m^-/p^+ mutants to achieve NMDAR-independent LTP induction suggested that the LTP deficit, and importantly, the potential site of hippocampal synaptic dysfunction, resided downstream of calcium influx. The studies described earlier set the stage for the investigation into the molecular derangements that could be responsible for the cognitive loss in AS patients.

4.24.4 Molecular Changes in the AS Mouse

It has been hypothesized that the deficit in LTP and learning was biochemical in origin. The lack of any morphological changes in the CNS, coupled with the ability to rescue LTP, suggested that a disruption in a biochemical signal transduction pathway was the explanation. The rescue of LTP also suggested that the mechanisms responsible for proper synaptic function were present but that the system needed to be pushed, (i.e., during repeated HFS) in order for the system to function at a level for LTP induction to occur. More importantly, the in-depth electrophysiology described earlier reduced the number of proteins to be examined from the greater than 10 000 present in the central nervous system to just a handful. The proteins chosen to be examined closer had to meet the following specific criteria: (1) known to be involved in LTP induction processes, (2) known to be involved in learning and memory processes, and (3) known to be activated or modified in the presence of Ca^{2+}. Extensive research showed no changes in the major synaptic-associated protein kinases including the phosphorylation levels of PKC, PKA, or ERK (P42) isolated from hippocampal homogenates of *Ube3a*-m^-/p^+ mice. However, a significant increase was detected in phospho-αCaMKII at the autophosphorylation site threonine 286 (Thr^{286}) (**Figure 3**) and threonine 305/306 (Thr^{305}/Thr^{306}). This single observation represented the first identified biochemical alteration in the AS mouse model that could potentially explain the learning and LTP deficits in the mouse and the cognitive deficits in human AS (Weeber et al., 2003). To help explain the implications of CaMKII misregulation, the next section addresses what is currently known about CaMKII regulation and its importance in normal signaling in the mammalian CNS.

4.24.5 CaMKII in Synaptic Plasticity and Memory Formation

4.24.5.1 Activation and Regulation of CaMKII

Initial studies of CaMKII's involvement in memory formation were conducted using an *in vitro* model for memory formation: LTP of the Schaffer-collateral synapses in area CA1 of the hippocampus. The Schaffer-collateral synapses are excitatory and use L-glutamate as their excitatory neurotransmitter. It has long been appreciated that induction of LTP requires high-frequency synaptic activity (Bliss and Lomo, 1973; Alger and Teyler, 1976) that results in activation of two types of glutamate receptors on postsynaptic CA1 pyramidal neurons: alpha-amino-3-hydroxy-

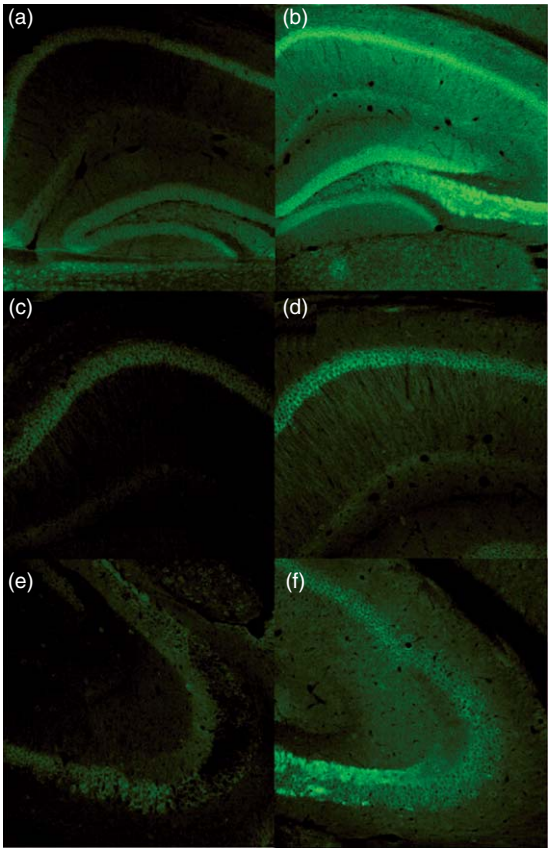


Figure 3 Increased threonine 286 Thr^{286} CaMKII phosphorylation in the Angelman Syndrome mouse model. Increased immunoreactivity to phosphorylated CaMKII at Thr^{286} in Angelman mouse hippocampus. Phosphorylated CaMKII at Thr^{286} was detected immunohistochemically in the hippocampi of wild type (a, c, e) and Angelman (b, d, f) mice. Increases in immunoreactivity are seen in the stratum pyramidale, stratum oriens, and stratum radiatum of CA1 and CA3 as well as the molecular layer and granule cell layer of the dentate gyrus. a, b, 100× magnification of the hippocampus. c, d, 200× magnification of area CA1. e, f, 200× magnification of area CA3.

5-methyl-4-isoxazole propionic acid (AMPA) and NMDA receptors. AMPA receptors have an ion channel that, when activated by glutamate, allows Na^+ to enter the cell, resulting in depolarization of the postsynaptic membrane (*See* Chapter 4.30). NMDARs are also coupled to an ion channel; however, under resting conditions this ion channel is blocked by a molecule of Mg^{2+} (*See* Chapter 4.20). Once the membrane is depolarized, the Mg^{2+} block is removed and Ca^{2+} ions flow through the NMDAR ion channel into the cytoplasm. The resulting increase in intracellular Ca^{2+} mediated by the NMDAR is the primary signal for induction of LTP (Lynch et al., 1983; Harris et al., 1984; Morris et al., 1986; Malenka et al., 1988). The increase in cytoplasmic Ca^{2+} concentration mediated by NMDARs is relatively short-lived, and therefore it cannot account for the long-term increase in synaptic efficacy observed after induction of LTP (*See* Chapter 4.21). Therefore, it has been proposed that the initial increase in Ca^{2+} leads to the activation of several downstream kinases, which modify the function of the various synaptic proteins and induce long-lasting changes in synaptic efficacy. This intimate relationship between NMDAR-mediated Ca^{2+} influx and CaMKII activation brings us back to the LTP deficit seen in the AS mouse. Saturating amounts of high-frequency stimulation, causing an optimum amount of Ca^{2+} influx, were used to overcome the LTP deficit. The signaling and autoregulatory aspects of Ca^{2+} on CaMKII will become more apparent in the following section.

The regulation of CaMKII through autophosphorylation mechanisms is extraordinarily dynamic and is a subject of review in a corresponding chapter of this volume (*See* Chapter 4.23). Briefly, Ca^{2+}-calmodulin activates CaMKII by disrupting the association between the catalytic and inhibitory domains (Colbran et al., 1989) (**Figure 4**). Once bound to Ca^{2+}-calmodulin, CaMKII undergoes autophosphorylation at amino acid residue Thr^{286}, a residue located within the inhibitory domain. Phosphorylation of Thr^{286} has three consequences for CaMKII function: the kinase becomes autonomously active (Saitoh and Schwartz, 1985), the affinity of Ca^{2+}-calmodulin for CaMKII increases 1000-fold, and a site becomes exposed, allowing CaMKII to bind NMDA glutamate receptors (Strack and Colbran, 1998; Leonard et al., 1999; Bayer et al., 2001). There exists another autophosphorylation site within the regulatory domain of the enzyme at threonine 305 and 306 ($Thr^{305/306}$) of the alpha and beta subunits, respectively. The potential functional consequences of phosphorylation at $Thr^{305/306}$ are poorly understood. However, it has been shown that $Thr^{305/306}$ phosphorylation can render the CaMKII enzyme inactive (Colbran, 1993). In addition, the $Thr^{305/306}$ resides in the Ca^{2+}-calmodulin binding site of CaMKII and inhibits subsequent binding of Ca^{2+}-calmodulin to CaMKII. Phosphorylation of $Thr^{305/306}$ can also occur in the absence of activation of CaMKII and possibly prevents subsequent activation by Ca^{2+}-calmodulin. This management of activation, alterations in Ca^{2+} and calmodulin sensitivity, and autophosphorylation is highly orchestrated.

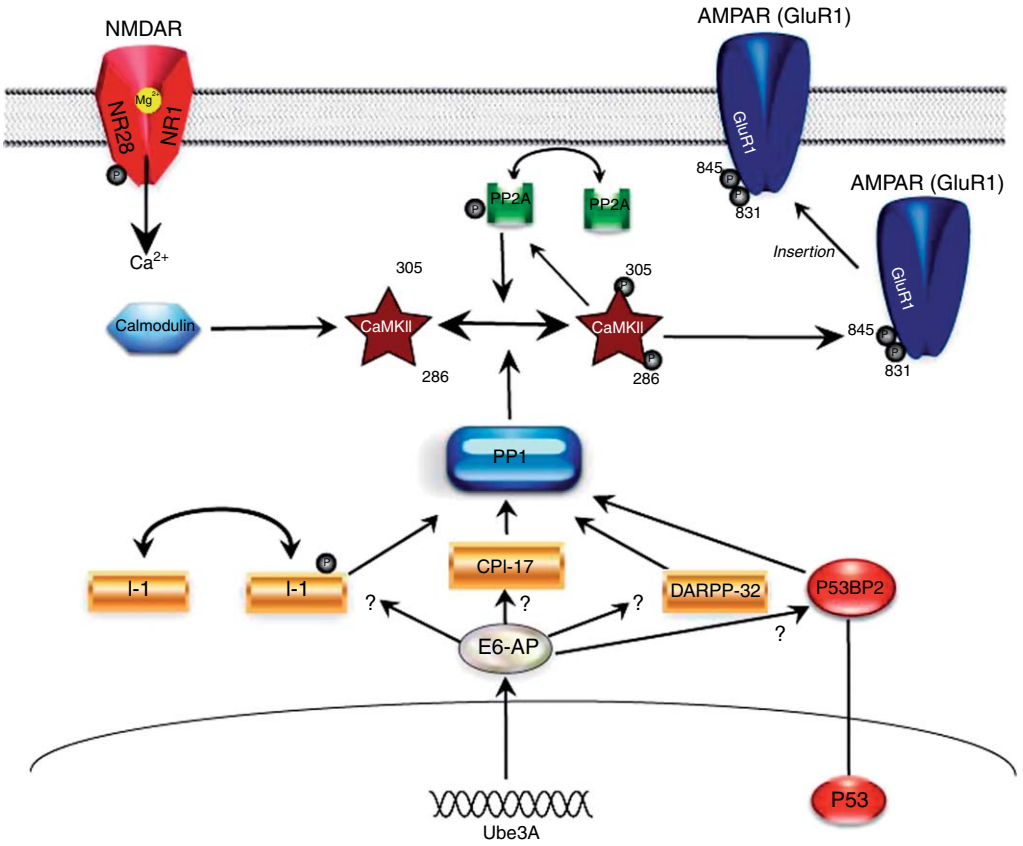


Figure 4 Schematic model of CaMKII regulation in the hippocampus. Inactivated CaMKII is located in the cytoplasm. Once activated by calcium-calmodulin and phosphorylated at threonine 286 (Thr^{286}), CaMKII translocates to the postsynaptic density, where it associates with *N*-methyl-D-aspartate receptors and is involved in alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor insertion. Autophosphorylation at $Thr^{305/306}$ decreases the affinity of CaMKII for *N*-methyl-D-aspartate receptors and signals for CaMKII translocation away from the postsynaptic density. Dephosphorylation of CaMKII by phosphatase activity once again allows sensitivity of CaMKII to future signaling via calcium influx.

Thus, even slight disruptions of this process can have devastating affects on synaptic function and cognitive ability (Colbran and Brown, 2004).

4.24.5.2 Regulation of CaMKII Activity in Synaptic Plasticity and Memory Formation

How is autophosphorylation tied to the regulation of synaptic plasticity? Recent studies have examined the role of the Thr^{286} and $Thr^{305/306}$ autophosphorylation in CaMKII-dependent synaptic function. Genetically modifying the Thr^{286} site to an alanine (T286A), which prevents phosphorylation at the Thr^{286} site, results in complete loss of LTP induction. Genetically modifying the Thr^{286} site to an aspartate (T286D), which mimics phosphorylation, also leads to impairment of LTP induction. More refined studies suggest that low levels of the autonomously active kinase facilitate, while high levels of autonomously active kinase inhibit, LTP induction. Alternatively, mutating the $Thr^{305/306}$ sites from a threonine to an alanine (T305/306A) increases the amount of CaMKII in the postsynaptic density (PSD) (*See* Chapter 4.32) and enhances LTP induction (Elgersma et al., 2002). Furthermore, mutating the threonine to an aspartate (T305/306D) decreases the amount of CaMKII in the PSD and blocks induction of LTP (Elgersma et al., 2002). Together, these results indicate that CaMKII is critical in the induction of LTP and must be active in a precise spatiotemporal pattern. Synaptic plasticity in the hippocampus is believed to be involved in the formation of spatial memories. Disruption of CaMKII function has profound effects on hippocampal LTP; therefore, one would expect disruption of CaMKII to have dramatic effects on long-term memory

formation. Animals lacking the CaMKII gene show profound deficits in spatial learning tasks (Silva et al., 1992; Bach et al., 1995). In addition, both sites of autophosphorylation appear to be involved in the formation of long-term memory. Deficits in spatial learning tasks are observed in animals that have either the T286A or T286D mutation. Moreover, animals possessing the T305/6A mutation exhibited enhanced spatial learning, while animals with the T305D mutation had severe deficits in spatial learning. All of these observations indicate that CaMKII is critical for the proper formation of hippocampus-dependent spatial memory.

4.24.6 Genetic Rescue of the AS Phenotype

The identified alterations in CaMKII raise the question of which alteration in phosphorylation, Thr^{286}, $Thr^{305/306}$, or both, is responsible for the observed AS phenotype. Due to the severe detrimental effects of CaMKII $Thr^{305/306}$ phosphorylation on synaptic plasticity and memory formation, as well as the similarities in the phenotypes with the AS mouse, the $Thr^{305/306}$ site was hypothesized to be responsible for the major phenotypes seen in AS. Studies in the laboratories of Weeber and Elgersma utilized female AS mice crossed with heterozygous αCaMKII males that carried the targeted *αCaMKII-T305V/T306A* mutation (CaMKII-TT305/6AV). This point mutation essentially prevents CaMKII inhibitory phosphorylation , but the use of heterozygotes allows a subset of endogenous CaMKII to be present without the mutation. Thus, the resulting F1 offspring yielded mutants with four different genotypes: wild-type (WT) mice, mutants carrying the single $\alpha CaMKII\text{-}305/6^{+/-}$ or AS mutation, and mice carrying the double AS/CaMKII-$305/6^{+/-}$ mutation. Hippocampal-dependent learning was assessed using the contextual fear-conditioning paradigm (*See* Chapter 4.11), utilizing the same protocol of two US–CS pairings employed in the original AS mouse characterization. The genetic prevention of $Thr^{305/306}$ phosphorylation is sufficient to rescue the defect in learning and memory. The CaMKII-$305/6^{+/-}$ mice can learn to associate the context and the foot shock to the same extent as wild-type littermates tested 24 h or 7 days following training (**Figure 5**). These results support the hypothesis that the underlying hippocampal learning deficit is due primarily to αCaMKII inhibitory phosphorylation.

Interestingly, αCaMKII inhibitory phosphorylation can influence the threshold for the induction of LTP. This is nicely demonstrated in the homozygous CaMKII-TT305/6AV mouse that exhibits increased LTP induction when a subthreshold stimulation is given, but in which normal LTP is induced when a strong stimulation is applied (*See* Chapter 4.16). In contrast, AS mice exhibit a significant LTP deficit utilizing a modest stimulation protocol, which is rescued using multiple trains of HFS. Consistent with the associative learning rescue experiments, AS mice showed a severe LTP deficit compared to wild-type mice and CaMKII-$305/6^{+/-}$ mice. However, the AS/CaMKII-$305/6^{+/-}$ double mutants show comparable LTP to that of wild-type mice and lack the LTP deficit seen in their AS mouse littermates (**Figure 5**). These studies dramatically show that the synaptic plasticity deficits established in the AS mice are the result of increased CaMKII inhibitory phosphorylation. Thus, genetic manipulation of the phosphorylation state of the inhibitory site of CaMKII effectively rescues both the synaptic plasticity deficit and cognitive disruption in the *Ube3a*-null mutation mouse model of AS.

The studies described here strongly suggest that the increased inhibitory phosphorylation of CaMKII is the site of molecular dysfunction underlying the cognitive and synaptic plasticity deficits associated with AS. This validates the great amount of research showing CaMKII dependence for normal synaptic plasticity and memory formation. Astonishingly, AS represents the lone human disorder that to date presents with a disruption in cognition that is associated with CaMKII dysfunction. This is likely attributed to both the importance of CaMKII substrates in peripheral cellular function and the number of developmental pathways in which CaMKII is enlisted. If not for the limited region-specific deletion of Ube3a, it is likely AS would result in embryonic lethality. This consideration raises the obvious question of whether the genetic rescue with the CaMKII-$305/6^{+/-}$ mutation is due to the normalization of the CaMKII signaling capacity in the adult, the ability for normal CaMKII-dependent development of the central nervous system, or both. The observation of occasional abnormalities in AS patients including scoliosis, hyperreflexia, hypopigmentation, strabismus, myopia or hypermetropia, and nystagmus would suggest a development component resulting from a CaMKII dysfunction.

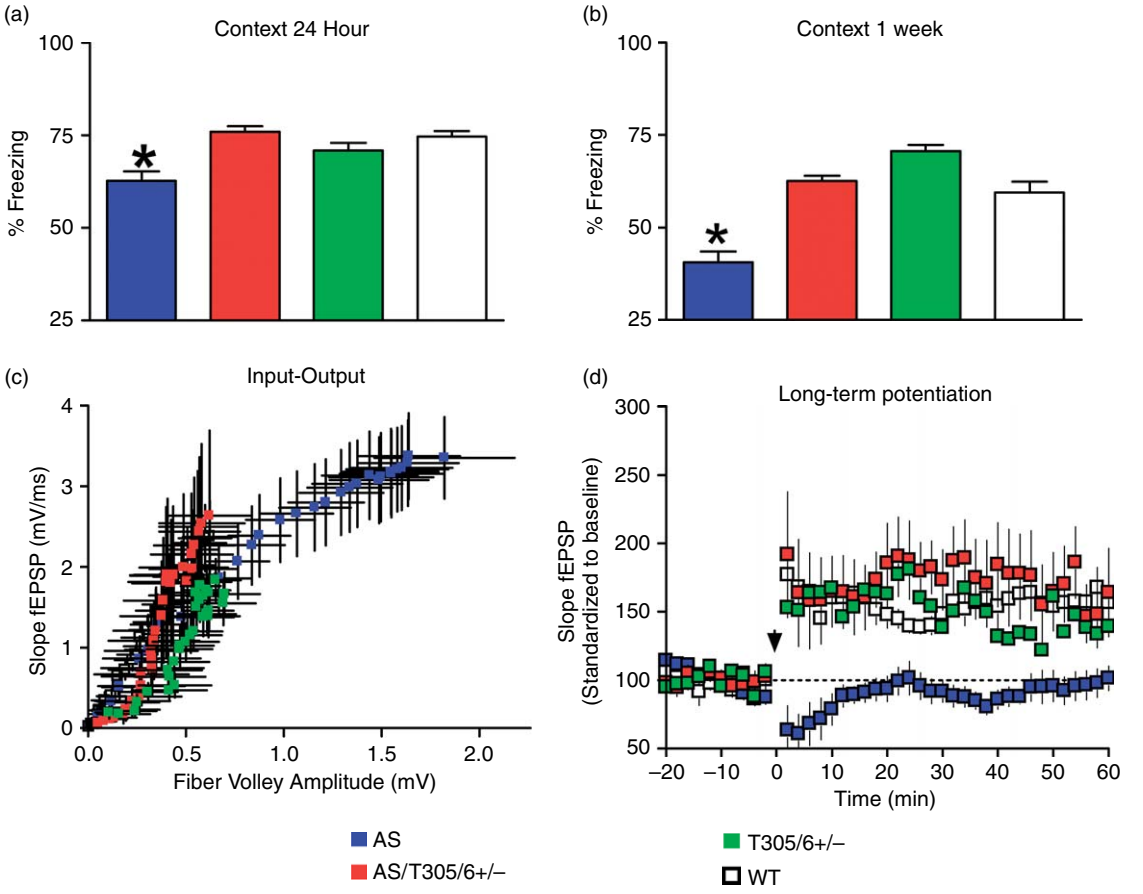


Figure 5 Hippocampal learning and synaptic plasticity in AS mice is rescued by the T305/6-CaMKII mutation. Mice trained in a conditioning chamber through the association with the context and two mild foot shocks were later reintroduced to the context. Freezing was assessed during a 3-min time period, and the amount of freezing represents the strength of the associative memory formed. Angelman Syndrome (AS) mutants show impaired contextual conditioning 24 h (a) and 1 week (b) after training, which is rescued in the AS/T305/6-CaMKII$^{+/-}$ mutants. Increased synaptic transmission (c) and disruption of LTP (d) is rescued in *Ube-3a*-m^{-}/p^{+}/T305/6-CaMKII$^{+/-}$ double mutants compared to AS mice. LTP was induced with two trains of 100-Hz stimulation delivered 20 s apart (represented by arrow) in hippocampus area CA1.

4.24.7 Proposed Mechanisms Underlying CaMKII Misregulation

The genetic rescue of the AS phenotype is a tremendous step forward, but the molecular connection between ubiquitin deficiency and alterations in CaMKII phosphorylation remains unclear. As discussed above, CaMKII cycles between phosphorylated and nonphosphorylated states. The cycling state of CaMKII is not unlike many other proteins involved in signal transduction processes that undergo endless cycles of phosphorylation and dephosphorylation. Evolution has perfected this process in the CNS in order to provide an exceptionally rapid and potent mechanism for the regulation of protein function. The phosphorylation of numerous proteins is kept in check by three major protein phosphatases (PP) found in the brain: PP1, PP2A, and PP2B (calcineurin). PP1 and PP2A appear to be primarily responsible for dephosphorylation of CaMKII at Thr^{286}, with PP2A regulating CaMKII phosphorylation in the cytoplasm and PP1 in the postsynaptic density (Mansuy, 2003) (**Figure 4**). Through the actions of PP1 and PP2A, the activity of CaMKII is held in check and allows the enzyme to return to a state where Ca^{2+} influx can again activate the proper signal transduction cascades involving CaMKII. The presence of PP1 and PP2A causes an apparent conundrum in the regulation of CaMKII. CaMKII is a Ca^{2+}-dependent kinase; however, under

conditions conducive to induction of long-term synaptic plasticity or memory formation, CaMKII undergoes autophosphorylation and becomes a Ca^{2+}-independent autonomous enzyme. How does this occur in the presence of PP1 and PP2A? Several proteins have been identified that act as inhibitors of PP activity. One example is the Inhibitor 1 (I1) protein (Huang and Glinsmann, 1976). When phosphorylated via protein kinase A, I1 can bind to and inhibit the activity of PP1. Inhibition of PP1 via I1 facilitates the transition of CaMKII from a Ca^{2+}-dependent to an autonomous state. The functional consequences of PPs on CaMKII activity are not solely negative. As noted above, CaMKII contains an inhibitory autophosphorylation site at $Thr^{305/306}$. Phosphorylation at this site downregulates CaMKII activity and prevents binding by Ca^{2+}-calmodulin. PP1 can dephosphorylate $Thr^{305/306}$, which then allows for reactivation of CaMKII by Ca^{2+}-calmodulin (Patton et al., 1990). **Figure 4** shows the highly regulated action of protein phosphatases, and the proteins that control their activity as well. Thus, the changes in the phosphorylation state of AS mouse hippocampal CaMKII could be due to changes in protein levels in one or more of these proteins. Interestingly, PP1 and/or PP2A phosphatase activity is significantly reduced in the AS mutants by a prodigious 2.5-fold decrease (Weeber et al., 2003). This finding strongly suggests that the aberrant state of P-Thr^{286} and P-Thr^{305} αCaMKII phosphorylation is due to changes in the activity of one or both of these important phosphatases. This also suggests that the increase in Thr^{305} αCaMKII is due to both the increase in P-Thr^{286} αCaMKII, since phosphorylation at Thr^{286} precedes phosphorylation at Thr^{305} (Colbran and Soderling, 1990; Hanson and Schulman, 1992), and reduced PP1 and/or PP2A activity, which are known to dephosphorylate P-Thr^{305} αCaMKII *in vitro* (Patton et al., 1990).

4.24.8 Concluding Remarks

The studies discussed here have set the stage for the next phase of research that will work toward uncovering the molecular pathways and mechanisms linking Ube3a maternal deficiency and CaMKII regulation. Future discoveries of E6-AP targets will likely shed the most light on this puzzling connection. Regardless, important lessons have been learned from the discovery of AS allele and its regulation. First, the production of the AS mouse model reemphasizes the utility and importance of mouse models for human disorders (*See* Chapters 4.19, 4.33, 4.42). Second, AS was one of the first human mental retardation disorders to bring to light the potential deleterious effects of epigenetic changes. The identification that the AS gene expresses a housekeeping-type protein dramatically illustrates the unexpected importance of proteins such as ubiquitin ligases once considered minor for normal synaptic function. Finally, these studies illustrate a compelling example of a human learning and memory disorder associated with deficits in hippocampal synaptic plasticity and CaMKII function.

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4.25 Mitogen-Activated Protein Kinases in Synaptic Plasticity and Memory

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4.25.1 Introduction

Mitogen-activated protein kinases (MAPK) make up an evolutionarily conserved family that regulates cellular growth and stress responses in mitotic cells throughout eukaryotic phyla (reviewed in Chang and Karin, 2001; Pearson et al., 2001). Mammalian cells express three major subfamilies of MAPK: The extracellular-regulated kinases (ERKs), the c-Jun amino-terminal kinases (JNKs), and p38 MAPKs. Each of these subfamilies of MAPKs forms part of a modular kinase cascade in which a MAPK is activated by dedicated MAPK kinases (MAPKKs or MAP2Ks, also designated MKKs or MEKs), which are in turn activated by multiple MAPK kinase kinases (MAPKKKs or MAP3Ks, also designated MKKKs or MEKKs). Sequential phosphorylation within these modules allows for integration of extracellular signals as well as increased specificity and amplification of MAPK-mediated downstream responses. Scaffold proteins that facilitate the interaction of components of these tripartite MAPK modules further enhance specificity with respect to both activating stimuli and substrates, allowing for precise spatial and temporal control of signaling activity.

Each MAPK subfamily or module is characterized by the existence of multiple isoforms at each hierarchical level: ERK1-2 are activated by MEK1-2, JNK1-3 are activated by MEK4 and MEK7, and p38α-δ are activated by MEK3 and MEK6. In contrast to the essentially monogamous relationship between MAP2Ks and their substrate MAPKs,

MAP3Ks can activate multiple MAP2Ks, and conversely, a given MAP2K can be activated by multiple MAP3Ks, depending on the nature of the extracellular signal. MAPKs are proline-directed serine/threonine kinases that phosphorylate substrates preferentially at (S/T)P target sequences. MAP2Ks are dual specificity kinases that phosphorylate both threonine and tyrosine residues in a conserved TXY target sequence in the activation loop of MAPK substrates. Dual phosphorylation by a MAPKK is absolutely required for MAPK activity under most conditions, and experimental detection of dually phosphorylated MAPK therefore provides a useful marker for MAPK activity. MAPK activation is antagonized by a group of protein phosphatases that dephosphorylate the critical threonine and/or tyrosine residues in the activation loop (reviewed in Keyse, 2000; Barr and Knapp, 2006).

The prototype and best-studied of the mammalian MAPK subfamilies is the ERK cascade. ERK1/2 are activated downstream of Ras-coupled tyrosine kinase receptors (RTKs) in response to growth, proliferation, or differentiation signals. GTP-bound Ras activates the MAP3Ks B-Raf and Raf-1, which leads to sequential activation of MEK1/2 and ERK1/2. The signaling activity of Ras is activated by guanine nucleotide exchange factors (GEFs), which stimulate replacement of Ras-bound GDP with GTP; conversely, Ras activity is suppressed by GTPase-activating proteins (GAPs), which stimulate the intrinsically weak GTPase activity of Ras. Consistent with the role of the ERK pathway in cell growth and proliferation, activating mutations in multiple components of this cascade, including Ras, Raf, and MEK, confer oncogenic properties and have been associated with human cancers (Schubbert et al., 2007). Furthermore, germline mutations in Ras, Raf, and MEK, which are associated with upregulation of ERK activity, are responsible for several developmental disorders, including Noonan, Costello, and cardiofaciocutaneous syndrome (Schubbert et al., 2007).

In addition to the well-described role of RTKs and Ras in activating the Raf-MEK-ERK module, ERK activity can also be regulated by seven-transmembrane domain G protein-coupled receptors (GPCRs) through G protein-dependent and -independent mechanisms. Agonist binding to GPCRs results in G protein activation and stimulation of adenylyl cyclase. Elevated intracellular cyclic adenosine monophosphate (cAMP) levels activate the small GTPase Rap1, and activated Rap1 can either stimulate or inhibit Ras-dependent ERK activation in a cell type-specific manner (Stork and Schmitt, 2002). Agonist binding to GPCRs also induces phosphorylation of the receptors by G protein–coupled receptor kinases (GRKs), promoting recruitment of β-arrestins and receptor desensitization. β-arrestins uncouple GPCRs from G proteins by blocking their interaction, but at the same time β-arrestins provide a scaffold for assembly of Src tyrosine kinases with components of the Raf-MEK-ERK family, resulting in ERK activation (Miller and Lefkowitz, 2001; Morrison and Davis, 2003). In contrast to the strong, transient ERK activation induced by Ras-coupled RTKs, which often elicits nuclear translocation of ERKs, GPCR-dependent ERK activation exhibits a more modest, sustained and localized profile.

Transcriptional regulation is a major downstream effector mechanism by which the ERK pathway transduces mitogenic signals into growth responses (Treisman, 1996). Two principal routes to transcriptional regulation by the ERK pathway have been characterized. First, activated ERK translocates to the nucleus and directly phosphorylates a number of transcription factor substrates, including Elk-1 and c-Myc. Phosphorylation of Elk-1 by the ERK pathway has been shown to stimulate serum response element (SRE)-dependent transcription. Second, ERK can activate several intermediary kinases, which then phosphorylate transcription factors and modulate their activity. For example, the ERK substrates Rsk2, MAPKAP kinase 2, and Msk1/2 have all been reported to phosphorylate the cAMP response element-binding protein (CREB) on a serine residue (Ser133) critical for its transcriptional activation function. Expression of the immediate-early gene *c-fos* is highly responsive to ERK activation due to the presence of both SRE and cAMP response element (CRE) sequences in its proximal promoter.

Although there is some overlap among the ERK, JNK, and p38 pathways with respect to activating stimuli in mitotic cells, the JNK and p38 cascades respond primarily to physical and chemical stress. The JNKs, which are also known as stress-activated kinases (SAPKs), are activated in response to ultraviolet (UV) irradiation, growth factor deprivation, cytokines, and inhibition of macromolecular synthesis. The p38 MAPKs can be activated by cytokines, UV irradiation, and osmotic and oxidative stress. Transcriptional regulation is a major effector mechanism by which the JNK and p38 cascades induce specific cellular responses, such as JNK-dependent apoptosis and p38-dependent cytokine production and inflammatory responses.

Components of MAPK cascades exhibit widespread and abundant neuronal expression throughout the central nervous system (Fiore et al., 1993a). In view of the well-characterized role of the ERK cascade in cellular growth and proliferation, it was initially unclear what functional role they might serve in postmitotic neurons. Fundamentally, however, MAPK cascades enable cells to produce appropriate and adaptive responses to their extracellular environment. Therefore, it is perhaps not surprising that MAPK cascades have been co-opted in neurons to mediate cellular responses to synaptic stimulation. In particular, MAPK signaling plays a central role in regulating modifications of synaptic strength and structure, which may represent the neuronal analog of growth in mitotic cells.

A number of excellent reviews have previously summarized the accumulating evidence for essential roles of MAPK in synaptic plasticity, learning, and memory in both mammals and invertebrates (Sweatt, 2001, 2004; Sharma and Carew, 2004; Thomas and Huganir, 2004; Davis and Laroche, 2006). Our objective here is to provide an up-to-date picture of the involvement of MAPK pathways in these processes, highlighting emerging evidence for a selective contribution of the ERK pathway to protein synthesis-dependent synaptic plasticity and memory consolidation. Owing to constraints of space, we focus on the role of the ERK pathway in synaptic plasticity and memory in mammalian systems while touching on important findings relating to the other MAPK cascades.

4.25.2 Mitogen-Activated Protein Kinase Activation in Neurons

The ERK cascade is activated in neurons in response to a wide variety of stimuli associated with synaptic plasticity. The mechanism of ERK activation most reminiscent of its action in mitotic cells occurs through stimulation of the tyrosine kinase Trk receptors by neurotrophins (Marsh et al., 1993; Segal and Greenberg, 1996). Treatment of cortical neurons with brain-derived neurotrophic factor (BDNF) elicits tyrosine autophosphorylation of the trkB receptor, recruitment of Ras, and activation of the canonical Raf/MEK/ERK cascade. Stimulation of *N*-methyl-D-aspartate (NMDA) receptors elicits ERK activation in hippocampal neurons (Bading and Greenberg, 1991; Kurino et al., 1995; English and Sweatt, 1996). Overexpression of dominant-negative Ras or a Ras GAP suppresses NMDA-induced ERK activation (Iida et al., 2001; Zhu et al., 2002), implying that calcium influx through NMDA receptors (NMDARs) impinges on mechanisms that increase the levels of GTP-bound Ras. Similarly, membrane depolarization and calcium influx through voltage-gated calcium channel (VGCC) stimulate Ras-dependent ERK activation in neuronal cells (Rosen et al., 1994; Farnsworth et al., 1995; Rosen and Greenberg, 1996; Iida et al., 2001).

At least five routes leading from elevated neuronal calcium levels to Ras activation have been characterized. First, calcium-dependent activation of Ras can be mediated by two highly related neuron-specific GEFs (also known as GRFs, guanine-nucleotide releasing factors), Ras-GRF1, and Ras-GRF2. Binding of calcium-calmodulin to an IQ motif in the Ras-GRFs strongly enhances their ability to activate Ras. Analysis of single and double Ras-GRF knockout mice demonstrated that Ras-GRF2 makes the major contribution to ERK activation in response to NMDAR activation in mature hippocampal slices, while Ras-GRF1 makes a relatively minor contribution (Li et al., 2006). The use of pharmacologic inhibitors specific for NMDARs containing either the NR2A subunit or NR2B subunit further suggested that Ras-GRF2 selectively couples NR2A-containing receptors to ERK activation in the mature hippocampus. In contrast, Ras-GRF1 couples to NR2B-containing NMDARs to activate p38 MAPK (Li et al., 2006). Consistent with these findings, an independent study employing NR2 subunit-specific inhibitors and RNA interference identified a dominant role for NR2A NMDARs in mediating NMDA-induced ERK activation in mature neurons, whereas NR2B NMDARs functioned to inhibit ERK activation, shortening its duration (Kim et al., 2005).

The relative contributions of Ras-GRFs, and NR2A- and NR2B-containing NMDAR, to NMDA-induced activation of the Ras-ERK pathway are subject to significant developmental regulation. Ras-GRFs are present at low levels in the neonatal mouse brain and gradually increase during the first few postnatal weeks, with persistently high levels of expression in the adult brain. Accordingly, Shc- and GRB2-mediated recruitment of Son-of-Sevenless (SOS) GEFs may support NMDA-induced activation of the Ras-ERK cascade in immature neurons (Tian et al., 2004). Similarly, NR2B appears to play a more important role in mediating NMDAR-induced ERK activation in immature neurons, followed by a developmental shift in which NR2B acts to limit ERK activation (Krapivinsky et al., 2003; Kim et al.,

2005). Several lines of evidence suggest that SynGAP, a neuron-specific Ras GAP, may act to limit ERK activity in response to NR2B NMDAR activation. Both Ras-GRF1 and SynGAP associate with NR2B, Ras-GRF1 through a direct interaction and with SynGAP through indirect interaction mediated by PSD-95 (Krapivinsky et al., 2003; Kim et al., 2005). The GAP activity of SynGAP is reportedly stimulated by phosphorylation by calcium/calmodulin-dependent protein kinase II (CaMKII), which also interacts with the NR2B subunit, suggesting a mechanism for calcium-induced Ras inhibition following NR2B NMDAR activation (Oh et al., 2004). Consistent with this notion, NMDAR-mediated ERK activation is depressed by SynGAP overexpression and accentuated by reduced SynGAP expression (Kim et al., 2005; Rumbaugh et al., 2006). In contrast, p38 MAPK activation is potentiated by SynGAP overexpression and depressed by SynGAP inactivation, but these effects are observed only in the absence of synaptic activity (Rumbaugh et al., 2006). Interestingly, it has also been observed that ERK is activated by stimulation of the synaptic pool of NMDARs, which contain both NR2A and NR2B subunits, whereas ERK is inactivated by stimulation of extrasynaptic NMDARs, which contain primarily NR2B subunits (Ivanov et al., 2006). Parallel examination of p38 MAPK has suggested opposing regulation of ERK and p38 activity mediated by SynGAP (Rumbaugh et al., 2006).

A second calcium-dependent route to Ras-ERK activation involves the cytoplasmic tyrosine kinases Src and Pyk2. Pyk2 is highly enriched in brain, and its tyrosine kinase activity is strongly stimulated by membrane depolarization and calcium influx through VGCC, as well as by activation of protein kinase C (PKC) (Lev et al., 1995). Autophosphorylated PYK2 can recruit the Grb2-Sos1 complex directly and indirectly through Shc, leading to calcium- and PKC-induced activation of Ras and MAPK. Tyrosine autophosphorylation of PYK2 also promotes direct interaction with Src, which activates Src kinase activity (Thomas and Brugge, 1997). Src has been implicated in Ras activation and downstream neuronal events in response to calcium influx through VGCCs (Rusanescu et al., 1995), suggesting an additional mechanism by which calcium-induced PYK2 activation can recruit Ras-ERK signaling.

Third, calcium signals can be relayed to the Ras-ERK cascade through calcium-calmodulin-dependent kinases. Distinct roles for both CaMKI and CaMKII in Ras-ERK activation have been proposed. NMDAR-induced ERK activation in hippocampal neurons and slices is blocked by inhibition of CaMKK and its downstream target CaMKI, whereas inhibition of CaMKIV and CaMKII activity had no effect (Schmitt et al., 2005). ERK activation in response to membrane depolarization in neuronal cells has also been reported to require CaMKK and CaMKI acting upstream of Ras (Schmitt et al., 2004). NMDAR-dependent long-term potentiation (LTP) induced by TBS activates ERK in a CaMKK-dependent manner, and inhibition of CaMKK and MEK cause mutually occlusive impairments in LTP. These findings suggest that activation of Ras-ERK signaling by calcium influx through NMDARs or VGCCs occurs through sequential activation of CaMKK and CaMKI. However, other findings suggest that NMDAR-mediated calcium influx during LTP induction can activate the Ras-ERK cascade through CaMKII. In cultured rat hippocampal slices, expression of a constitutively active form of CaMKII produces a Ras-dependent potentiation of AMPAR-mediated responses that occludes pairing-induced LTP (Zhu et al., 2002). The mechanism linking CaMKII to Ras in this context, which is presently undefined, would seem to oppose the inhibition of Ras-ERK signaling mediated by the action of CaMKII on SynGAP. A fourth mechanism by which calcium influx is coupled to ERK activation relies on the calcium-stimulated adenylyl cyclases AC1 and AC8 (Ferguson and Storm, 2004). Overexpression of AC1 enhances levels of ERK activation in hippocampal neurons, and hippocampal ERK activation is impaired by deletion of AC1 and AC8 (Wang et al., 2004; Sindreu et al., 2007). Finally, a fifth pathway conveying calcium signals to ERK involves the activation of Ras and Rap1 by a family of calcium and diacylglycerol-regulated GEFs, known as CalDAG-GEFs (Ebinu et al., 1998; Kawasaki et al., 1998).

Adenylyl cyclase activation and elevated levels of cAMP have been widely reported to activate ERK in PC12 cells and hippocampal neurons (Vossler et al., 1997; Impey et al., 1998; Roberson et al., 1999; Ambrosini et al., 2000; Iida et al., 2001; Patterson et al., 2001). However, the mechanism coupling elevated levels of cAMP to the ERK cascade remain unsettled, with evidence in neuronal cells supporting either Ras- or Rap1-dependent pathways, which can function in protein kinase A (PKA)-dependent or PKA-independent manners. The complexity of ERK activation by cAMP appears to derive from the differing subcellular localizations of Ras and Rap1 and their respective GEFs, as well as their

differential ability to activate Raf isoforms (reviewed in Stork, 2003). Ras can activate both of the neuronal Raf isoforms, Raf-1 and B-Raf. In contrast, Rap1 is capable of activating B-Raf, whereas it binds to but does not activate Raf-1, which may account for its ability to antagonize Ras function in some contexts. Ras is primarily localized to the plasma membrane, while Rap1 localizes to a variety of membrane compartments, including Golgi, endosomes, lysosomes, plasma membrane, and perinuclear structures. PKA-independent activation of Rap1 by cAMP is mediated by Epac1/2, Rap1-GEFs that directly bind cAMP. Epacs activate a perinuclear pool of Rap1 that does not result in activation of B-Raf or ERK. In contrast, PKA-dependent activation of Rap1 by the Rap1-GEF C3G enables activation of plasma membrane-localized Rap1 and B-Raf, which appears to be the major pathway responsible for forskolin-induced ERK activation in PC12 cells (Wang et al., 2006). The mechanisms linking cAMP to ERK have been widely found to exhibit cell type-specific differences; for example, cAMP-induced ERK activation is accompanied by Ras activation in cortical neurons but not in PC12 cells (Ambrosini et al., 2000). Highlighting the significance of restricted patterns of subcellular localization, inhibition of hippocampal Rap1 activity in transgenic mice causes a selective reduction in the activation of a membrane-associated pool of ERK, while overall levels of cytosolic ERK activation remain unaffected (Morozov et al., 2003).

A role for Rap1 and Rap2 in activation of the p38 and JNK cascades, respectively, has emerged from studies of LTP and AMPAR trafficking in cultured rat hippocampal slices. Overexpression of constitutively active Rap1 stimulates p38 but not ERK activation, and overexpression of dominant-negative Rap1 suppresses p38 but not ERK activation (Zhu et al., 2002). Similarly, overexpression of constitutively active Rap2 stimulates JNK but not ERK or p38 activation, and overexpression of dominant-negative Rap2 suppresses JNK but not ERK or p38 activation (Zhu et al., 2005).

The ERK pathway is also potently activated in cultured neurons and hippocampal slices by agonist binding to several classes of neuromodulatory receptors that play important roles in synaptic plasticity, including group 1 metabotropic glutamate receptors, muscarinic acetylcholine receptors, dopamine receptors, and β-adrenergic receptors (Fiore et al., 1993b; Roberson et al., 1999; Ambrosini et al., 2000; Rosenblum et al., 2000). Consistent with the role of the cAMP/PKA second messenger pathway in transducing signals from G-protein coupled receptors, ERK activation in hippocampal slices in response to dopamine and the β-adrenergic agonist isoproterenol is blocked by a PKA inhibitor. Activation of PKC by phorbol ester elicits ERK activation, and ERK activation in response to specific agonists of group I mGluRs requires PKC. The role of PKC in conveying signals from muscarinic acetylcholine receptors to the ERK pathway is less clear. Stimulation of muscarinic receptors with carbachol reliably induces ERK activation in hippocampal slices and cultured cortical neurons, but variable blockade of carbachol-induced ERK activation by PKC inhibitors has been reported (Roberson et al., 1999; Rosenblum et al., 2000). The scaffolding protein KSR1 (kinase suppressor of Ras 1), which promotes association of Raf-1 with MEK and ERK, specifically couples type 1 mGluRs and PKC to activation of a membrane-associated pool of ERK, which appears to target only a subset of ERK substrates in the hippocampus (Shalin et al., 2006). In contrast, KSR1 does not influence the coupling of cAMP/PKA to hippocampal ERK activation.

Induction of CA1 LTP and hippocampal long-term memory are accompanied by NMDAR-dependent ERK activation (English and Sweatt, 1996; Atkins et al., 1998). A large number of studies subsequently confirmed and extended these findings by documenting ERK activation in response to the induction of various forms of ERK-dependent LTP, long-term depression (LTD) and long-term memory in the hippocampus, amygdala, and neocortex (e.g., Berman et al., 1998; Blum et al., 1999; Winder et al., 1999; Kanterewicz et al., 2000; Schafe et al., 2000; Dudek and Fields, 2001; Patterson et al., 2001; Thiels et al., 2002; Ying et al., 2002; Kelly et al., 2003; Gallagher et al., 2004; Kelleher et al., 2004a; Levenson et al., 2004; Wang et al., 2004; Kushner et al., 2005; Banko et al., 2006; Chen et al., 2006; Paul et al., 2007; Sindreu et al., 2007). Where examined, ERK activation in NMDAR-dependent forms of synaptic plasticity and learning is blocked by NMDAR antagonists. Similarly, ERK activation in NMDAR-independent forms of synaptic plasticity is blocked by VGCC antagonists (e.g., Kanterewicz et al., 2000; Dudek and Fields, 2001). In addition, an important role for action potential generation in recruitment of ERK activation during LTP induction has been proposed, based on the observation that action potentials are sufficient for ERK activation in the absence of synaptic activity (Dudek and

Fields, 2002; Zhao et al., 2005). Interestingly, stimulation with TBS and varying frequencies (5–100 Hz) of tetanus strongly activates ERK throughout dendrites, soma, and nucleus of CA1 neurons, suggesting that ERK-dependent processes may be activated on a cell-wide basis during LTP (Dudek and Fields, 2001; Patterson et al., 2001).

4.25.3 Mitogen-Activated Protein Kinases in Long-Term Potentiation and Long-Term Depression

Considerable evidence now supports the importance of bidirectional modifications of synaptic strength as cellular correlates of learning and memory. The opposing processes of synaptic strengthening and weakening, embodied experimentally by LTP and LTD, have been most intensively studied at hippocampal area CA1 synapses. Given the wealth of experimental evidence concerning synaptic plasticity at CA1 synapses, and the likely importance of plasticity at this synapse for hippocampal learning and memory, our discussion will be largely confined to CA1 LTP and LTD. Both LTP and LTD exhibit a transient early phase (E-LTP, E-LTD), which is independent of new protein synthesis, and a longer-lasting late phase (L-LTP, L-LTD), which requires new protein synthesis (reviewed in Kelleher et al., 2004b). In addition, NMDAR-dependent and NMDAR-independent forms of both LTP and LTD have been described (*See* Chapter 4.16). The expression of early-phase LTP and LTD is mediated by synaptic activity-dependent insertion or removal of AMPARs into the postsynaptic membrane (reviewed in Malinow and Malenka, 2002; Bredt and Nicoll, 2003; Malenka and Bear, 2004). As outlined below, the ERK pathway has been implicated in essentially all of these processes.

The availability of highly specific pharmacologic inhibitors has allowed the consequences of acute inhibition of MAPK activation on various forms of synaptic plasticity to be determined. In the case of the ERK cascade, cell-permeable inhibitors of MEK1/2 have been widely used as specific probes of ERK function, revealing an important role for ERK in many, but not all, forms of LTP and LTD. Genetic methods of interfering with MAPK function provide an important complement to pharmacologic approaches, allowing inhibition of cascade components for which pharmacologic agents are not available, and also permitting analysis of the effects of partial or complete inactivation of one or more isoforms of a particular component.

4.25.3.1 Essential Roles for Extracellular-Regulated Kinase in *N*-Methyl-D-Aspartate Receptor–Dependent and –Independent Long-Term Potentiation

A requirement for ERK activation in synaptic plasticity was first documented for NMDAR-dependent LTP induced by high-frequency tetanic stimulation (100 Hz) in acute hippocampal slices (English and Sweatt, 1997; Atkins et al., 1998; Impey et al., 1998). While MEK inhibitors significantly attenuated expression of LTP induced by modest or strong tetanization protocols, MEK inhibition had no effect on basal synaptic transmission, short-term forms of synaptic plasticity, or LTP maintenance. Inhibition of tetanus-induced CA1 LTP has been observed with multiple, structurally distinct MEK inhibitors (PD98059, U0126, SL327) (English and Sweatt, 1997; Atkins et al., 1998; Impey et al., 1998; Rosenblum et al., 2002; Selcher et al., 2003). Comparison of results obtained in rat and mouse hippocampal slices, however, has revealed a curious species-specific difference in the ERK dependence of LTP induced by modest tetanic stimulation. Specifically, LTP induced by a pair of closely spaced tetanic trains is inhibited by MEK antagonists in the rat hippocampus but not in the mouse hippocampus (English and Sweatt, 1997; Atkins et al., 1998; Watabe et al., 2000; Selcher et al., 2003). Similarly, LTP induced at mouse CA1 synapses by a single tetanic train is unaffected by MEK inhibition (Winder et al., 1999). The differing ERK requirements of various forms of CA1 LTP induced by patterns of 100-Hz tetanization may relate to their dependence on protein synthesis, with ERK activation selectively required for protein synthesis–dependent LTP (see Section 4.25.3.4).

ERK activation also contributes to other well-characterized forms of NMDAR-dependent LTP. CA1 LTP can be effectively induced by stimulation at the frequency of the hippocampal theta rhythm (3–12 Hz). This form of LTP is thought to be particularly relevant to behavior, since CA1 pyramidal cells in the rodent hippocampus fire spikes or complex spike bursts at theta frequency during spatial exploration (O'Keefe, 1993). MEK inhibitors suppress CA1 LTP induced in the mouse hippocampus

by 5-Hz stimulation (theta frequency stimulation, TFS) or brief high-frequency bursts of stimulation delivered at 5 Hz (theta burst stimulation, TBS) (Winder et al., 1999; Watabe et al., 2000; Patterson et al., 2001; Selcher et al., 2003). Increases in postsynaptic excitability and the firing of complex action potential bursts have been implicated in the induction of LTP by TFS and TBS, and ERK activation can modulate these neuronal properties in the context of TFS and TBS (Thomas et al., 1998; Winder et al., 1999; Watabe et al., 2000; Selcher et al., 2003). One mechanism through which ERK may influence postsynaptic excitability during theta-like stimulation, particularly with regard to dendritic backpropagation of action potentials, is through regulation of A-type potassium channel function (Adams et al., 2000; Yuan et al., 2002).

Induction of NMDAR-dependent LTP can be facilitated or enhanced by neuromodulatory agents under certain conditions (e.g., when combined with subthreshold afferent stimulation). Activation of β-adrenergic receptors, either alone or in conjunction with muscarinic acetylcholine receptors, stimulates ERK activation and facilitates CA1 LTP induction in an ERK-dependent manner (Winder et al., 1999; Watabe et al., 2000; Giovannini et al., 2001; Gelinas and Nguyen, 2005).

Using intracellular recordings, NMDAR-dependent CA1 LTP can be induced by pairing postsynaptic depolarization with low-frequency (typically 2 Hz) presynaptic stimulation. A MEK inhibitor (PD98059) blocks such pairing-induced LTP at CA1 synapses in acute and cultured rat hippocampal slices (Bolshakov et al., 2000; Zhu et al., 2002). Moreover, virus-mediated expression of dominant-negative or constitutively active forms of Ras in slice culture demonstrated that Ras activation is necessary and sufficient to induce LTP (Zhu et al., 2002). Synaptic potentiation produced by constitutively active Ras occludes pairing-induced LTP and is prevented by MEK inhibitors, indicating that ERK activation downstream of Ras is essential for LTP.

Investigation of the molecular mechanisms underlying LTP has focused largely on NMDAR-dependent forms, since hippocampal learning and memory characteristically depend on NMDAR activation (Morris et al., 1986, 1990; Miserendino et al., 1990; Kim et al., 1991). However, synapses in the rodent hippocampus and other brain regions also exhibit NMDAR-independent LTP, in which postsynaptic calcium influx depends on activation of voltage-gated calcium channels (Grover and Teyler, 1990; Aniksztejn and Ben-Ari, 1991; Aroniadou and Teyler, 1992; Huang and Malenka, 1993; Weisskopf et al., 1999). Although the behavioral significance of NMDAR-independent LTP is somewhat unclear, VGCCs have been implicated in certain forms of learning and memory, particularly aspects of amygdala-dependent fear memory (Bauer et al., 2002; Cain et al., 2002; Moosmang et al., 2005; McKinney and Murphy, 2006). Consistent with the potent activation of the ERK cascade evoked by calcium influx through VGCC, two well-studied forms of NMDAR-independent CA1 LTP have been shown to depend on ERK activation. High-frequency tetanization (200 Hz) recruits a substantial component of NMDAR-independent LTP, and treatment with the potassium channel blocker tetraethylammonium (TEA) induces LTP (termed TEA-LTP or LTP_K) that is insensitive to NMDAR antagonists (Grover and Teyler, 1990; Aniksztejn and Ben-Ari, 1991; Huang and Malenka, 1993). Induction of CA1 LTP with either 200-Hz tetanization or TEA treatment activates ERK in a VGCC-dependent manner, and MEK inhibitors block the induction of both forms of LTP (Kanterewicz et al., 2000). BDNF treatment also induces an NMDAR-independent form of LTP at CA1 synapses in acute hippocampal slices and at perforant path-dentate granule cell synapses *in vivo* (Kang and Schuman, 1995; Messaoudi et al., 2002); in the latter case, BDNF-induced LTP has been shown to require ERK activation (Ying et al., 2002). Enhanced presynaptic release of neurotransmitter has been implicated in the expression of BDNF-dependent LTP and LTP induced by TEA or 200-Hz stimulation (Zakharenko et al., 2001, 2003), suggesting that postsynaptic ERK activation may contribute to the generation of a retrograde messenger.

4.25.3.2 Different Forms of Long-Term Depression Mediated by Extracellular-Regulated Kinase and p38 Mitogen-Activated Protein Kinase at CA1 Synapses

Several distinct forms of LTD have been characterized at CA1 synapses in hippocampal slices (Dudek and Bear, 1992; Bolshakov and Siegelbaum, 1994; Oliet et al., 1997; Palmer et al., 1997; Kemp and Bashir, 1999; Huber et al., 2000; reviewed in Malenka and Bear, 2004). NMDAR-dependent LTD can be induced in the neonatal hippocampus by prolonged periods of low-frequency stimulation (LFS, typically at 1 Hz). In comparison with the

higher frequencies of stimulation required to induce NMDAR-dependent LTP, the lower levels of calcium influx generated in response to low-frequency stimulation are thought to activate protein phosphatases and favor the production of LTD. Analysis of the mechanisms underlying NMDAR-dependent LTD induced by LFS in neonatal hippocampal slice culture demonstrated an essential role for p38 MAPK activation downstream of Rap1 (Zhu et al., 2002). Viral expression of dominant-negative Rap1 blocked LTD, while expression of constitutively active Rap1 depressed AMPA-mediated synaptic transmission and occluded LTD. Furthermore, LTD and the synaptic depression induced by constitutively active Rap1 were blocked by a p38 MAPK inhibitor (SB203580), but not by MEK inhibitor (PD98059) or a JNK inhibitor (SP600125).

At least two forms of NMDAR-independent LTD that depend on the activation of group I mGluRs have been described at CA1 synapses, with the mechanistic features apparently differing depending on the developmental stage and/or induction protocol. mGluR-dependent LTD induced in neonatal hippocampal slices by prolonged 5-Hz stimulation is suppressed by a p38 MAPK inhibitor (SB203580), but not by a MEK inhibitor (PD98059) (Bolshakov et al., 2000). LTD induction is associated with mGluR-dependent p38 MAPK activation (though possible concomitant ERK activation was not assessed in this study). Further supporting an essential role for p38 MAPK, intracellular dialysis of CA1 neurons with activated p38 MAPK induces LTD, which occludes subsequent LTD in response to 5-Hz stimulation. The induction of this form of LTD depends on postsynaptic mGluR activation, while its expression appears to be mediated presynaptically through a decrease in transmitter release (Bolshakov and Siegelbaum, 1994). These mechanistic features suggest that p38 MAPK is recruited downstream of mGluR activation in postsynaptic neurons and participates in the production of a retrograde messenger.

In contrast, mGluR-dependent LTD induced in more mature hippocampal slices by paired-pulse low-frequency stimulation or direct application of a type I mGluR agonist (e.g., DHPG) requires postsynaptic protein synthesis and is expressed postsynaptically through internalization of synaptic AMPARs (Huber et al., 2000; Snyder et al., 2001; Xiao et al., 2001). This form of mGluR-dependent LTD is inhibited by antagonists of MEK (PD98059, U0126) but not p38 MAPK (SB203580) (Gallagher et al., 2004; Banko et al., 2006). In addition, mGluR stimulation sufficient to induce LTD activates ERK but not p38 MAPK. Thus, both NMDAR-dependent and mGluR-dependent forms of LTD in the neonatal hippocampus appear to depend on p38 MAPK, while the mGluR-dependent LTD that predominates at later developmental stages depends instead on ERK. The available evidence suggests that both p38 MAPK and ERK can promote the synaptic removal of AMPARs in different contexts, with ERK possibly playing a specific role in protein synthesis–dependent LTD.

A role for the remaining MAPK family pathway, JNK, has been described in NMDAR-dependent synaptic depotentiation. LTD and depotentiation share many features and are generally thought to represent closely related processes mechanistically. However, one possible asymmetry pertains to the identities of the synaptic AMPARs that are removed by each process, since LTP is mediated by insertion of GluR1- and GluR4-containing AMPARs, while LTD is mediated by removal of GluR2/3-containing AMPARs (Malinow and Malenka, 2002; Bredt and Nicoll, 2003; Malenka and Bear, 2004). In cultured rat hippocampal slices, Rap2-dependent activation of JNK mediates depotentiation of pairing-induced LTP and removal of synaptic GluR1-containing AMPARs (Zhu et al., 2005).

4.25.3.3 Coupling of NR2A to Extracellular-Regulated Kinase and NR2B to p38 in Long-Term Potentiation and Long-Term Depression

As outlined above, analysis of LTP and LTD in cultured rat hippocampal slices, combined with pharmacologic inhibition and viral expression of mutant forms of Ras and Rap, has indicated specific roles for Ras-ERK signaling in NMDAR-dependent LTP and Rap1-p38 MAPK signaling in NMDAR-dependent LTD at CA1 synapses (Zhu et al., 2002). Other data have suggested opposing patterns of recruitment of ERK and p38 MAPK activation by NMDARs containing the NR2A and NR2B subunits. Specifically, association of NR2B-containing NMDARs with Ras-GRF1, CaMKII, and SynGAP appears to limit ERK activation and promote p38 MAPK activation (Zhu et al., 2002; Krapivinsky et al., 2003; Oh et al., 2004; Kim et al., 2005; Rumbaugh et al., 2006). Conversely, Ras-GRF2 and NR2A-containing NMDARs are functionally coupled to ERK activation. Interestingly, germline deletion of Ras-GRF1 and Ras-GRF2 produces opposing effects on

NMDAR-dependent LTP and LTD at CA1 synapses (Li et al., 2006). Ras-GRF1 inactivation significantly impairs LFS-induced LTD but has no effect on LTP induced by TBS and a minor effect on LTP two trains of 100-Hz tetanization separated by 20 s. In contrast, inactivation of Ras-GRF2 impairs LTP induced by both TBS and paired 100-Hz tetanization but leaves LTD unaffected. Although the apparent requirement for ERK in LTP induced with a pair of closely spaced 100-Hz tetanic trains is at odds with other data showing this form of LTP to be ERK independent in the mouse hippocampus (Selcher et al., 2003; Kelleher et al., 2004a), these findings nevertheless suggest a specific role for NR2B and p38 in LTD and for NR2A and ERK in LTP. The foregoing observations are further consistent with the reported dependence of CA1 LTP on NR2A NMDARs and CA1 LTD on NR2B NMDARs (Liu et al., 2004). However, this model has been challenged by data from several groups demonstrating that induction of CA1 LTP can be mediated by NMDARs containing either NR2B or NR2A (Kiyama et al., 1998; Kohr et al., 2003; Berberich et al., 2005, 2007; Weitlauf et al., 2005). Several groups have similarly shown that activation of NR2B-containing NMDARs is not required for CA1 LTD. These data argue that NR2 subunit composition does not directly specify the induction of LTP or LTD (Morishita et al., 2007). Rather, they support an alternate model in which NR2 subunit composition regulates the threshold for induction of LTD versus LTP – a form of 'metaplasticity' – by modulating the degree of postsynaptic calcium influx and the activation of signaling mechanisms favoring either LTD or LTP.

4.25.3.4 A Specific Role for Extracellular-Regulated Kinase in the Protein Synthesis-Dependent Phase of Synaptic Plasticity

Increasing evidence suggests a specific role for ERK activation in the protein synthesis–dependent phases of LTP and LTD. Genetic inhibition of ERK activation in the postnatal hippocampus impairs L-LTP but not E-LTP, and the effect of ERK inhibition on L-LTP is indistinguishable from the effect of the translational inhibitor anisomycin (Kelleher et al., 2004a). Surprisingly, transcriptional inhibition with actinomycin-D exerts a distinct, delayed kinetic pattern of inhibition of L-LTP. ERK inhibition also abolishes the enhancement of translation and translation factor phosphorylation in CA1 neurons that accompanies the induction of L-LTP. Taken together, these findings suggest that upregulation of ERK-dependent translation plays a dominant role in the establishment of protein synthesis–dependent LTP, and transcriptional induction is required for the subsequent maintenance of these synaptic changes (Kelleher et al., 2004a). ERK is also required for a translation-dependent and transcription-independent form of LTD induced by mGluR activation (Huber et al., 2000; Gallagher et al., 2004; Banko et al., 2006). This selective involvement of the ERK pathway in protein synthesis–dependent synaptic plasticity likely underlies its selective role in the protein synthesis–dependent process of memory consolidation (see following).

Further arguing for a specific role of ERK in protein synthesis–dependent LTP, the differing ERK dependence of tetanus-induced forms of LTP in the mouse hippocampus parallels the distinction between E-LTP and L-LTP, suggesting a selective contribution of ERK to L-LTP. MEK inhibition blocks LTP induced by multiple 100-Hz tetanic trains separated by several minutes, a protocol which has been shown to produce protein synthesis–dependent LTP. In contrast, LTP induced by a single or by a pair of 100-Hz tetanic trains separated by 10–20 seconds is unaffected by MEK inhibition, and both of these induction protocols have been shown to evoke protein synthesis–independent LTP (Huang and Kandel, 1994; Huang et al., 1996; Winder et al., 1998). The sensitivity of LTP induced by a pair of closely spaced tetanic trains to MEK inhibition in the rat hippocampus could be reconciled with this hypothesis if this protocol induced a protein synthesis–dependent form of LTP in the rat (but not the mouse). Data suggesting that this is in fact the case have recently been presented: LTP induced by a pair of 100-Hz tetanic trains separated by 20 s persists for more than 3 h and is sensitive to transcriptional inhibition (Levenson et al., 2004). Moreover, early-phase LTP induced with a standard TBS protocol exhibits a protein synthesis–dependent component that is entirely blocked by MEK inhibition with U0126, while the protein synthesis–independent component is unaffected by MEK inhibition (Schmitt et al., 2005). These observations suggest that the ERK pathway exerts its effects on TBS-induced LTP primarily through a protein synthesis–dependent mechanism. Further supporting the generality of this proposed role for the ERK pathway, ERK activation is required for forskolin-induced LTP, which is entirely dependent on new protein synthesis (Frey et al., 1993; Patterson et al., 2001), and protein synthesis–dependent L-LTP in the amygdala requires ERK activation (Huang et al., 2000). It should be noted,

however, that some forms of TBS can produce a protein synthesis–independent LTP that is nevertheless sensitive to MEK inhibition, indicating that ERK can facilitate induction of TBS-LTP through additional mechanisms, such as modulation of neuronal excitability (Winder et al., 1999; Selcher et al., 2003).

4.25.4 Mitogen-Activated Protein Kinase in Learning and Memory

4.25.4.1 ERK Activation in Hippocampus- and Amygdala-Dependent Learning and Memory

Consistent with the central role of the ERK pathway in many forms of synaptic plasticity, a variety of studies in experimental rodents over the past decade have established the importance of the ERK pathway in mammalian learning and memory. As with its role in synaptic plasticity, the responsiveness of the ERK pathway to excitatory and neuromodulatory synaptic transmission, and particularly its position as a convergence point for a variety of neuronal activity–induced signaling mechanisms, makes it well-suited to function as a key mediator of associative memory. A requirement for ERK in associative memory was first demonstrated in fear conditioning, in which rodents learn to associate an unconditioned stimulus (an experimental chamber in contextual conditioning or an experimental tone in cued conditioning) with a conditioned stimulus (an aversive foot shock). Formation of an associative fear memory in cued conditioning depends on the integrity of the amygdala, whereas associative memory in contextual conditioning depends on both the hippocampus and the amygdala (Kim and Fanselow, 1992; Phillips and LeDoux, 1992). Training of rats in contextual fear conditioning elicits heightened ERK activation in the hippocampus within 1 h, while exposure to the unconditioned or conditioned stimuli without formation of an associative memory fails to activate ERK (Atkins et al., 1998). Pretraining systemic administration of an NMDAR antagonist, which is known to block the acquisition of conditioned fear memory (Miserendino et al., 1990; Kim et al., 1991), suppressed the specific increase in hippocampal ERK phosphorylation. Similarly, ERK activation is transiently and specifically increased in the amygdala 1 h after fear conditioning (Schafe et al., 2000). Systemic administration of a MEK inhibitor (SL327) 1 h prior to training causes a dose-dependent reduction in associative memory for both contextual and cued conditioning in rats and mice when tested 24 h later (Atkins et al., 1998; Selcher et al., 1999). Contextual and cued fear conditioning are also impaired in a dose-dependent manner following pretraining intraventricular infusion of a MEK inhibitor (PD98059) (Schafe et al., 1999). A specific requirement for ERK activation in the amygdala was subsequently demonstrated by targeted infusion of MEK inhibitor in the amygdala, which caused a dose-dependent impairment in long-term memory for cued fear conditioning (Schafe et al., 2000). Similarly, intraamygdalar infusion of a cell-permeable form of the ERK phosphatase STEP causes amnesia for cued fear conditioning (Paul et al., 2007).

These findings made the important initial demonstration that ERK activation downstream of NMDARs is essential for the formation of associative memory. The impairment in both cued and contextual conditioning imposed by MEK inhibition indicates a requirement for ERK activation in the amygdala, but its possible role in the hippocampus could not be distinguished. Subsequent studies using a hippocampus-dependent task, the hidden platform Morris water maze, demonstrated an essential role for ERK activation in the formation of hippocampal memory. In this task, rodents learn through a series of trials to locate a submerged platform in a pool using distal visual cures, thereby acquiring NMDAR-dependent spatial reference memory for the platform location (Morris et al., 1982). Acquisition of spatial reference memory in rats is associated with transient ERK activation in the dorsal hippocampus (Blum et al., 1999). Systemic administration of a MEK inhibitor (SL327) in mice prior to training on 5 consecutive days interferes with acquisition of spatial reference memory in the hidden platform water maze; control experiments excluded effects of the drug on memory recall and sensorimotor performance (Selcher et al., 1999). The role of hippocampal ERK activation in spatial reference memory was addressed more directly using a stereotactic approach (Blum et al., 1999). Intrahippocampal infusion of a MEK inhibitor (PD98059) 20 min prior to or immediately following training significantly impaired memory retention when tested 48 h later. In contrast, posttraining infusion of a p38 MAPK inhibitor (SB203580) did not interfere with memory retention (Blum et al., 1999). Spatial reference memory following training in the hidden platform water maze and long-term memory for contextual but not cued fear conditioning are

impaired by genetic inhibition of ERK activation in the postnatal hippocampus (Kelleher et al., 2004a). Contextual fear conditioning was also impaired in another transgenic mouse with neuron-specific inhibition of ERK activation (Shalin et al., 2004).

4.25.4.2 Upstream Regulators of Extracellular-Regulated Kinase in Learning and Memory

Studies of mice bearing mutations in upstream regulators of the ERK cascade have provided supporting evidence that ERK is an essential component of the molecular machinery underlying hippocampus- and amygdala-dependent memory. Heterozygous disruption of the K-Ras isoform causes deficient hippocampus-dependent memory in the hidden platform Morris water maze (Costa et al., 2002). Conditional inactivation of the B-Raf isoform in excitatory neurons of the postnatal mouse forebrain (which bypasses the embryonic lethality caused by germline disruption) attenuates hippocampal ERK activation following contextual fear conditioning and impairs spatial reference memory in the hidden platform Morris water maze, but surprisingly leaves contextual and cued fear conditioning unaffected (Chen et al., 2006). Analysis of two independent lines of knockout mice lacking Ras-GRF1 have been analyzed; in one case, hippocampus-dependent but not amygdala-dependent memory was found to be impaired, while in the other case, essentially the reverse pattern was observed (Brambilla et al., 1997; Giese et al., 2001). The basis for this discrepancy is not clear but may relate to methodological differences.

Several studies have examined the consequences of upregulation of Ras-ERK signaling. Transgenic mice overexpressing an activated form of H-Ras display enhanced hippocampus-dependent learning (Kushner et al., 2005). Acquisition of reference memory in the hidden platform water maze was accelerated, and memory for contextual fear conditioning was enhanced both 2 h and 24 h following training. In wild-type mice, contextual fear conditioning induced parallel increases in ERK-mediated phosphorylation of synapsin I. Synapsin I phosphorylation was potentiated in H-Ras transgenic mice, and inactivation of synapsin I prevented enhancement of contextual fear memory by H-Ras overexpression. In conjunction with electrophysiological findings indicating augmented presynaptic function and plasticity in H-Ras mice, these observations implicate Ras-ERK signaling in the activity-dependent regulation of neurotransmitter release (Kushner et al., 2005). The specificity of this genetic manipulation for presynaptic function may relate, at least in part, to the subcellular distribution of Ras-ERK activation, as both endogenous and transgene-derived H-Ras were found to be predominantly localized to excitatory presynaptic terminals, with no detectable dendritic localization. In contrast to the enhancement of hippocampal LTP and memory observed with H-Ras overexpression, other genetic manipulations that elevate Ras-ERK signaling activity, such as heterozygous disruption of the Ras GAPs Syn-GAP and NF1, appear to have a deleterious effect (Silva et al., 1997; Komiyama et al., 2002). Furthermore, restoration of normal levels of Ras-ERK signaling in *NF1* mutant mice (through mutation of K-Ras or N-Ras isoforms) rescues the hippocampal LTP and memory defects (Costa et al., 2002). These findings are consistent with the presence of cognitive impairment in human developmental disorders caused by hyperactive Ras-ERK signaling (Schubbert et al., 2007).

Two recent studies have highlighted the importance of the subcellular localization of ERK activation for synaptic plasticity and memory. Transgenic overexpression of a dominant-negative form of Rap1 in excitatory forebrain neurons decreased the basal activities of B-Raf and a membrane-associated pool of ERK in the hippocampus, while increasing basal levels of Raf-1 and leaving total and cytosolic hippocampal ERK activity unaltered (Morozov et al., 2003). This reduction in basal membrane-associated ERK activity was sufficient to impair hippocampal memory and two ERK-dependent forms of CA1 LTP (theta frequency– and forskolin-induced LTP). Impaired ERK-dependent LTP was associated with reduced basal and tetanus-induced phosphorylation of the ERK substrate Kv4.2 and reduced complex spiking of CA1 neurons during LTP induction. The activity of a membrane-associated pool of ERK was also compromised by germline disruption of KSR1, a scaffold protein that facilitates interaction of components of the ERK cascade (Shalin et al., 2006). Interestingly, KSR1 inactivation interfered with the coupling of PKC to membrane-associated ERK activation, without altering the responsiveness of membrane-associated ERK to adenylyl cyclase/cAMP. Moreover, ERK activation in the cytosol in response to stimulation of either PKC- or adenylyl cyclase/cAMP pathways was unaffected. A corresponding dissociation of ERK-dependent forms of LTP was observed, with impaired PKC-mediated LTP (TBS-induced LTP) but normal cAMP-mediated LTP (LTP induced

by TFS plus isoproteronol). Long-term memory formation was impaired in the hidden platform water maze as well as contextual and cued conditioning, suggesting that PKC-dependent recruitment of membrane-associated ERK activity is essential for hippocampus- and amygdala-dependent memory, possibly acting *via* regulation of synaptic activity-dependent neuronal excitability (Shalin et al., 2006).

4.25.4.3 A Specific Role for Extracellular-Regulated Kinase Activation in Memory Consolidation

Analysis of the contribution of ERK activation to the temporal phases of memory has highlighted a specific role in the process of memory consolidation. Short-term memory is insensitive to inhibitors of protein synthesis, whereas long-term memory is disrupted by such agents. The transition from short-term to long-term memory, or memory consolidation, thus depends on new gene expression (reviewed in Davis and Squire, 1984). Analysis of the role of ERK activation in fear conditioning has revealed that intraventricular or intraamygdalar infusion of MEK inhibitor blocks long-term memory assessed 24 h after training, but short-term memory assessed 30–60 min after training remains intact (Schafe et al., 1999, 2000). Similarly, infusion of a cell-permeable ERK phosphatase into the amygdala selectively disrupts consolidation of fear memory (Paul et al., 2007). Genetic inhibition of hippocampal ERK activation through expression of a dominant-negative form of MEK1 causes a significant impairment of long-term memory for contextual fear conditioning 24 h after training, while short-term memory 1 h after training is unaffected (Kelleher et al., 2004b). The temporal pattern of memory impairment in fear conditioning caused by MEK inhibition in these studies corresponds closely to the temporal pattern observed with inhibition of protein synthesis (Abel et al., 1997; Schafe and LeDoux, 2000), suggesting that ERK activation plays an important role in the new gene expression required for memory consolidation. Investigation of conditioned taste aversion (CTA) memory, which is encoded in the insular cortex, has demonstrated the importance of ERK activation in protein synthesis–dependent memory consolidation beyond the hippocampus and amygdala. The formation of conditioned taste aversion memory for an unfamiliar taste is accompanied by ERK activation in the insular cortex, and infusion of a MEK inhibitor (PD98059) into the insular cortex interferes with long-term CTA memory, but not short-term CTA memory, similar to the effect of translational inhibition (Rosenblum et al., 1993; Berman et al., 1998). In addition to its role in memory consolidation, the ERK cascade also appears to be required for the related protein synthesis–dependent phenomenon of memory reconsolidation (Kelly et al., 2003; Duvarci et al., 2005).

Recent evidence suggests that ERK-mediated translational upregulation is a crucial mechanism underlying memory consolidation (Kelleher et al., 2004b). The impaired memory consolidation caused by genetic inhibition of hippocampal ERK activation in the postnatal hippocampus is associated with reduced site-specific phosphorylation of translation initiation factors (eIF4E and S6), consistent with a reduced translational response during long-term memory formation. MEK inhibition in this study caused parallel reductions in neuronal activity–dependent translation (including dendritic protein synthesis) and translation factor phosphorylation in cultured hippocampal neurons and hippocampal slices. Transcriptional regulation is an alternate mechanism by which ERK may regulate the new gene expression needed for memory consolidation. Fear conditioning elicits coincident activation of ERK, CREB, and the CREB kinase MSK1 in CA1 neurons downstream of calcium-stimulated adenylyl cyclases, and MSK1 activation is blocked by systemic administration of a MEK inhibitor (SL327) (Sindreu et al., 2007). Fear conditioning also induces ERK-dependent acetylation of histone H3 in CA1 neurons, and increased histone acetylation is associated with enhanced long-term memory for contextual fear conditioning (Levenson et al., 2004).

There has been comparatively little investigation of the role of the other MAPK families, p38 and JNK, in learning and memory. Inhibition of p38 MAPK in the hippocampus has been reported to have no effect on hippocampal learning and memory in the hidden platform Morris water maze. However, requirements for all three MAPK families – ERK, p38 MAPK, and JNK – have been reported in another hippocampus-dependent task, one-trial inhibitory avoidance learning (Walz et al., 1999; Alonso et al., 2003; Bevilaqua et al., 2003). Definition of the role played by the stress-induced MAPK pathways in learning and memory, relative to the widely demonstrated importance of the ERK pathway, awaits further exploration.

4.25.5 Mitogen-Activated Protein Kinase Effector Mechanisms in Synaptic Plasticity and Memory

4.25.5.1 Neuronal Excitability and A-Type Potassium Channels

Modulation of membrane excitability, particularly in dendrites, makes an important contribution to the extent of membrane depolarization and calcium influx during LTP induction, thereby influencing the probability of LTP. For example, complex bursting of CA1 pyramidal neurons and dendritic backpropagation of action potentials have been implicated in efficient induction of LTP using particular stimulation protocols (Magee and Johnston, 1997; Thomas et al., 1998; Watanabe et al., 2002).The ERK cascade appears to regulate both of these manifestations of neuronal excitability. During TBS, complex spike bursting of CA1 pyramidal neurons is augmented, and MEK inhibition suppresses both LTP and increased spiking induced by TBS (Winder et al., 1999; Selcher et al., 2003). The ability of backpropagating action potentials to invade dendrites and amplify excitatory postsynaptic potentials (EPSPs) is regulated primarily by dendritic A-type potassium channels (reviewed in Johnston et al., 2000). A gradient of increasing density from proximal to distal dendrite of the A-type channel, particularly the Kv4.2 subunit, effectively dampens backpropagation of action potentials and limits dendritic excitability.

Treatment of hippocampal slices with isoproterenol or activators of PKA and PKC increases the amplitude of action potentials in distal dendrites of CA1 pyramidal neurons, consistent with downregulation of dendritic A-type currents (Yuan et al., 2002). These increases in action potential amplitude are abolished by MEK inhibition, indicating that downregulation of A-type channel function by PKA and PKC is mediated by the ERK pathway. Moreover, isoproterenol, PKA activation, and PKC activation all produced ERK-dependent increases in the phosphorylation of the Kv4.2 subunit. ERK can directly phosphorylate Kv4.2, and phosphorylation of one of these sites appears to suppress the A-type current (Adams et al., 2000; Schrader et al., 2006). Thus, direct phosphorylation of Kv4.2 by ERK is likely responsible for the heightened dendritic excitability associated with ERK activation, which may in turn account for the dual dependence of TBS-LTP on ERK activation and complex spike bursting. Increased ERK-mediated susceptibility of dendrites to backpropagation of action potentials generated by complex spiking may enhance activation of NMDARs and VGCCs and facilitate LTP induction.

4.25.5.2 AMPAR Trafficking

Parallel roles for all three MAPK families in the synaptic activity–dependent trafficking of AMPARs that underlies bidirectional synaptic modifications have emerged from a series of recent studies. The expression of NMDAR-dependent LTP and LTD is mediated by the synaptic insertion and removal, respectively, of AMPARs, which account in large part for the resulting changes in synaptic strength (reviewed in Malinow and Malenka, 2002; Bredt and Nicoll, 2003; Malenka and Bear, 2004). Thus, LTP is associated with the conversion of silent synapses lacking AMPARs to active synapses, while LTD is associated with synaptic silencing due to net loss of AMPARs. These dynamic changes in the synaptic content of AMPARs occur as a result of the endocytosis and exocytosis of specific populations of AMPARs, defined by their subunit composition. LTP is mediated by the synaptic insertion of AMPARs containing GluR1, which contains a long cytoplasmic tail, whereas LTD is mediated by the synaptic removal of AMPARs containing GluR2 and GluR3, which contain short cytoplasmic tails. Synaptic depotentiation appears to occur through a reversal of LTP, namely, synaptic removal of GluR1-containing AMPARs.

As described above, Ras was found to control synaptic potentiation in an ERK-dependent manner in cultured hippocampal slices, while Rap1 controlled synaptic depression in a p38-dependent manner (Zhu et al., 2002). Furthermore, constitutively active Ras mimics LTP by driving the synaptic insertion of GluR1-containing AMPARs, and constitutively active Rap1 mimics LTD by removing GluR2-containing AMPARs (Zhu et al., 2002). Consistent with the selective coupling of ERK activation to NR2A-containing NMDARs, NR2A promotes the surface expression and delivery of GluR1 in cultured hippocampal neurons, whereas NR2B opposes synaptic delivery of GluR1 (Kim et al., 2005). Consistent with the proposed role of SynGAP acting through NR2B to antagonize ERK activity, SynGAP overexpression inhibits surface delivery of GluR1 in hippocampal neurons (Kim et al., 2005; Rumbaugh et al., 2006). Complementary electrophysiological studies in cultured hippocampal neurons showed that SynGAP overexpression

depresses AMPAR-mediated synaptic transmission, whereas SynGAP deficiency enhances synaptic transmission (Rumbaugh et al., 2006). In addition, SynGAP negatively regulates ERK activation and positively regulates p38 activation.

Taken together, these findings suggest a model in which Ras-ERK signaling acts downstream of NR2A NMDARs to drive GluR1 insertion and LTP, and Rap1-p38 signaling operates downstream of NR2B NMDARs to promote loss of AMPARs and LTD. This model is consistent with the reported dependence of CA1 LTP on activation of NR2A-containing NMDARs, and CA1 LTD on activation of NR2B-containing NMDARs (Liu et al., 2004). However, the hypothesis that NR2A or NR2B containing AMPARs directly specify LTP or LTD is challenged by data from several groups showing that activation of NR2A-containing NMDARs is not obligatory for CA1 LTP, nor is activation of NR2B-containing NMDARs necessary for CA1 LTD (Kiyama et al., 1998; Kohr et al., 2003; Berberich et al., 2005; Weitlauf et al., 2005; Berberich et al., 2007; Morishita et al., 2007). As one possible resolution of this apparent discrepancy, differential coupling of NMDARs containing NR2A or NR2B to distinct MAPK cascades, which can in turn promote the synaptic insertion or removal or AMPARs, may contribute to metaplastic regulation of the threshold for induction of LTD or LTP.

However, there is some apparent uncertainty concerning the AMPAR subtype whose synaptic levels are reduced by NR2B NMDARs, SynGAP, and p38. Some data suggest that Rap1-p38 promotes Ras-ERK-independent synaptic removal of GluR2 (Zhu et al., 2002), whereas other results suggest that NR2B and SynGAP, which appear to inhibit ERK activity and stimulate p38 activity, instead antagonize GluR1 insertion (Kim et al., 2005; Rumbaugh et al., 2006). Synaptic depotentiation would appear to be the process properly associated with reversal of LTP-induced AMPAR trafficking and removal of surface GluR1. Recent data suggest that depotentiation of pairing-induced LTP in a hippocampal slice culture system is mediated by activation of a Rap2-JNK pathway downstream of NR2A NMDARs, leading to internalization of GluR1 (Zhu et al., 2005). In this study, depotentiation was induced using the same LFS protocol that induces LTD in naïve slices, combined with a p38 inhibitor to block LTD and isolate depotentiation. The situation is further complicated by the observation that AMPARs containing subunits with long cytoplasmic tails, such as GluR1, can undergo activity-independent synaptic exchange with AMPARs containing short cytoplasmic tails, such as GluR2/3. Further investigation will help to clarify the relative roles of NR2 subunits and MAPK pathways in AMPAR trafficking mechanisms in these systems.

4.25.5.3 Transcriptional Regulation

MAPK pathways, principally the ERK pathway, influence the transcriptional response to neuronal activity by regulating the activity of the transcription factors Elk-1 and CREB. Transcriptional regulation is a major mechanism by which MAPKs, especially ERK, mediate the growth responses of mitotic cells to growth factor stimulation (Treisman, 1996). Transcription of so-called immediate early genes is rapidly upregulated in response to extracellular stimulation as a result of the interaction of transcriptional activator proteins with *cis*-acting elements located upstream of minimal promoter sequences. One of the best characterized immediate-early genes encodes the c-Fos protein, which together with c-Jun makes up the AP-1 transcription factor. The *c-fos* gene contains two types of upstream *cis*-acting element that mediate its transcriptional induction in response to cellular stimulation, designated the serum response element (SRE) and the cAMP-response element (CRE). SRE-dependent transcription is mediated by the formation of a ternary complex in which serum response factor (SRF) and members of the TCF (ternary complex factor) family of transcription factors simultaneously interact with the SRE. ERK directly phosphorylates TCFs, such as Elk-1, in response to growth factor stimulation, resulting in enhanced ternary complex formation and SRE-dependent transcription (Wasylyk et al., 1998). p38 MAPK and JNK have also been reported to phosphorylate TCFs under some conditions. CRE-dependent transcription in response to elevated intracellular levels of cAMP and calcium is mediated by CREB, which binds the CRE and activates transcription (Shaywitz and Greenberg, 1999; Mayr and Montminy, 2001). The ability of CREB to activate CRE-dependent transcription is dependent on the inducible phosphorylation of a specific residue (Ser133), as well as its interaction with the co-activator CBP (CREB-binding protein), which possesses histone acetylase (HAT) activity. ERK activation enhances Ser133-phosphorylation of CREB and CRE-dependent gene

expression through MAPK-activated protein kinases such as ribosomal S6 kinase (RSK) and MSK1, ERK substrates that can phosphorylate CREB on Ser133 (Xing et al., 1996; Arthur et al., 2004).

Neuronal activity–dependent gene expression plays an essential role in many long-lasting physiological and morphological responses to experience in the developing and adult nervous systems. CREB is the transcription factor most widely implicated in such responses, particularly synaptic plasticity and learning and memory (Lonze and Ginty, 2002). CREB function is essential for long-term forms of synaptic facilitation and memory in invertebrate systems, and mutant mice bearing loss-of-function CREB mutations display variable defects in LTP and memory (Silva et al., 1998). CREB is activated via Ser133 phosphorylation in response to diverse neuronal stimuli, including calcium influx through NMDARs and VGCCs, activation of Trk receptors by neurotrophins, and increases in cAMP levels through activation of G protein–coupled receptors (Shaywitz and Greenberg, 1999).

The Ras/ERK pathway has been implicated in CREB phosphorylation and CRE-dependent transcription in response to neurotrophins and calcium influx. For example, induction of LTP with both NMDAR- and VGCC-dependent components by repeated high-frequency tetanization produced ERK-dependent stimulation of CRE-dependent gene expression (Impey et al., 1996, 1998). BDNF-induced LTP in the dentate gyrus *in vivo* requires ERK activation and is associated with ERK-dependent CREB phosphorylation and transcription of the immediate-early gene *Arc* (Ying et al., 2002). The neurotrophins nerve growth factor (NGF) and BDNF stimulate CREB phosphorylation and CRE-dependent transcription through a Ras-ERK-RSK pathway in neuronal cells (Ginty et al., 1994; Xing et al., 1996, 1998; Finkbeiner et al., 1997; Pizzorusso et al., 2000). Following neurotrophin stimulation of neuronal cells, activated ERK directly phosphorylates RSK isoforms (RSK1/2/3), which translocate to the nucleus and act as Ser133 CREB kinases. An alternate ERK-dependent pathway from neurotrophins to CREB phosphorylation involves the nuclear ERK substrate MSK1 (mitogen- and stress-activated kinase 1), which also functions as a CREB kinase (Arthur et al., 2004). In this study, neurotrophin-induced CREB phosphorylation and CRE-dependent transcription in cortical neurons were not affected by inhibition of RSK1 and RSK2 activity but were markedly reduced by inhibition or genetic disruption of MSK1 activity.

CREB phosphorylation and CRE-dependent transcription in response to calcium influx through NMDAR and VGCC have been reported to involve both CaMKIV and ERK (Bito et al., 1996; Impey et al., 1998; Hardingham et al., 1999; Dolmetsch et al., 2001; Wu et al., 2001; Impey et al., 2002). The ERK cascade appears to be the major kinase pathway conveying calcium signals to the nucleus and CREB following activation of both NMDARs and VGCCs. Calcium influx through VGCCs evokes a rapid and transient CaMKIV-dependent increase in CREB phosphorylation, as well as a slower and more persistent ERK-dependent increase (Wu et al., 2001). ERK activation occurs in response to localized increases in calcium in the vicinity of VGCCs, followed by nuclear translocation of ERK and/or RSK (Dolmetsch et al., 2001). Similarly, NMDAR-mediated ERK activation leads to persistent CREB phosphorylation and appears to involve local microdomains of elevated calcium (Hardingham et al., 2001). Calcium-dependent ERK activation is necessary but not sufficient for CRE-induced transcription, which additionally requires elevated nuclear calcium, activation of CaMKIV, and CaMKIV-mediated phosphorylation of CBP (Hardingham et al., 1997; Chawla et al., 1998; Impey et al., 2002).

Much less is known about the mechanisms regulating Elk1 phosphorylation and Elk1-dependent transcription in neurons. Stimulation of Elk1-dependent transcription in PC12 cells by glutamate and NGF has been reported to require ERK activation (Xia et al., 1996; Johnson et al., 1997). Similarly, glutamate-induced Elk1 phosphorylation in striatal slices is blocked by a MEK inhibitor (Vanhoutte et al., 1999). LTP in the dentate gyrus *in vivo* stimulates phosphorylation of ERK, CREB, and Elk1, as well as transcription of the immediate-early gene *zif268*; all of these effects are prevented by infusion of a MEK inhibitor, which produces a rapid decay of LTP (Davis et al., 2000). ERK activation is also required for LTD and LTD-induced enhancement of Elk1 phosphorylation in hippocampal area CA1 *in vivo* (Thiels et al., 2002). A possible role for Elk1 in synaptic plasticity and learning and memory has not yet been investigated.

Chromatin modification provides an additional mechanism through which ERK may regulate transcription. Acetylation and phosphorylation of histones, particularly histone 3, facilitate transcriptional activation (Cheung et al., 2000; Nowak and

Corces, 2004). Recent studies have implicated the ERK pathway in neuronal activity–induced acetylation and phosphorylation of histone H3. Contextual fear conditioning stimulated H3 acetylation and phosphorylation in an NMDAR- and ERK-dependent manner in hippocampal area CA1, and ERK activation similarly mediated enhanced H3 phosphoacetylation in area CA1 *in vitro* (Levenson et al., 2004; Chwang et al., 2006). In striatal slices, glutamate acts through the ERK pathway and MSK1/2 to stimulate H3 phosphorylation (Brami-Cherrier et al., 2007).

4.25.5.4 Translational Regulation

It has long been known that long-term memory formation and persistent forms of synaptic plasticity in both vertebrate and invertebrate systems can be disrupted by inhibitors of mRNA and protein synthesis (Davis and Squire, 1984; Kandel, 2001; Kelleher et al., 2004b). Investigation of the mechanisms enabling these processes focused for many years on transcriptional regulation. However, several lines of evidence support a crucial role for translational control in the consolidation of synaptic plasticity and memory. First, the observation that transcriptional and translational inhibitors impair L-LTP with significantly different kinetics highlighted a role for translational control independent of transcription in protein synthesis–dependent LTP (Kelleher et al., 2004a). Translational inhibition causes an early and progressive impairment of L-LTP, whereas transcriptional inhibition has a delayed effect, leaving L-LTP unaffected for the initial 60–90 min, and thereafter causing a progressive inhibition. These observations imply that the initial expression of L-LTP is mediated by the enhanced translation of preexisting mRNAs, and translation of newly synthesized mRNAs contributes to the subsequent maintenance of protein synthesis–dependent LTP. Second, the recognition that a subset of neuronal mRNAs is localized to dendrites, and that dendrites can support translation independent of the cell body, suggested that local translation could provide a mechanism for rapid synthesis of new protein products in response to synaptic activity (Sutton and Schuman, 2006). Third, dendritic translation is sufficient for certain forms of protein synthesis–dependent LTP, such as mGluR-dependent LTD and BDNF-induced LTP (Kang and Schuman, 1996; Huber et al., 2000).

Translational control by the ERK pathway has recently emerged as a central mechanism in the establishment of long-lasting forms of synaptic plasticity and memory (reviewed in Kelleher et al., 2004b; Klann and Dever, 2004). Eukaryotic translation is primarily regulated at the level of initiation through a cap-dependent mechanism. Recognition of the 5′ mRNA cap by eIF4E promotes the formation of a translation initiation complex and ribosomal recruitment. The rate-limiting step of cap recognition is regulated by the availability of eIF4E, which is in turn regulated by a family of inhibitory eIF4E binding proteins, 4E-BPs. 4E-BPs and eIF4E are phosphorylated in response to mitogenic stimulation in mitotic cells, which results in increased availability of eIF4E and an increased rate of translation initiation. Mitogen-induced activation of S6 kinases and phosphorylation of ribosomal protein S6 are also highly correlated with increased translation rates, although it is unclear how these events may contribute to translational induction. In mitotic cells, ERK has been implicated in mitogen-induced eIF4E phosphorylation, which is mediated by the ERK substrates and eIF4E kinases, Mnk1/2 (Waskiewicz et al., 1997; Wang et al., 1998). The mTOR and ERK pathways appear to cooperate to effect sequential hyperphosphorylation of 4E-BPs in response to mitogenic stimulation (Gingras et al., 2001; Herbert et al., 2002). However, until recently, little was known about translational regulation in response to neuronal activity or its possible role in synaptic plasticity and memory.

A recent study demonstrated a crucial role for translational control by the ERK pathway in the consolidation of LTP and memory (Kelleher et al., 2004a). Translation of transfected reporter mRNAs and endogenous mRNAs in hippocampal neurons was stimulated in an ERK-dependent manner by multiple forms of neuronal activity, including BDNF, membrane depolarization, and excitatory synaptic activity. Investigation of the underlying mechanisms revealed that BDNF, membrane depolarization, and excitatory synaptic activity elicited ERK-dependent site-specific phosphorylation of components of the translational machinery, specifically eIF4E, 4EBPs, and ribosomal protein S6. Excitatory synaptic activity also induced ERK-dependent translation of endogenous mRNAs and phosphorylation of eIF4E, 4E-BPs, and ribosomal protein S6 in synaptoneurosomes, indicating that similar ERK-dependent mechanisms operate at local dendritic and neuron-wide levels. Supporting the physiological relevance of neuronal activity–dependent translation mediated by the ERK pathway

to synaptic plasticity and memory, induction of L-LTP in area CA1 of hippocampal slices was accompanied by a rapid increase in the translation of endogenous mRNAs and the phosphorylation of eIF4E and ribosomal protein S6, and these translational stimulatory effects were abolished by inhibition of ERK activation in CA1 neurons by conditional expression of a dominant-negative form of MEK1. Similarly, contextual fear conditioning likewise stimulated phosphorylation of hippocampal eIF4E and ribosomal protein S6, and these effects were abolished by conditional genetic inhibition of hippocampal ERK activation. These biochemical defects were correlated with specific impairments of L-LTP and hippocampal long-term memory, while E-LTP and short-term memory were unaffected.

Additional lines of evidence have further substantiated a central role for the ERK pathway in control of neuronal activity–dependent translation. Treatment of cortical neurons with BDNF stimulated ERK-dependent translation of endogenous mRNAs and phosphorylation of eIF4E (Takei et al., 2001). Activation of NMDARs, adenylyl cyclase (forskolin), PKC (PDA) or group I mGluRs (DHPG) in hippocampal slices stimulated ERK-dependent phosphorylation of Mnk1 and eIF4E in area CA1 (Banko et al., 2004, 2006). Interestingly, p38 MAPK appears to contribute to basal levels of Mnk1 and eIF4E phosphorylation in hippocampal area CA1, but not to DHPG-induced increases, which are mediated instead by ERK (Banko et al., 2006). Phosphorylation of eIF4E and 4E-BPs is also stimulated in an ERK-dependent manner by induction of a form of TBS-LTP that requires both ERK activation and protein synthesis (Schmitt et al., 2005). Stimulation of the phosphorylation of ERK, Mnk1, and eIF4E by NMDAR activation in area CA1 requires PKA, while stimulation of ERK, eIF4E, and 4E-BP phosphorylation by induction of TBS-LTP requires CaMKK (Banko et al., 2004; Schmitt et al., 2005). These observations suggest that ERK-dependent translational control during some forms of LTP can occur downstream of cAMP/PKA and CaMKK/CaMKI second messenger pathways.

4.25.5.5 Implications for Synaptic Tagging and Capture

To account for the preservation of input specificity in protein synthesis–dependent forms of synaptic plasticity, an attractive model has been proposed in which stimulated synapses are marked by an immobile synaptic tag, which can function to capture newly synthesized gene products needed for the expression of the appropriate synaptic change (Frey and Morris, 1997). In essence, the model implies that the expression of protein synthesis–dependent synaptic plasticity involves two parallel processes operating downstream of NMDAR activation: (1) protein synthesis–independent generation of a synaptic tag, and (2) protein synthesis–dependent generation of new protein products. On the basis of the evidence outlined above, it has been suggested that the ERK pathway participates primarily in the second process, and specifically that ERK-dependent translation supplies the new products needed for the capture of L-LTP or L-LTD by tagged synapses (Kelleher et al., 2004b). In view of their similar requirements for ERK-dependent translation, L-LTP and L-LTD are likely distinguished by the setting of LTP- or LTD-specific synaptic tags, respectively, rather than the synthesis of a pool of proteins dedicated to either LTP or LTD. These LTP- and LTD-specific tags may then recruit from the available pool of proteins the components needed for the consolidation of the corresponding synaptic change. According to this proposal, the spatial extent of activation of ERK-dependent translation will define a local domain within which tagged synapses can share and compete for the available pool of new proteins, which will determine the ultimate pattern of consolidated synaptic weight changes. Such a mechanism may be advantageous for the efficient storage and reactivation of memory engrams (Govindarajan et al., 2006).

4.25.6 Conclusions

As described in this review, research over the past decade has revealed that MAPK signaling cascades are key regulators of synaptic plasticity and memory consolidation in the adult brain. Regulation of long-term synaptic modifications in nondividing, mature neurons reprises the general role for these cascades in controlling committed changes during development, such as cell division and terminal differentiation. Moreover, MAPK cascades in the adult brain are pluripotent regulators of diverse biochemical processes, including transcription, translation, receptor trafficking and ion channel function. These neuronal functions recapitulate the broader biological role of MAPK signaling mechanisms as high-order coordinators of cellular responses to extracellular signals.

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4.26 Proteolysis and Synaptic Plasticity

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4.26.1 Introduction

Understanding mechanisms underlying synaptic plasticity has been an intense area of research in neuroscience. As a result of many years of research, we now know that short-term synaptic plasticity requires posttranslational modification of existing proteins, whereas long-term synaptic plasticity entails new gene expression and protein synthesis. In recent years, proteolysis by the ubiquitin–proteasome pathway has attained prominence as a new molecular mechanism underlying synaptic plasticity.

4.26.2 The Ubiquitin–Proteasome Pathway

Although the ubiquitin–proteasome pathway was originally thought to function in degradation of abnormal proteins, a large number of studies indicate that this pathway plays a major role in regulated proteolysis of short-lived regulatory protein substrates under physiological conditions. In this pathway, the process of degradation of a substrate protein can be divided into two steps: (1) covalent attachment of ubiquitin to the substrate protein, forming the polyubiquitin chain attached to the substrate (ubiquitin conjugation), and (2) degradation of the polyubiquitinated substrate and disassembly of the polyubiquitin chain and recycling of free ubiquitin (**Figure 1**).

When a protein is to be specifically degraded in the cell, it is marked by covalent attachment of ubiquitin to an ε amino group of lysine residues. The ubiquitin conjugation step is a highly regulated step catalyzed by the action of three classes of enzymes called E1, E2, and E3. E1, the ubiquitin-activating enzyme, activates the free ubiquitin, a small protein of 76 amino acids, in an adenosine triphosphate (ATP)-dependent reaction. Activated ubiquitin is then transferred to an E2, which is generally referred to as a ubiquitin-carrier enzyme. An enzyme belonging to a class of enzymes called E3s (ubiquitin ligases) then ligates the activated ubiquitin to the substrate. A second ubiquitin is attached to an internal lysine residue in the first ubiquitin (Lys-48) and thus by sequential linkages of monoubiquitins, a polyubiquitin chain grows (Glickman et al., 2002; Hegde, 2004).

The polyubiquitinated substrate is then recognized by a large multisubunit proteolytic complex called the 26S proteasome and is degraded to small peptides and amino acids (Hegde and DiAntonio, 2002). Ubiquitination also plays a role in endocytosis. Attachment of a single ubiquitin to a lysine residue (monoubiquitination) or single ubiquitin molecules to multiple lysine residues in a substrate (multiple monoubiquitination) usually marks the protein substrates in the plasma membrane for endocytosis. The disassembly of polyubiquitin chains or removal of monoubiquitins is carried out by deubiquitinating enzymes (DUBs) (Hegde, 2004).

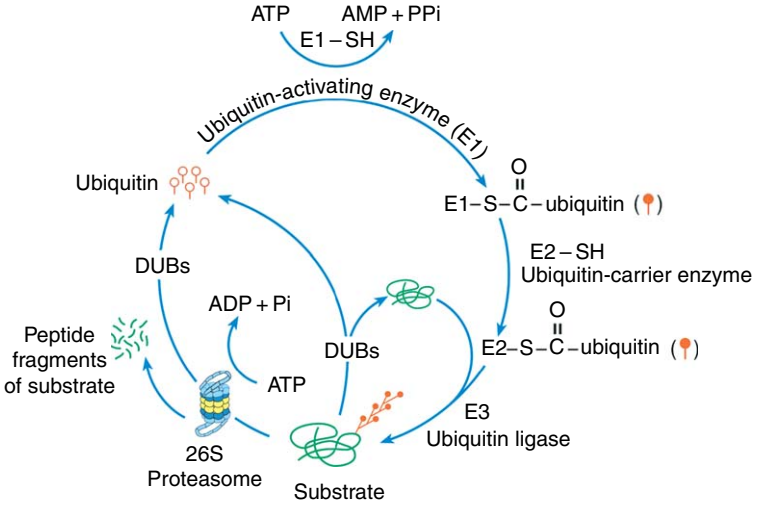


Figure 1 The ubiquitin–proteasome pathway. In this proteolytic pathway, ubiquitin (a single ubiquitin molecule is represented by open circles with straight tails) is selectively and covalently linked to the substrate. The enzymatic process of attaching ubiquitin to substrates is called ubiquitination, or ubiquitin conjugation, and depends on the action of three different classes of enzymes: E1, E2, and E3. First, ubiquitin is activated by E1 to form a ubiquitin-adenosine monophosphate (AMP) intermediate. Activated ubiquitin (closed circles with straight tails) is passed onto E2 (ubiquitin carrier enzymes). E2s transfer ubiquitin to an E3 (ubiquitin ligase), which ligates the activated ubiquitin to the substrate. To the ubiquitin attached to substrate, another ubiquitin is attached and thus through successive linkages of ubiquitin a polyubiquitin chain forms. Polyubiquitinated substrates are degraded by a proteolytic complex called the 26S proteasome in an adenosine triphosphate (ATP)-dependent reaction. Ubiquitin is not degraded but the polyubiquitin chain is disassembled and ubiquitin is recycled by deubiquitinating enzymes (DUBs). Before being committed to being degraded by the proteasome, ubiquitination is reversible. DUBs can disassemble the polyubiquitin chain if a substrate is ubiquitinated erroneously and prevent the degradation of the substrate.

4.26.3 Ubiquitin-Conjugating Enzymes: E1, E2, and E3

Among the three classes of ubiquitin-conjugating enzymes, E1 is the least physiologically regulated. E1 activates ubiquitin in an ATP-dependent step. Activated ubiquitin is then attached to all the substrates degraded by the ubiquitin–proteasome pathway; there is no substrate specificity in this process. E2s are more selective and are believed to interact with specific E3s. Based on our knowledge of ubiquitination reaction, it can be asserted that E3s are the enzymes that possess substrate specificity.

Originally, E2s were believed only to carry the activated ubiquitin and transfer it onto the E3s. Recent studies, however, suggest that at least some E2s can directly conjugate ubiquitin to substrates. E2s are structurally and functionally diverse. Simple eukaryotes such as yeast (*Saccharomyces cerevisiae*) have 13 genes potentially encoding E2s. The number of E2s in mammals is estimated to be in the range of 25–30. Most E2s have a core domain of around 14 K that is approximately 35% conserved between different E2s. The other part of the enzymes appears to be variable. Although most E2s are small, some notable exceptions exist. For example, an E2 called BIR-repeat-containing ubiquitin-conjugating enzyme (BRUCE) is a gigantic 528-K protein. The diversity of E2s generates some degree of specificity in the ubiquitin-conjugating reaction. E2s bind to E3s selectively. Since the diversity of E3s is even greater than that of E2s, the combination of E2s and E3s potentially can generate a high degree of specificity. The heterogeneity of E2s is reflected in their subcellular localization as well. Although several E2s are present in the cytosol, some E2s are localized to other subcellular compartments. For example, BRUCE is localized to the Golgi apparatus and a yeast E2 called Ubc6 is anchored to the membrane of endoplasmic reticulum (Glickman et al., 2002).

E3s are the enzymes that specifically recognize the substrates. E3s can be single proteins or a complex of proteins. Single-subunit E3s can accept ubiquitin in a thioester linkage from E2s and ligate ubiquitin to the substrate. When E3 contains multiple subunits, it is generally believed that the enzyme brings the E2 and the substrate together and facilitates the transfer of ubiquitin to substrate. However, E3s are the most diverse among the ubiquitin-conjugating enzymes and the least characterized. Therefore, it is possible that E3 catalytic mechanisms other than those described below might exist in nature. There are two major classes of E3s: (1) HECT (homologous to E6-AP carboxyl-terminus) domain E3s and (2) RING (really interesting new gene) finger E3s (**Figure 2**).

4.26.3.1 HECT Domain E3s

The typical example in this class of E3 is the ubiquitin ligase called E6-AP, which ligates ubiquitin to the tumor suppressor protein p53. Viral protein E6 associates with a cellular protein called E6-AP (E6-associated protein). The C-terminal region of E6-AP contains the catalytic domain of the ubiquitin ligase. E6-AP ligase can function with either of the E2s called UbcH5 and UbcH7 (**Figure 2**). Later studies found that a family of proteins, ubiquitin ligases, with homology to the catalytic domain of E6-AP exists. These ubiquitin ligases are called HECT domain E3s. In addition to the HECT domain, there is another domain in many E3s called the WW domain. The WW domain-containing E3s also tend to have a C2 domain. The presence of the C2 domain is highly relevant to nervous system function because the C2 domain responds to elevation of intracellular Ca^{2+} and helps in translocation to the plasma membrane. Therefore, presence of this domain in neuronal HECT E3s might be critical in ligating ubiquitin to neurotransmitter receptors or proteins associated with them (Hegde, 2004).

4.26.3.2 RING Finger E3s

These E3s are called RING finger E3s because they contain a RING finger domain, which consists of seven cysteine residues and one histidine residue, forming a single folded domain binding two zinc ions. The arrangement of metal binding residues in the RING finger domain contrasts with the tandem arrangement in the zinc finger domain found in many proteins. Although numerous other proteins were found to have the RING finger motif, the biological function of these proteins remained elusive. During the past few years, several ubiquitin ligases were found to contain the RING finger. The RING finger motif in ubiquitin ligases is critical for transfer of ubiquitin to substrates or to RING finger proteins themselves. The RING finger category of E3s can be subdivided into: (1) single-subunit RING finger E3s, and (2) multisubunit RING finger E3s.

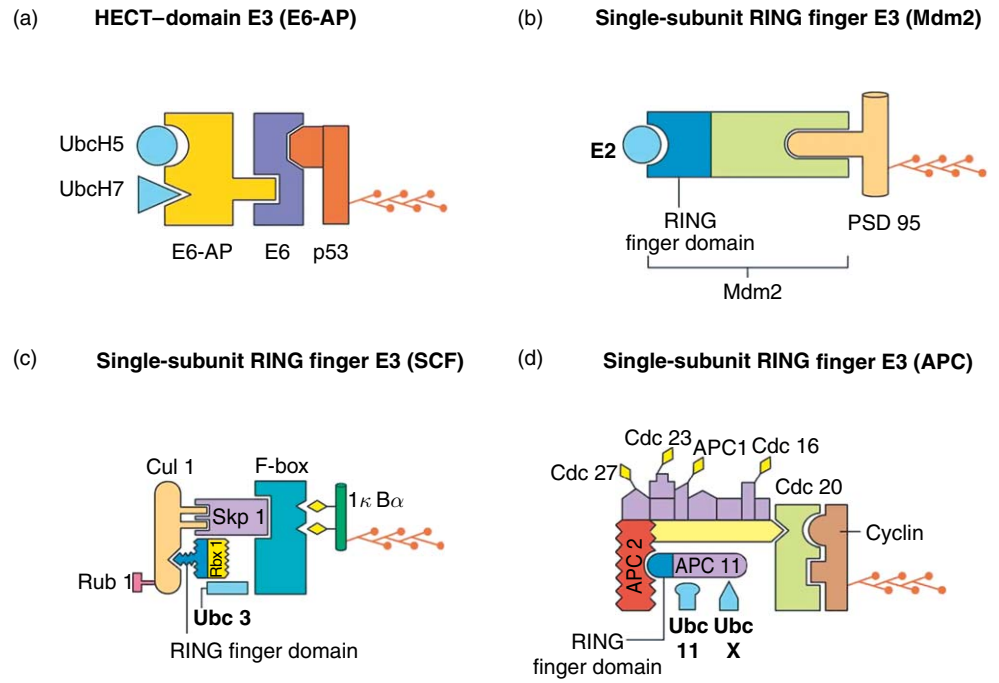


Figure 2 Classes of ubiquitin ligases (E3s). (a) HECT-domain E3. E6-AP ubiquitin ligase in combination with E6 protein and one of the two E2s (UbcH5 or UbcH7) ligates ubiquitin to the p53 tumor suppressor protein. (b) Single-subunit RING finger E3. Mdm2 ligates ubiquitin to postsynaptic density 95 (PSD 95) with the help of an E2 enzyme. (c) Multisubunit RING finger E3. SKP1-cullin-F-Box protein (SCF) ligases contain the substrate recognition site on an F-box protein. Skp1 is an adaptor that joins the F-box protein to Cul1. Ring finger domain is on Rbx 1. The E2 is Ubc3. Cul 1 is modified by Rub1, another ubiquitin-like protein, leading to an increase in the activity of the ligase complex. The substrate is phosphorylated (diamonds) IκBα. (d) Multisubunit RING finger E3. Anaphase-promoting complex (APC) is a more complex example of multisubunit RING finger E3s and has a subunit composition distinct from that of SCF. Cdc20 protein in APC has the substrate (Cyclin) recognition site. The RING finger domain is on APC11. The E2s Ubc 11 or UbcX can function with the APC ligase. In addition, several adaptor proteins, some labeled (Cdc27, Cdc23, APC 1, Cdc16) and some unlabeled, interact with Cdc20 and APC11. Diamonds on the adaptor subunits indicate phosphorylation. The polyubiquitin chain is shown on the substrates in each panel.

4.26.3.2.1 Single-subunit RING finger E3s

Single-subunit RING finger E3s contain the RING finger domain and the substrate recognition site in the same protein. One of the well-characterized single-subunit RING finger E3s is Mdm2, which ubiquitinates p53 in normal cells. As discussed above, E6-AP, a HECT ubiquitin ligase, ubiquitinates p53 in human papilloma virus (HPV)-infected cells. A recent study showed that in HPV-infected cells, E6-AP ubiquitinates p53. Although Mdm2 is present in HPV-infected cells, it does not mediate ubiquitination of p53. Other studies using antisense oligonucleotides directed against E6-AP showed that E6-AP is essential for degradation of HPV-positive cells but not in HPV-negative cells. Conversely, decreasing Mdm2 expression or expression of Mdm2-inactivating peptides decreased p53 degradation in HPV-negative cells but not in HPV-positive cells. Interestingly, the structural determinants on p53 that are recognized by E6-AP and Mdm2 are different from each other. For example, p53 that is a substrate for E6-AP has an asparagine at position 268, whereas p53 that is a substrate for Mdm2 has an aspartate at position 268 (Hengstermann et al., 2001).

4.26.3.2.2 Multisubunit RING finger E3s

4.26.3.2.2.(i) The SKP1-cullin-F-Box protein complex The SKP1-cullin-F-Box protein (SCF) complex contains at least four proteins: Skp1, Cul1, Roc1/Rbx1/Hrt1, and an F-box protein (**Figure 2**). In the center of the SCF complex is the RING finger domain-containing protein Rbx1. The SCF type ligases have another invariant protein called cullin. The theme appears to be that the cullins interact with linker proteins such as Skp1 to recruit substrate-interacting proteins such as the F-box proteins. There are at least five different cullins in mammals. There are several F-box proteins as well. Although it

is not clear how many F-box proteins exist in mammals, the budding yeast genome comprises 17 F-box proteins. Therefore, with just with the cullin and F-box combination alone, it would be theoretically possible to generate close to 100 E3s with differing specificities. Although the regulation of SCF ligases is not completely understood, two mechanisms of posttranslational regulation have been discovered thus far. One is the covalent linkage of ubiquitin-related protein Rub1 to a cullin (Cul1 in **Figure 2**). The second mode of regulation appears to be regulation of levels of F-box proteins through ubiquitin-mediated degradation via an autocatalytic mechanism (Deshaies, 1999). A well-characterized substrate of the SCF complex is IκBα.

4.26.3.2.2.(ii) Anaphase-promoting complex

Although anaphase-promoting complex (APC) has a subunit with a RING finger domain (APC 11), this ubiquitin ligase is distinct from the SCF ligase in overall subunit combination (**Figure 2**). For example, instead of one adaptor found in SCF ligases (such as Skp1), APC has multiple subunits that serve as adaptors. Also, unlike SCF ligases, substrate phosphorylation is not an important determinant for specific substrate recognition by the APC ligase. Rather, substrate specificity of APC ligases appears to be modulated by incorporation of specificity factors into the ligase complex. For example, Cdc20 (**Figure 2**) enables APC to degrade substrates at the onset of anaphase such as the anaphase inhibitor Pds1p, whereas substitution of Cdc20 with another specificity factor called Hct1 enables APC to degrade a different set of substrates such as mitotic cyclins late in the anaphase. APC acts together with an E2 Ubc11 or Ubc X. One of the most studied substrates of APC is mitotic cyclin. This substrate has a short stretch of nine amino acids called the destruction box, which is critical for recognition by the APC ubiquitin ligase (Page and Hieter, 1999).

4.26.3.2.2.(iii) Von Hippel-Lindau-elongin B-elongin C complex

The VBC (VHL-elongin B-elongin C) complex was identified in association with the VHL (von Hippel-Lindau) tumor-suppressor protein. The structure of this complex is similar to that of the SCF complex. The substrate (transcription factor hypoxia-inducible factor 1α, HIF1-α) binds to the VHL protein or SOCS box containing proteins. The VHL protein binds to the Skp1 homolog elongin C and a ubiquitin homolog elongin B. Cul2 and the Roc1/Rbx1 RING finger protein are associated with the complex (Jackson et al., 2000).

Another class of proteins called E4s that elongate the polyubiquitin chain has been discovered (Koegl et al., 1999). The protein product of a gene previously known as ubiquitin fusion degradation protein 2 (UFD2) in yeast was found to catalyze ubiquitin chain assembly along with E1, E2, and E3 and was named E4. E4s contain a modified version of the RING finger, designated as U-box. Since there have not been many studies on E4s and the first E4 discovered functions as cofactor for an E3, it might be premature to conclude that E4s belong to a special class of E3s.

4.26.4 The Proteasome

The term proteasome is used to describe two kinds of multisubunit proteolytic complexes, the 26S and 20S, based on their sedimentation coefficient. The 26S proteasome degrades ubiquitinated protein substrates. The 26S complex contains the 20S as a core and regulatory caps on either end like a dumbbell. Each cap of the 26S proteasome is called the 19S regulatory complex (19S RC). The 20S core is a cylindrical structure consisting of the catalytic part of the proteasome (Hegde, 2004; Pickart and Cohen, 2004).

4.26.4.1 The Catalytic 20S Core

Our knowledge of the proteasome organization comes from the studies on the crystal structure of the proteasome from the archaebacterium *Thermoplasma acidophilum* (Lowe et al., 1995) and the yeast *S. cerevisiae* (Groll et al., 1997). It appears that the proteasome is more ancient than ubiquitin because archaebacteria have the proteasome but not ubiquitin. *T. acidophilum* has two genes encoding α and β subunits. The subunits are arranged in four stacked rings to form the catalytic cylinder with the two middle rings consisting of β subunits, which are sandwiched between two rings of α subunits. In the *T. acidophilum* proteasome, both the α and β subunits are present in seven copies each and assembled in a symmetrical fashion $\alpha_7\beta_7\beta_7\alpha_7$. This general structure is preserved in eukaryotes but the α and β subunits have diverged into seven different subunits each. In yeast, the 20S core is made up of two outer rings with seven α subunits (α1–α7) in each ring and two inner rings consisting of seven β subunits each (β1–β7).

The catalytic core of the proteasome is a threonine protease. Based on the crystal structure of the proteasome, it was concluded that proteasome functions through a new kind of proteolytic mechanism. In this mechanism, the active site nucleophile is the hydroxyl group on the threonine residue at the N-terminus of the β subunit. This catalytic mechanism seems to be conserved across evolution. The antibiotic lactacystin and the active form β-lactone, which are specific and irreversible inhibitors of the proteasome, bind to the N-terminal threonine residue in the β subunit of the mammalian proteasome. The 20S proteasome can exist not only as a core of 26S, but also as a separate population that cannot degrade ubiquitinated proteins. However, the 20S proteasome by itself has chymotrypsin-like, trypsin-like, and postglutamyl peptidase activities which cleave after hydrophobic, basic, and acidic residues, respectively. The peptide hydrolyzing activity of the 20S proteasome can itself be modulated by an 11S regulatory cap (Glickman et al., 2002; Hegde, 2004).

4.26.4.2 19S Regulatory Complex

The 19S RC recognizes the polyubiquitinated substrate, and channels the substrate into the catalytic 20S core of the proteasome. It also has the capacity to regulate the activity of the catalytic core and determine the nature of the degradation process. Usually one 19S RC is attached to either end of the catalytic core. The subunits of the 19S RC are highly conserved across evolution. Two subcomplexes can be recognized within the 19S RC called the base and the lid.

4.26.4.2.1 The base of the proteasome

The base consists of six ATPase subunits (Rpt1–Rpt6) and two non-ATPase subunits (Rpn1 and Rpn2). The Rpt subunits are highly conserved through evolution (up to 75% identical between yeast and humans). The ATPase subunits have a domain called the AAA (ATPases associated with different cellular activities) domain in the center. These subunits are homologous to each other, with the highest degree of homology in the AAA domain. Some of the Rpt subunits contact the α ring in the catalytic core of the proteasome and are believed to channel the substrate into the catalytic chamber for degradation (Hegde, 2004).

4.26.4.2.2 The lid of the proteasome

The lid, which comprises eight subunits, can attach itself to the base of the 19S RC or detach as a complex from the base. All the subunits in the lid are non-ATPase subunits. The exact function of the lid subunits is not known. Degradation of polyubiquitinated proteins requires lid attachment, indicating that the lid performs an essential function in ubiquitin–proteasome-mediated degradation. Also, in archaebacteria that lack ubiquitin, the proteasome is devoid of the lid. The lid subunits of the proteasome share a characteristic sequence of 200 amino acids with COP9 signaling complex and translation initiation factor 3. This sequence is called the PCI (proteasome, COP9, initiation factor 3) domain. In addition, the subunits of the lid have a 120-amino-acid-long MPN (Mpr1p/Pad1p N-terminus) domain that is important for the structure of Rpn8 and Rpn11 subunits. In addition, a subset of MPN domain-containing proteins have a motif of five polar residues called the MPN+ motif. The MPN+ motif has been shown to be critical for the function of Rpn11 (Hegde, 2004).

4.26.5 Deubiquitinating Enzymes

Ubiquitination reaction is reversible before the ubiquitinated protein is committed to degradation by the proteasome. The reversibility is less clear with respect to endocytotic degradation, i.e., internalization of plasma membrane proteins through endocytosis and their degradation through the lysosome. Based on knowledge of the endocytotic pathway, it is reasonable to assume that ubiquitination is reversible until the endocytosed membrane proteins such as neurotransmitter receptors are routed to the multivesicular body (MVB) for lysomal degradation or prior to its entry into invaginating vesicles of MVB. However, if the machinery responsible for recognizing ubiquitinated cargo and initiating its entry into the MVB vesicles is defective, recycling of activated receptors might occur because of the action of DOA4, a DUB required for the removal of ubiquitin from the MVB cargo proteins.

Ubiquitin is removed from substrates by enzymes called DUBs. Based on protein sequence and the molecular size, DUBs can be classified into two general classes: (1) Low-molecular-weight (20–30 K) ubiquitin C-terminal hydrolases (UCHs), and (2) high-molecular-weight (approximately 100 K) ubiquitin-specific proteases (UBPs; also called USPs) (Hegde, 2004). There are numerous DUBs in almost every eukaryotic organism studied. Among the DUBs, UBPs belong to a large family containing diverse genes, whereas the UCH family has fewer genes. For example, in yeast

S. cerevisiae there are 17 UBPs and one UCH (Amerik et al., 2000). In the human genome there are 63 genes encoding UBPs and four genes that code for UCHs. UCHs and UBPs subserve different functions in the eukaryotic cell. Although the current name for these enzymes – DUBs – emphasizes the removal of ubiquitin from substrates, some DUBs, particularly UCHs, function to process linearly linked ubiquitin precursors and generate monoubiquitin. UCHs are cysteine proteases in that the critical residue in the catalytic site is a cysteine. In addition, histidine and aspartate residues are critical for catalytic activity. All UCHs contain these residues even if they do not share a high degree of homology elsewhere in the sequence. UCHs cleave small peptide chains linked to the carboxyl-terminus of ubiquitin. UBPs can cleave the isopeptide bond between ubiquitins in a polyubiquitin chain as well as the isopeptide bond between ubiquitin and the substrate. DUBs are important for generating free ubiquitin at various steps of the ubiquitin–proteasome pathway. Ubiquitin is encoded by the tandemly linked polyubiquitin gene. In the cell, linear polyubiquitin protein molecules are not detected. When polyubiquitin and UCH-L1 are co-expressed in bacterial cells, polyubiquitin is co-translationally processed to generate monoubiquitin. Therefore it is possible that polyubiquitin is processed by UCH or other DUBs to generate monoubiquitin in eukaryotic cells as well. In addition to the polyubiquitin gene, ubiquitin is also encoded by fusion to two ribosomal subunits called L40 and S27. These gene products are also believed to be processed by DUBs. Cleavage of isopeptide bond in the ubiquitin chains linked through Lys-48 of ubiquitin serves two purposes. One is to recycle ubiquitin after it has been used for marking a substrate for ubiquitination. Another function is to edit the errors made by the ubiquitin-conjugating enzymes and reverse the ubiquitination reaction so that the substrate is no longer degraded (Wilkinson, 2000). Also, the editing function of DUBs probably serves to reverse the monoubiquitin attachment that marks membrane proteins for endocytosis.

4.26.6 Regulation of the Ubiquitin–Proteasome Pathway

Proteolysis by the ubiquitin–proteasome pathway can be regulated at the ubiquitin-conjugation step or at the proteasome step. Since the specificity of ubiquitination lies at the conjugation step, clearly regulation of the conjugation process is important in determining whether or not a substrate is targeted for degradation. Regulation of proteasome has a global effect on degradation of cellular substrates.

4.26.6.1 Regulation of Ubiquitin Conjugation

Ubiquitin needs to be conjugated to the right substrate, at the right place in the cell, and at the right time in order to control physiological processes properly. Commitment of a substrate protein to ubiquitin–proteasome-mediated degradation is regulated by (1) modification of the substrate; (2) modulation of ubiquitin ligase activity; and (3) removal of ubiquitins.

4.26.6.1.1 Modification of the substrate

Protein substrates are degraded in the cell at specific times in response to physiological stimuli. In addition, degradation of substrates is likely to be spatially restricted within a cell. Based on the accumulated evidence, it appears that the vulnerability or resistance to ubiquitin–proteasome-mediated degradation is regulated usually by a posttranslational modification. The protein substrates are modified in two main ways: (1) by posttranslational modification such as phosphorylation or (2) by allosteric modifications.

4.26.6.1.1.(i) Phosphorylation of the substrate

Phosphorylation of a substrate can make it vulnerable to ubiquitination or resistant to ubiquitination. For example, yeast cyclins Cln2 and Cln3, the cyclin-dependent kinase inhibitor p27Kip1, and transcriptional regulators IκBα and β-catenin are ubiquitinated after phosphorylation. In neurons, ubiquitination of p35, a neuronal-specific activator of cyclin-dependent kinase 5 (Cdk5), is stimulated when the protein is phosphorylated by Cdk5 within the active kinase complex (Patrick et al., 1998).

It is instructive to consider examples of some substrates as to how the regulation at the level of substrate works. The transcription factor nuclear factor kappa B (NF-κB) is inhibited by IκBα, which binds to NF-κB and keeps it in an inactive form in the cytosol. NF-κB is activated by numerous external stimuli such as cytokines, ionizing radiation, or neuronal injury. NF-κB is activated by proteolysis of IκBα, which releases NF-κB to be translocated to the nucleus, where it initiates transcription. Ubiquitination of IκBα requires phosphorylation on Ser-32 and Ser-36. Upon phosphorylation, IκBα is recognized by a specific

multisubunit RING finger ligase complex called $SCF^{\beta\text{-TrCP}}$. Activity of $SCF^{\beta\text{-TrCP}}$ ligase seems to be constitutive, while the kinases that phosphorylate IκBα are activated by the stimuli known to induce NF-κB-mediated transcription. It must be noted that phosphorylation of a substrate can make a stable protein vulnerable to degradation, as we saw above, or phosphorylation can have the opposite effect of stabilizing a short-lived protein. In the *Aplysia* nervous system, for example, CCAAT/enhancer binding protein (C/EBP), a transcription factor critical for long-term synaptic plasticity, is made resistant to ubiquitin–proteasome-mediated degradation when it is phosphorylated by mitogen-activated protein kinase (MAPK) (Yamamoto et al., 1999).

4.26.6.1.1.(ii) Allosteric modification of the substrate Although less well studied, a mechanism for making a substrate susceptible to ubiquitination is allosteric modification by ligands. A physiological example is that of degradation of regulatory (R) subunits of cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) (Hegde et al., 1993). R subunits are substrates for ubiquitination and degradation by the proteasome. Degradation of R subunits leads to persistent activation of PKA without persistence in cAMP elevation and bridges the short-term action of the neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) to gene expression (Hegde et al., 1997). The R subunit has two cAMP-binding sites. Without cAMP binding, R subunits are resistant to ubiquitination. Mutation studies have reveled that for ubiquitination of R subunits, binding of cAMP to both sites is essential. For example, R subunit mutants that bind cAMP to only one site are not efficiently degraded (Chain et al., 1999).

4.26.6.1.2 Modulating the activity of ubiquitin ligases

Ubiquitin ligases largely control the substrate specificity of the ubiquitin conjugation reaction. The temporal specificity of ubiquitin conjugation to substrates by these enzymes is provided by regulation of the ligase activity. The activity of ubiquitin ligases can be modulated by posttranslational modification such as phosphorylation and by allosteric modification of the enzyme, or by attachment to ubiquitin-like (UbL) proteins.

4.26.6.1.2.(i) Modulation of ubiquitin ligases by phosphorylation Regulation of ubiquitin ligase activity by phosphorylation has been shown in the studies on a multisubunit ligase, APC. As the name implies, this complex is critical for cell cycle progression into anaphase. A recent study shows that a form of APC is also expressed in postmitotic neurons.

APC can be activated by Cdc2 kinase, which appears to exert its effect by activating another protein kinase called polo-like kinase. In *Xenopus* and humans, phosphorylation of four different APC subunits, APC1, CDC16, CDC23, and CDC27, has been shown to be increased during mitosis. In neurons, APC might have a role in ubiquitinating different substrates from the ones ubiquitinated during cell cycle progression. An observation that lends credence to this notion is that levels of polo-like kinases Fnk and Snk dramatically increase with stimuli that produce long-term potentiation (LTP) and other forms of synaptic plasticity (Kauselmann et al., 1999). Activity of at least one single-subunit RING finger ubiquitin ligase, c-Cbl, is known to be regulated by phosphorylation. c-Cbl ubiquitinates the epidermal growth factor receptor (EGFR). Tyrosine phosphorylation of c-Cbl stimulates the ligase to ubiquitinate the EGFR at a site next to the RING finger domain (Glickman et al., 2002).

Phosphorylation of ubiquitin ligases could have an inhibitory effect as well. In the fission yeast *Schizosaccharomyces pombe*, PKA blocks APC activity. Moreover, the inhibitory effect of PKA seems to be dominant over the stimulatory effect of the polo-like kinase. Even if APC has been activated by polo-like kinase, addition of mammalian PKA to APC-containing fractions inhibits ubiquitination of the substrate cyclin B. Phosphorylation of ubiquitin ligases is also regulated by phosphatases. For example, type I protein phosphatases (PP1) are necessary for progression into anaphase. Also, in *S. pombe*, mutations in *dis2+*, a gene that encodes a catalytic subunit of PP1, have a deleterious effect. Although the exact mechanism of phosphatase action in promoting activity of APC is not clear, perhaps the phosphatases act by counteracting the negatively regulating protein kinases such as PKA (Hegde, 2004).

4.26.6.1.2.(ii) Allosteric modification of ubiquitin ligases In addition to phosphorylation, ubiquitin ligase activity can be stimulated by allosteric activation. A ubiquitin ligase called Ubr1 targets transcription factor Cup9, which is a negative regulator of di/tri peptide transporter *Ptr1* gene. Ubr1 has three sites at which it can bind other molecules. It is believed that site III on Ubr1 binds to the substrate

Cup9. Peptides that bind to site I or II can allosterically stimulate ligase activity of Ubr1 toward Cup9 (Turner et al., 2000).

4.26.6.1.2.(iii) Modulation of ubiquitin ligase activity by attachment of ubiquitin-like proteins

Activity of the ligases is also modified by posttranslational modification by covalent linkage of UbL proteins. Linkage of a UbL protein to an E3 ubiquitin ligase appears to modulate the activity of the ligase. For example, a UbL protein called Rub1 (related to ubiquitin 1) is conjugated to proteins of the cullin family, which are part of the SCF ligase complex. Conjugation to Rub1 is required for maximal activity of the SCF ligase 14 (Hegde, 2004).

4.26.6.1.3 Removal of ubiquitins

Ubiquitin–proteasome-mediated degradation can be regulated by removal of ubiquitin. When a protein is polyubiquitinated, it is targeted to the proteasome for degradation unless the ubiquitin chains are removed by the action of DUBs. Deubiquitination by DUBs serves two purposes: (1) Reversing ubiquitination of a protein or (2) disassembling the polyubiquitin chains before the ubiquitinated proteins are channeled to the 26S proteasome. Disassembly of polyubiquitin chains at the proteasome step is likely to be a rate-limiting step for degradation. Since the pore of the proteasome catalytic chamber is small (13 Å), the polyubiquitin tag needs to be removed before the substrate is fed into the catalytic core. Otherwise the catalytic chamber is likely to be clogged, thereby reducing the rate of degradation. The DUBs, called UBP6 (called USP14 in yeast) and UCH37, are associated with the proteasome. Also, a subunit in the lid of the 19S RC, Rpn 11, has been shown to possess deubiquitinating activity (Yao and Cohen, 2002).

4.26.7 Achieving Specificity of Ubiquitin Linkage: Combinatorial Coding by E2s and E3s

Specificity of conjugation of ubiquitin to a substrate requires the action of three enzymes: E1, E2, and E3. E1 is common to all ubiquitination reactions because this enzyme activates ubiquitin. There is some degree of specificity at the E2 step. The E3s are the most specific to a given substrate, however. Initially it was thought that there was a specific E3 for each substrate. This situation would be untenable because of the coding burden it places on the genome. Rather, it is likely that the specificity is derived from combination of recognition modules. A given single or multisubunit E3 has a domain specific to a given substrate. In some instances, an E3 ligates ubiquitin to only one substrate. In other cases, an E3 ligates ubiquitin to more than one substrate. When an E3 has activity toward more than one substrate, it is generally thought that the domain on an E3 that interacts with one substrate is distinct from the domain that an E3 uses to interact with another substrate. In some cases, however, all of the different substrates bind to the same site through the same interaction motif. It is estimated that the human genome contains approximately 1000 genes encoding E3s (Ciechanover and Brundin, 2003). The estimate for number of genes coding for E2s is at least 25 (Weissman, 2001). Considering that there are roughly 30,000 genes in the human genome, theoretically E2s and E3s together could potentially generate a unique combination for every protein encoded by the human genome! Besides the unique E2–E3 combinations, specificity can be generated by the state of the substrate (vulnerable or resistant to degradation) as well as regulation of E3s in many ways, as described. Thus, the ubiquitin conjugation machinery can be highly specific to a given substrate (Hegde, 2004).

4.26.8 Regulation of the Proteasome

Proteasome activity can be regulated in two main ways. One is regulation by cofactors or proteins that are loosely associated with it and by induction or phosphorylation of the subunits, particularly those of the 19S RC. The proteasome can also be regulated by a change in the composition of the intrinsic subunits.

4.26.8.1 Regulation by Cofactors and Loosely Associated Factors

In addition to the intrinsic subunits, proteins that interact with the proteasome complex regulate its activity. Often the cofactors are components of the ubiquitin pathway. Proteins such as chaperones and heat shock proteins also assist in proteasome-mediated degradation of proteins. Both E2s and E3s have been found to interact with the proteasome. For example, ubiquitin conjugating enzymes (E2s) Ubc1, Ubc2, and Ubc4 coimmunoprecipitate with the proteasome. E3s such as Ubr1 and Ufd4 have been shown to physically interact with subunits of the 19S RC.

APC and SCF ubiquitin ligases co-purify with the 19S RC. A ubiquitin ligase called Hul5 is associated with the proteasome as well. The cofactors interact with the proteasome directly or via other proteins such as Cic1 and hPLICs that recruit the cofactors to the proteasome. For example, hPLIC-1 and hPLIC-2, the human counterparts of yeast Dsk2, interact with the proteasome as well as specific E3s E6-AP and β-TrCP. Two other proteins, Rad23 and BAG1, are also known to interact with the proteasome (Hegde, 2004).

In addition, the DUBs interact with the proteasome. A DUB called Ubp6 is known to interact with the regulatory complex of the proteasome. Since binding to the proteasome increases the activity of Ubp6 300-fold, Ubp6 is thought to assist in the removal of polyubiquitin chain just before the substrate is degraded and aid in recycling ubiquitin for further use. Other DUBs have also been reported to associate with the proteasome. A yeast DUB which is related to the human tre-2 oncogene associates with the proteasome and assists in the disassembly of the polyubiquitin chain. The *Aplysia* homolog of UCH-L1 (Ap-uch) associates with the proteasome and improves proteolytic activity. In *in vitro* experiments, it was shown that Ap-uch cleaves the first ubiquitin attached to the substrate (Hegde et al., 1997). Among the factors that interact with the proteasome are heat shock proteins and chaperones. It has been known for a long time that the ubiquitin–proteasome pathway degrades misfolded or unfolded proteins. The general belief is that the heat shock proteins refold the misfolded proteins into the right conformation. Misfolded proteins not rescued by the heat shock proteins are thought to be substrates for the ubiquitin–proteasome pathway. It was not clear, however, which ubiquitin ligase (E3) specifically recognizes misfolded proteins. Recently, a chaperone called BAG1 has been shown to interact both with the proteasome and the heat shock protein Hsp70. CHIP (carboxyl terminus of the Hsc70-interacting protein) is known to ubiquitinate unfolded proteins. CHIP contains a protein sequence motif called the U-Box and a RING finger domain. CHIP can interact with either Hsp70 or Hsp90, and together with either of the heat shock proteins can ligate ubiquitin to misfolded proteins. The cooperation between heat shock proteins and the proteasome is also required to degrade aberrant membrane proteins. For example, the cystic fibrosis transmembrane conductance regulator (CFTR) is a membrane protein which is often misfolded. CHIP and Hsc70 together recognize the CFTR protein and target it to proteasome-mediated degradation (Hegde, 2004).

Proteasome activity can also be regulated by substitution of subunits of the 20S core with subunits such as the ones induced by interferon γ (IFNγ). Furthermore, alternative regulatory complexes with different composition than that of the 19S RC can be attached to the 20S core, which alters the substrate specificity and activity of the proteasome (Glickman et al., 2002).

4.26.8.2 Regulation of the Proteasome by Induction and Phosphorylation of Subunits and Subcellular Distribution

The subunits of the 26S proteasome are not fixed but change in response to the physiological condition of the cell. The capacity of the 26S proteasome to degrade ubiquitinated proteins can be regulated by changes in (1) the total amount of the proteasome, (2) its subunit composition, and (3) its subcellular distribution. The changes in the total amount of 26S can be brought about by the extent of 19S cap binding to the 20S core. This could occur by an increase in the amount of 19S as well as by increased association of the existing 19S with the 20S core. During metamorphosis of *Manduca*, flight muscles develop and intersegmental muscles are destroyed. The destruction of intersegmental muscles is brought about by an increase in ubiquitin-dependent proteolysis as a result of extensive hormone-dependent reprogramming of the 19S RC. It has been shown that the multiubiquitin binding subunit (MBP/S5a) and the ATPases MSS1 (S7) and S4 are induced significantly during this period. An increase in the amount of 26S by enhanced binding of 19S to 20S without an increase in the total amount of 20S occurs during the metaphase-anaphase transition in the meiotic cell cycle. In addition to the regulation of total proteasome content, the activity of the proteasome also appears to be regulated by alterations in its subcellular distribution. For example, during ascidian (marine animals commonly called sea squirts) embryonic development, the distribution of the proteasome changes in a cell-cycle-dependent manner. The proteasome localized in the nucleus during interphase disappears from the nucleus during prophase, and in telophase the proteasome is again localized in the newly formed nucleus (Hegde, 2004).

Proteasome activity can also be regulated by phosphorylation. For example, it has been shown that phosphorylation events are necessary for the

assembly of the 26S proteasome. Several subunits, including MSS1, S4, S6, and S12 of the 19S RC, have been shown to be phosphorylated. Recently it was demonstrated that assembly of the proteasome requires phosphorylation of Rpt6, an ATPase subunit. Our recent studies showed modulation of the proteasome activity by protein kinases in the nucleus and synaptic terminals. Interestingly, we observed that protein kinases differentially modulate the proteasome activity in the two subcellular compartments of the neuron (Upadhya et al., 2006).

4.26.9 Ubiquitin–Proteasome Pathway and Synaptic Plasticity

Although ubiquitin was used a marker for brain pathology, no physiological or pathological role for ubiquitin in the nervous system was found until a little over a decade ago. The first discovery of ubiquitin–proteasome-mediated degradation of a physiologically relevant substrate in the nervous system was that of R subunits of PKA (Hegde et al., 1993). Since then several substrates of the ubiquitin–proteasome pathway in the nervous system have been identified (**Table 1**).

4.26.9.1 Degradation R Subunits of PKA and Proteolytic Removal of a cAMP Response Element Binding Protein Repressor

A role for the ubiquitin–proteasome pathway in synaptic plasticity was discovered during the investigation of persistent activation of PKA. Studies on the biochemical mechanism of long-term facilitation (Greenberg et al., 1987) in *Aplysia* indicated that PKA was persistently activated in the absence of elevated cAMP. How is PKA activated in the absence of sustained increase in cAMP? It was found that the R subunits of PKA were decreased without any change in the catalytic (C) subunit during induction of long-term facilitation. Since there was no change in mRNA for either the R subunit or the C subunit, it was concluded that R subunits were diminished perhaps through proteolysis. What is the mechanism of R subunit degradation? In a

Table 1 Components of the ubiquitin–proteasome pathway with known physiological function in the nervous system[a]

Molecule	*Description*	*Reference*
R subunit	Subunit of cAMP-dependent protein kinase—crucial for synaptic plasticity in *Aplysia*	Hegde et al., 1993
CREB1b	A transcription repressor that antagonizes CREB during long-term facilitation in *Aplysia*	Upadhya et al., 2004
GluR1	Transmitter receptor critical for regulating synaptic strength	Burbea et al., 2002
β2-adrenergic receptor	Receptor that functions to modulate synaptic activity	Shenoy et al., 2001
PSD-95	A structural protein in PSD important for synaptic plasticity	Colledge et al., 2003
Dunc-13	Presynaptic protein in *Drosophila* that primes synaptic vesicle release	Speese et al., 2003
Epsin15	Monoubiquitination of epsin bridges transmembrane receptors to clathrin-coated pits for endocytosis	Carbone et al., 1997
E6-AP	Ubiquitin ligase (E3) important for synaptic plasticity, contextual memory	Jiang et al., 1998
Ap-uch	Deubiquitinating enzyme with a role in long-term synaptic plasticity in *Aplysia*	Hegde et al., 1997
Bendless	Ubiquitin-conjugating enzyme (E2) that controls synapse formation in *Drosophila*	Muralidhar and Thomas, 1993; Oh et. al., 1994
APC	A multisubunit RING finger ligase critical for regulating axonal morphogenesis, synaptic size and activity	Juo and Kaplan, 2004; van Roessel et al., 2004; Stegmuller et al., 2006
LIN-23	The substrate-binding part (F-Box) of SCF ligase that regulates glutamate receptor abundance	Dreier et al., 2005
Fat facets	Deubiquitinating enzyme that regulates glutamate receptor abundance	DiAntonio et al., 2001

[a]This list includes only some examples and is not meant to be comprehensive.

series of biochemical experiments, Hegde et al. (1993) found that R subunits were substrates for ubiquitination and proteasome-mediated degradation. Moreover, a UCH (Ap-uch) that interacts with the proteasome was found to be induced by 5-HT, the neurotransmitter that induced long-term facilitation. Ap-uch was found to be critical for induction of long-term facilitation (Hegde et al., 1997) (**Figure 3**). Subsequently, Chain et al. (1999) showed that at sensorimotor neuron synapses, injection of lactacystin, a specific proteasome inhibitor, blocked induction of long-term facilitation. Since the R subunit inhibits the activity of C subunits of PKA, the results were interpreted to suggest that the ubiquitin–proteasome pathway operates to remove inhibitory constraints on formation of long-term memory. This has been corroborated by work carried out on the rat hippocampus. Lopez-Salon and coworkers (Lopez-Salon et al., 2001) demonstrated that bilateral infusion of lactacystin to the CA1 region of the rat hippocampus caused total retrograde amnesia for a one-trial avoidance learning. They also showed that total ubiquitination increases in the hippocampus 4 h after the training. These results are consistent with the idea that a decrease in some critical inhibitory proteins during long-term memory formation is mediated by the ubiquitin–proteasome pathway.

Additional evidence that the ubiquitin–proteasome pathway might function to degrade proteins that normally inhibit long-term synaptic plasticity has also been obtained using the *Aplysia* model. Stimulation protocols that induce long-term facilitation in *Aplysia* cause ubiquitination and degradation of a cAMP response element binding protein (CREB) repressor called CREB1b. Both ubiquitination and degradation of CREB1b are increased by protein kinase C (**Figure 3**). It remains to be seen whether CREB1b or the ligase that targets CREB1b for ubiquitination is modulated by PKC (Upadhya et al., 2004).

4.26.9.2 Modulation and Essential Function of a Deubiquitinating Enzyme in Synaptic Plasticity

Subsequent to the finding on ubiquitin–proteasome-mediated degradation of R subunits of PKA, a crucial role in long-term facilitation for a neuronal-specific UCH was discovered. *Aplysia* UCH (Ap-uch) is the homolog of human UCHL1 and is induced by stimuli that induce long-term facilitation but not stimuli that induce short-term facilitation. Injection of antibodies or antisense oligonucleotides specific to Ap-uch into sensory neurons that synapse onto motor neurons in

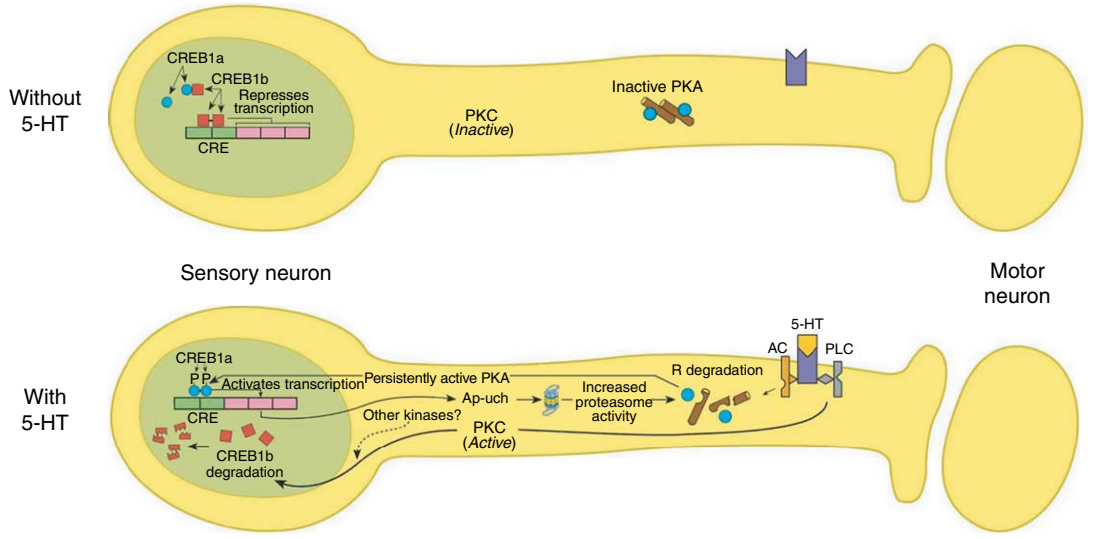


Figure 3 Role of the ubiquitin–proteasome pathway in long-term facilitation. When sensory neurons are stimulated with the neurotransmitter serotonin (5-HT), which induces long-term facilitation, R subunits of protein kinase A (PKA) are ubiquitinated and degraded by the proteasome, making the kinase persistently active. The catalytic subunit of PKA (blue circle) translocates to the nucleus and phosphorylates cAMP response element binding protein 1a (CREB1a), the activator form of CREB. Concomitantly, the repressor form of CREB, CREB1b, is degraded by the ubiquitin–proteasome pathway. Protein kinase C (PKC), which is also activated by 5-HT-mediated signaling, stimulates ubiquitin conjugation to CREB1b and subsequent degradation. AC, adenylyl cyclase; PLC, phospholipase C.

culture blocked induction of long-term facilitation (Hegde et al., 1997). Investigation of biochemical functions of Ap-uch indicated that Ap-uch is capable of cleaving small attachments to linearly attached ubiquitin molecules such as ubiquitin–ubiquitin–cysteine but not large attachments such as glutathione-S-transferase (GST) in substrates such as ubiquitin–GST. More interestingly, additional biochemical analyses showed that Ap-uch associates with the proteasome. The association of Ap-uch increases the rate of degradation by the proteasome. For example, addition of recombinant Ap-uch to *in vitro* degradation systems showed that there was approximately double the degradation of the R subunit of PKA. Since persistent activation of PKA has been shown to be critical for induction of long-term facilitation and R subunits of PKA were found to be substrates for the ubiquitin–proteasome pathway, the experiments on Ap-uch provided some molecular explanation for the role of regulated proteolysis in long-term facilitation (Hegde et al., 1997). How does Ap-uch increase the rate of degradation by the proteasome? Using recombinant ubiquitin with its Lysine-48 mutated to Arg, which can support multiple monoubiquitin attachments to the protein substrate, it was shown that Ap-uch stimulates the release of ubiquitin from substrates (Hegde et al., 1997). Therefore, it can be inferred that Ap-uch perhaps cleaves the first ubiquitin in the polyubiquitin chain attached to the substrate. The function of UCHs in synaptic plasticity appears to be evolutionarily conserved. Recently, the mammalian homolog of Ap-uch, UCH-L1, has been shown to play a role in long-term memory in mice.

4.26.9.3 Possible Differential Role of the Proteasome in Different Compartments of Neurons

Although previous investigations found that proteasome inhibitors block induction of long-term facilitation, recent analysis of long-term facilitation at *Aplysia* synapses obtained different results. Bath application of the active form of lactacystin, *clasto*-lactacystin β-lactone, to sensorimotor neuron synapses resulted in strengthening the synapses and increasing neurite outgrowth in isolated sensory neurons. Furthermore, application of β-lactone increased 5-HT-induced long-term facilitation. The increase in neurite elongation is consistent with results obtained in PC12 and Neuro2A cells in which lactacystin induces neurite outgrowth. Interestingly, the increase in synaptic strength using the *Aplysia* sensorimotor neuron synapses observed by application of β-lactone was blocked in the presence of the translation inhibitor anisomycin but not by the transcription inhibitor actinomycin D (Zhao et al., 2003). This result is highly surprising, since innumerable studies have documented the requirement for transcription in the induction of long-term facilitation in *Aplysia* as well as several vertebrate forms of long-term memory (Chen and Tonegawa, 1997; Kandel, 2001). Both sets of results can be reconciled if one postulates that proteasome has different roles in different cellular compartments. In the same neuron, the proteasome is likely to carry out different tasks in different subcellular compartments, resulting in different physiological consequences at different loci. Therefore, blocking different roles of the proteasome during induction of memory would lead to distinct and even opposite effects on synaptic strength. For example, the proteasome is known to degrade transcription repressors. Degradation of transcription repressors should allow transcription activators to induce gene expression, which in turn leads to development of long-term facilitation. If the proteasome is inhibited only in the nucleus before the repressors are degraded, gene expression and hence induction of long-term facilitation should be blocked. On the other hand, if the degradation of proteins needed at the synapse for developing long-term facilitation is inhibited by the proteasome, long-term facilitation should be enhanced. Although it might sound radical, I think the main purpose of transcription during induction of long-term facilitation or other forms of long-term memory is to provide mRNAs for synthesis of the rapidly turning over proteins needed for memory formation. These proteins are likely to be degraded by the ubiquitin–proteasome pathway. If the degradation of these proteins is prevented, then long-term memory formation becomes independent of transcription. In support of this idea, Zhao et al. (2003) found that proteasome inhibitor-induced synaptic strengthening depends on translation but not transcription. Differential temporal regulation of the proteasome could occur within the same subcellular compartments. In the nucleus, the proteasome has to degrade transcription repressors to allow transcription activators to induce gene expression, which in turn leads to development of long-term facilitation. If the proteasome is inhibited during the time the repressors are degraded, gene expression and hence induction of long-term facilitation should be blocked. In support of this idea, we have found that a transcription repressor called CREB1b is degraded by the ubiquitin–proteasome pathway in response to long-term facilitation-inducing protocols (Upadhya et al., 2004).

Our biochemical experiments on the proteasome also support differential function of the proteasome in different neuronal compartments. Our results showed that in both *Aplysia* nervous system and mouse brain, proteasome activity in the synaptic terminals is significantly higher than that of the nuclear proteasome. Moreover, the proteasome activity in the two compartments is differentially regulated by protein kinases relevant to synaptic plasticity such as PKA, PKC, and MAPK (Upadhya et al., 2006).

As discussed, differential activity of the proteasome in *Aplysia* might explain conflicting results obtained in different studies. Does differential proteasomal activity affect synaptic plasticity differentially in vertebrates? Our preliminary studies indicate that proteasome has differential roles during induction and maintenance phases of the late phase of long-term potentiation (L-LTP). We have found that proteasome inhibition enhances the early phase of L-LTP but inhibits the late, maintenance phase of L-LTP (Hegde et al., 2006). Although two other studies have tested proteasome inhibition on LTP, neither study reported differential function of proteasome on LTP (Fonseca et al., 2006; Karpova et al., 2006). Both of these studies only showed inhibition of LTP with proteasome inhibitor. The study by Karpova et al. (2006) used MG132, which is not a highly specific proteasome inhibitor. MG132 has been known to strongly inhibit other proteases such as calpain and cathepsin B (Chain et al., 1999; Tang and Leppla, 1999). Because calpain inhibition is known to block LTP (del Cerro et al., 1990; Denny et al., 1990), the results of experiments that use only MG132 are not strictly attributable to proteasome inhibition. In the study by Fonseca et al. (2006), I think the main reason the authors failed to discover the enhancement of the early phase of LTP with proteasome inhibition is because they used nanomolar concentrations of lactacystin and epoxomycin. Numerous studies, including the one cited in the aforementioned study (Dick et al., 1996), have established that micromolar concentration of lactacystin is required for efficient proteasome inhibition. All three catalytic activities of the proteasome are inhibited only with 100 μmol l^{-1} lactacystin or 50 μmol l^{-1} β-lactone (Fenteany et al., 1995).

4.26.10 Roles of the Ubiquitin–Proteasome Pathway at the Synapse

Ubiquitin–proteasome-mediated proteolysis has been found to have both presynaptic and postsynaptic roles at the synapse.

4.26.10.1 Presynaptic Roles of Proteolysis: Degradation of Synaptic Vesicle Proteins

The earlier observations on the role of the ubiquitin–proteasome pathway in synaptic plasticity mainly focused on long-term effects. Recent studies, however, indicate that the ubiquitin–proteasome pathway also functions in regulating short-term synaptic plasticity. For example, a protein, Dunc-13, which is critical in priming the synaptic vesicles, is ubiquitinated and degraded by the proteasome in *Drosophila* neuromuscular synapse. Application of proteasome inhibitors and the dominant-negative mutation in a core subunit (β6) of the *Drosophila* proteasome both lead to an increase in presynaptic accumulation of Dunc-13 protein. Also, application of the proteasome inhibitors lactacystin and epoxomycin cause an increase in the excitatory junctional current, suggesting that accumulation of Dunc-13 and the resultant increase in the net Dunc-13 quantity leads to enhanced synaptic transmission (Speese et al., 2003).

Is it likely that the quantity of other synaptic proteins with a key role in neurotransmission is regulated by the ubiquitin–proteasome pathway? If so, regulated proteolysis may have a wider role in controlling short-term synaptic plasticity. As of now, two other synaptic vesicle proteins, synaptophysin and syntaxin, have been shown to be substrates for ubiquitin–proteasome-mediated degradation. Vulnerability of synaptophysin for ubiquitin–proteasome-mediated degradation was discovered indirectly by looking for proteins that bind to synaptophysin in the yeast two-hybrid system. Two genes encoding for proteins Siah-1A and Siah-2 were isolated from the rat brain two-hybrid cDNA library. These two genes are closely related to each other and are homologs of *Drosophila seven in absentia* gene. Siah proteins are single-subunit RING finger ubiquitin ligases with the RING finger domain in the N-terminal region. It is not clear which ligase (Siah1-A or Siah-2) is responsible for degradation of synaptophysins *in vivo* in neurons. Because the experiments were carried out in nonneuronal cells (Chinese hamster ovary cells) and PC12 cells, the physiological consequences of synaptophysin degradation remain to be determined.

Another presynaptic substrate for the ubiquitin–proteasome pathway is syntaxin 1. The group that isolated the Siah ligases also investigated ubiquitin–proteasome-mediated degradation of syntaxin 1 using the yeast two-hybrid strategy. The ligase that ubiquitinates syntaxin 1 is called staring (syntaxin 1-interacting

RING finger protein). Co-expression of staring with syntaxin 1 in HeLa cells increases the degradation of syntaxin 1, which can be inhibited by the proteasome inhibitor MG132. Again, the physiological effect of ubiquitin–proteasome-mediated degradation of syntaxin 1 remains to be determined.

4.26.10.2 Modulation of Postsynaptic Structure and Function by Proteolysis

Ubiquitin and proteasome appear to regulate proteins that control synaptic transmission such as neurotransmitter receptors and proteins that are part of the synaptic structure such as postsynaptic density proteins. Investigations in *Caenorhabditis elegans* revealed a role for ubiquitin in endocytosis of GLR-1 type of glutamate receptor (Burbea et al., 2002). Later studies in *C. elegans* showed a role for APC in regulating the abundance of GLR-1 glutamate receptor, although the APC substrate was not identified (Juo and Kaplan, 2004). Intriguingly, SCF ligase also appears to control GLR-1 glutamate receptor quantity in *C. elegans* (Dreier et al., 2005). In mammalian hippocampal neurons, treatment with the proteasome inhibitor MG132 blocks agonist-induced endocytosis of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type glutamate receptors (Patrick et al., 2003). The exact mechanism of AMPA receptor endocytosis in mammals is not clear. Patrick et al. (2003) argue that the AMPA receptor itself is not the target of proteasome action. Furthermore, apart from AMPA-induced internalization, *N*-methyl-D-aspartate (NMDA)-induced internalization is also prevented by application of the proteasome inhibitor. The authors speculate that a protein(s) that interacts with AMPA receptor might be the target (Patrick et al., 2003). Given that both NMDA and AMPA agonists stimulated internalization that is inhibited by the proteasome, I think that this is a case of passive internalization and not an instance of regulated AMPA receptor endocytosis. In support of this idea, a thorough study by others (Colledge et al., 2003) showed that postsynaptic density-95 (PSD-95) is regulated by ubiquitin–proteasome-mediated degradation, and degradation of PSD-95 leads to AMPA receptor internalization. In this study as well, proteasome inhibitors prevent AMPA receptor internalization. Furthermore, Colledge et al. (2003) demonstrated that application of the proteasome inhibitor MG132 to hippocampal slices reduces the magnitude of hippocampal long-term depression. A direct role for ubiquitin in regulated endocytotic degradation of AMPA receptors is still an open question. Given the *C. elegans* data of Burbea et al. (2002), it is possible that targeting of AMPA receptors for degradation after internalization depends on ubiquitination. Endocytosis of other neurotransmitter receptors might be directly or indirectly regulated by ubiquitination. Glycine receptor has been shown to be internalizedupon ubiquitination. A protein associated with gamma-aminobutyric acid A (GABAA) receptors, Plic-1, indirectly controls removal of GABAA through endocytosis. Interestingly, proteasome inhibitors prevent degradation of internalized GABAA receptors. Ubiquitin–proteasome-mediated proteolysis appears to regulate several other proteins in addition to PSD-95 in the PSD proteins, including several structural proteins. For example, Shank, GKAP, and AKAP79/150 are degraded through the ubiquitin–proteasome pathway (Ehlers, 2003). Unlike the degradation of PSD-95, the physiological relevance of proteolytic removal of Shank, GKAP, and AKAP79/150 is not clear because the studies were correlative and a direct link between ubiquitin–proteasome-mediated degradation of the PSD proteins and structural remodeling was not established. There is also evidence that the ubiquitin–proteasome pathway controls a protein that regulates spine shape. SPAR controls dendritic spine shape by reorganizing the actin cytoskeleton (Pak et al., 2001). During activity-dependent remodeling of synapses, SPAR was found to be degraded by the ubiquitin–proteasome pathway (Pak and Sheng, 2003). Degradation of SPAR is stimulated by serum-inducible kinase (SNK). Neuronal activity induces SNK mRNA in the cell body and the induced SNK is targeted to the dendritic spines. Because of the time required for SNK mRNA to travel to the spines, Pak and Sheng (2003) speculate that SPAR may function to oppose synaptic remodeling after elevated activity.

4.26.11 The Ubiquitin–Proteasome Pathway and Developmental Synaptic Plasticity

Support for a role for the ubiquitin–proteasome pathway in neuronal connectivity was obtained through investigations on a *Drosophila* mutation called bendless, which causes abnormal neuronal connectivity. The bendless gene encodes a ubiquitin-conjugating enzyme (Muralidhar and Thomas, 1993; Oh et al., 1994). Additional evidence for a function of the ubiquitin–proteasome pathway in synapse formation

came from a screen in *Drosophila* designed to identify the genes controlling size and strength of synapses. This screen found that fat facets (faf), a gene that encodes a DUB, functions in synapse formation in the neuromuscular junction. Overexpression of faf in the developing *Drosophila* nervous system causes synaptic overgrowth and perturbs synaptic transmission. Another genetic screen for identifying the mutants that enhance the faf overexpression phenotype found that a loss of function in a gene called highwire (hiw) causes a significant increase in number of synapses. The hiw gene encodes a huge protein with 5233 amino acids. The hiw protein contains a RING finger domain, a characteristic feature of some ubiquitin ligases. Therefore, it is conceivable that hiw and faf promote or inhibit the degradation of the same substrate by ubiquitinating or deubiquitinating it, respectively (Wan et al., 2000; DiAntonio et al., 2001). How does hiw regulate synaptic growth? It has been shown to negatively regulate signaling by bone morphogenetic protein (BMP)/transforming growth factor β (TGFβ), which stimulates presynaptic growth in *Drosophila* neuromuscular junction. Highwire binds to a protein in the BMP/TGFβ signaling pathway called Med. Although there is no direct evidence yet for ubiquitination of Med by hiw, the genetic data suggest that hiw functions to ubiquitinate Med for degradation. Moreover, synaptic overgrowth produced by overexpression of faf is suppressed by disrupting BMP signaling (McCabe et al., 2004). Regulation of the BMP/TGFβ signaling pathway may be only one way in which hiw controls synaptic growth. Another study using a screen for suppression of synaptic growth caused by hiw mutation identified mutation in wallenda, a MAP kinase kinase kinase. Hiw protein was shown to regulate synaptic levels of endogenous wallenda protein. It has been suggested that hiw might have multiple functions because of its vast size and multiple domains and therefore might be responsible for regulating BMP/TGFβ signaling as well as the MAPK pathway.

The ubiquitin–proteasome pathway also seems to regulate synaptic size. In *Drosophila* neuromuscular junction (NMJ), a mutation in APC2, a subunit of APC, greatly increases the number of boutons. APC in *Drosophila* NMJ regulates levels of Liprin-α in neurons. Liprin-α is a protein that interacts with tyrosine phosphatases. In *Drosophila*, Liprin-α is required for control of synaptic number by Dlar, a receptor tyrosine phosphatase (van Roessel et al., 2004). The exact mechanism by which regulation of Liprin-α by APC regulates synaptic size remains to be elucidated.

Protein degradation has been found to play a role in other major processes that establish neuronal connectivity such as axon guidance and dendrite sculpting. In neurons of *Xenopus* retina, netrin-1 causes the growth cones to turn and lysophosphatidic acid (LPA) causes the growth cone to collapse. Both netrin-1 and LPA effects on the growth cone are blocked by proteasome inhibitors. In addition, in cerebellar granule neurons, APC regulates axonal morphogenesis through its action on a transcription co-repressor called SnoN (Stegmuller et al., 2006). The evidence for ubiquitin–proteasome-mediated proteolytic molding of the dendrites came from work on *Drosophila*. During metamorphosis, specific sensory neurons in *Drosophila* larvae undergo extensive dendritic pruning and remodel their dendrites. Mutation in a ubiquitin-conjugating enzyme called UbcD1 blocks dendritic pruning. Additional experiments revealed that UbcD1 is likely to degrade a ubiquitin ligase called DIAP1 that antagonizes caspases. This study provides an elegant explanation for local activation of caspases which enables removal of dendrites while preserving the neurons.

4.26.12 Ubiquitination and Endocytosis

As a result of earlier research on nonneuronal cells and recent studies on neurons, it has become clear that ubiquitination plays a vital role in targeting membrane proteins for endocytosis. Some general principles regarding the role of ubiquitin in endocytosis have emerged. A membrane protein, say, a neurotransmitter receptor, becomes a target for endocytosis when it is ubiquitinated. Unlike the polyubiquitin chain that marks substrate for proteasome-mediated degradation, endocytosis appears to be mainly mediated by monoubiquitination. The ubiquitinated receptor binds to adaptor proteins called epsins that couple the receptor to the clathrin-coated pits (Carbone et al., 1997). The ubiquitinated receptor then undergoes endocytosis and is incorporated into endosomes, which in turn are sequestered into the multivesicular body. The membrane of the multivesicular bodies becomes continuous, with lysosomes leading to degradation of the receptor. It has been shown that targeting the internalized receptor to the multivesicular body also requires ubiquitination. Moreover, a 350-kDa complex called endosomal sorting complex that is

required for transport (ESCRT-I) and recognizes the ubiquitinated receptors has been identified. Function of ESCRT-I is essential for sorting the endocytosed receptor into the multivesicular body (**Figure 4**) (Katzmann et al., 2001). Two other complexes, ESCRT-II and ESCRT-III, are thought to be necessary for the continued sorting into the multivesicular body (Babst et al., 2002a,b). If the ubiquitin from the endocytosed receptor is removed the receptor recycles back to the plasma membrane. Although deubiquitination of endocytosed receptors is less well studied, UBPs are likely to carry out the ubiquitin removal function (**Figure 4**).

4.26.12.1 Endocytosis and Synaptic Function

Internalization through ubiquitin-mediated signaling and sorting into the multivesicular body is likely to play a critical role in controlling the neurotransmitter receptor number on the plasma membrane (hence synaptic function) in the nervous system. In support of this idea, Burbea et al. (2002) found in *Caenorhabditis elegans* that GLR-1, a homolog of the mammalian GluR1 which is part of the AMPA-type glutamate receptor, is internalized through a ubiquitin-mediated mechanism. GLR-1 was found to be ubiquitinated *in vivo*. When ubiquitin was overexpressed, the quantity of GLR-1 on the neuronal surface was reduced. The effect of ubiquitin overexpression was blocked by mutations in the unc-11 gene, which encodes a clathrin adaptin protein (AP180). Mutation of a specific lysine residue to arginine in the cytoplasmic tail of GLR-1 reduced internalization of GLR-1. All these results together suggest that GLR-1 is endocytosed through a ubiquitin- and clathrin-dependent mechanism. Furthermore, mutation of the lysine residues that prevent ubiquitination of GLR-1-affected locomotion in *C. elegans*. These observations indicate that prevention of ubiquitin-mediated GLR-1 endocytosis leads to an increase in synaptic strength resulting from

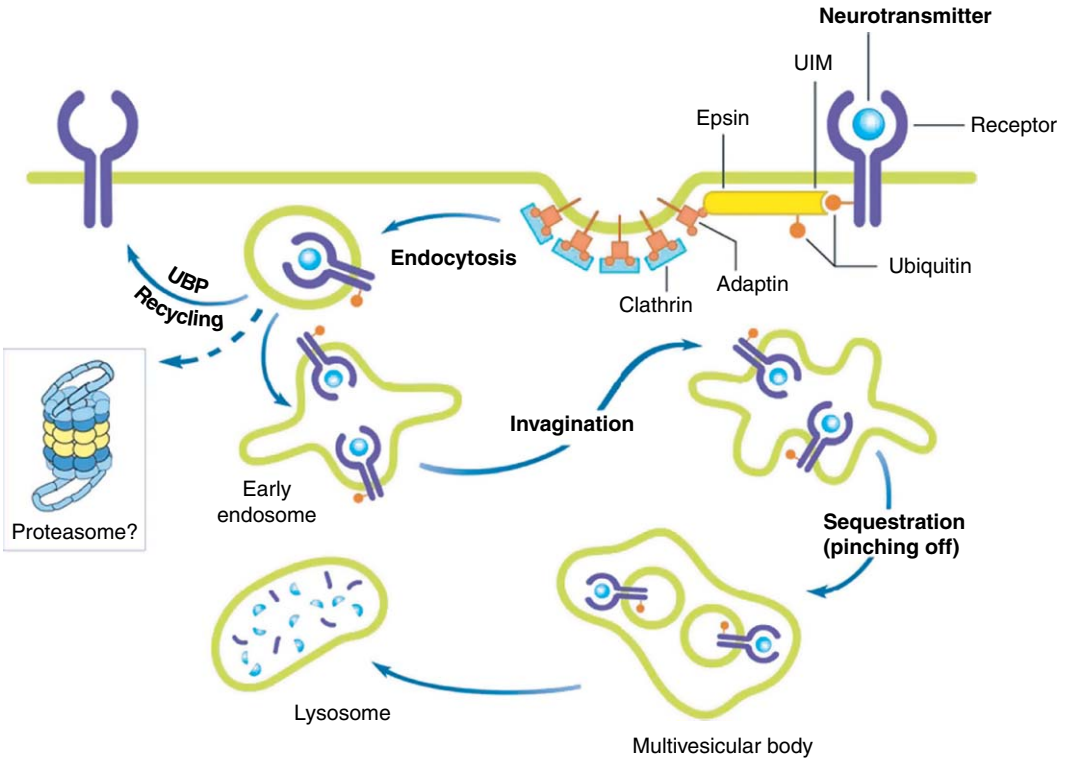


Figure 4 Ubiquitin and endocytosis. Receptors on the plasma membrane undergo monoubiquitination as a result of ligand (e.g., neurotransmitter). Ubiquitinated receptors bind to proteins called epsins, which in turn interact with adaptor proteins (adaptin) bound to clathrin-coated pits. Ubiquitination also functions to sort the internalized membrane protein into early endosomes, which directs them to degradation by lysosome via the multivesicular body. If ubiquitin from the endocytosed receptors is removed by a UBP, the receptor recycles back to the membrane. Proteasome inhibitors block endocytotic degradation of some proteins such as glutamate receptor subunits, indicating a possible role for the proteasome. UIM, ubiquitin-interacting motif.

a higher number of GLR-1 on the neuronal surface. Since the cytoplasmic tails of four mammalian AMPA receptor subunits (GluR1–GluR4) and *C. elegans* GLR-1 have a stretch of 16 conserved amino acids homologous to the region in the yeast Ste2p and Ste6p proteins that have been shown to be signals for ubiquitination and endocytosis, it is highly likely that GluR subunits in other species are endocytosed through a ubiquitin-dependent mechanism as well. Moreover, it has been found that internalization of GluR1 and GluR2 subunits of the AMPA receptor is inhibited by introduction of ubiquitin chain elongation mutant (Lys-48 mutated to Arg-48) (Patrick et al., 2003). Intriguingly, it was observed that a proteasome inhibitor, MG132, reduced internalization of AMPA receptors. One would expect the ubiquitinated GluRs to be either recycled back to the plasma membrane or to be routed to the lysosome for degradation. Involvement of the proteasome, if proven, would add a new twist to the process of endocytosis. A similar effect of MG132 on degradation of internalized vasopressin receptors (Martin et al., 2003) as well as the quantity of GABA receptors (Bedford et al., 2001) has been reported (see following for description). Other neurotransmitter receptors have been shown to be internalized through a ubiquitin-dependent process as well. Using the *Xenopus* oocyte expression system, Buttner and colleagues (Buttner et al., 2001) demonstrated that inhibitory glycine receptors are ubiquitinated at the plasma membrane and internalized. The internalization process generates fragments of 35 and 13 kDa. In their experiments, application of concanamycin, which blocks acidification of lysosomal and endosomal compartments by inhibiting vesicular H^+-ATPases, prevents cleavage, whereas the proteasome inhibitor lactacystin has no effect on generation of the smaller fragments of glycine receptor. The GABAA receptor number on the neuronal membrane appears to be determined by a ubiquitin-mediated process as well. Evidence for a possible role of ubiquitin in GABAA receptor internalization is indirect. The GABAA receptor interacts with the UbL protein Plic-1, which stabilizes the GABAA receptor. Application of the proteasome inhibitor lactacystin leads to a significant increase in the steady-state levels of a1 and b3 subunits of the GABAA receptor (Bedford et al., 2001). The inference drawn with respect to GABAA internalization is that association with Plic-1 prevents the receptor from being ubiquitinated and routed to either the lysosome or the proteasome for degradation. It is not clear what roles proteasome and lysosome play in GABAA receptor degradation (Luscher and Keller, 2001). The Plic-1 UbL domain contains a proteasome-interacting motif (Upadhya et al., 2003). Since Plic-1 also binds the GABAA receptor a and b subunits through a different domain (ubiquitin-associated [UBA] domain, which is different from UbL domain), it is possible that the UbL domain in Plic-1 is utilized for routing the internalized GABAA receptor subunits for degradation through the proteasome.

Ubiquitination of plasma membrane receptors is likely to have a widespread role in the brain. Recently, mammalian G-protein-coupled receptors (GPCRs) have been shown to be endocytosed through a ubiquitin-mediated mechanism. Two receptors, the β2-adrenergic receptor (β2-AR) and the V2-type vasopressin receptor (V2-VR), have been studied in detail (Shenoy et al., 2001; Martin et al., 2003). The endocytosis of GPCRs mediated by the ubiquitin signal differs from the endocytosis of other receptors in that an adaptor protein called β-arrestin plays a role. β-arrestin is ubiquitinated as well. The endocytosed β2-AR is routed to the lysosome for degradation if the β-arrestin is deubiquitinated or dissociated from β2-AR. If β-arrestin remains ubiquitinated, β2-AR recycles back to the plasma membrane. In contrast to the β2-AR example, endocytosis of V2-VR typifies the regulated ubiquitin-mediated endocytotic removal and destruction of the receptor, which effectively reduces the receptor number on the plasma membrane. In response to simulation by the agonist, arginine-vasopressin V2-VR is internalized. Agonist stimulation induces ubiquitination of V2-VR as well as ubiquitination of β-arrestin2. Ubiquitination of β-arrestin2 as well as ubiquitination of V2-VR is persistent. Unlike other receptors, V2-VR appears to be polyubiquitinated. How is endocytosed V2-VR degraded? Is it routed to the lysosome? Although earlier studies showed that V2-VRs were delivered to the lysosome after internalization, it was found that ubiquitinated V2-VR could only be detected in the presence of a proteasome inhibitor, MG132 (Martin et al., 2003), thus suggesting that the ubiquitinated V2-VR was degraded by the proteasome. Is it possible, however, that both the proteasome and lysosome have a role in degradation of V2-VR? Requirement for both proteasome- and lysosome-mediated degradation has been reported for other plasma membrane receptors such as insulin-like growth factor receptor (Vecchione et al., 2003), estrogen receptor (Nawaz et al., 1999), and growth hormone receptor (van Kerkhof et al., 2000).

4.26.12.2 Fate of Proteins after Endocytosis: Lysosomal Versus Proteasomal Degradation

The traditional view of endocytotic degradation is that the degradation occurs in the lysosome. Also, as discussed, ubiquitin appears to target the endocytosed proteins to the multivesicular body for eventual degradation in the lysosome. A role for the proteasome is also indicated based on several studies. For example, endocytotic degradation of interleukin-2 receptor complex (Yu and Malek, 2001), growth hormone receptor (van Kerkhof et al., 2000), δ opioid receptor (Chaturvedi et al., 2001), vasopressin receptor (Martin et al., 2003), and GABAA receptor subunits (Bedford et al., 2001) is blocked by proteasome inhibitors. In addition, proteasome inhibitors have been shown to block endosomal sorting of membrane proteins to the lysosome (van Kurkhof et al., 2001). There is a caveat to these studies, however. Prolonged application of proteasome inhibitors leads to depletion of the ubiquitin pool (Patrick et al., 2003). Therefore, proteasome inhibitors might have an adverse effect on ubiquitination of receptors and hence indirectly block lysosomal degradation of receptors. If there is a role for the proteasome, it might be to partially degrade some proteins which otherwise block routing to the lysosome through the multivesicular body. Since proteasome is known to act on retrotranslocated endoplasmic reticulum proteins, it might act on the proteins in the early endosome in a similar fashion. In support of this idea, the investigations by others suggest that proteasome inhibitors might have a global effect on intracellular trafficking (Rocca et al., 2001). The least likely possibility is that endocytosed proteins are degraded through the lysosome or the proteasome, with the choice determined by differential ubiquitination.

4.26.13 Unanswered Questions and Future Directions

The research on the role of the ubiquitin–proteasome pathway in synaptic plasticity and other physiological functions in the nervous system has only begun to scratch the surface. One of the major tasks is to understand how the spatial and temporal regulation of substrate stability occurs in the nervous system. It is likely that the local protein degradation may be responsible for strengthening or weakening specific synapses (Hegde, 2004). It would also be important to elucidate how protein degradation works hand-in-hand with protein synthesis during induction and maintenance of synaptic plasticity. In addition, researchers need to develop precise tools for manipulating specific components of the ubiquitin–proteasome pathway in the nervous system. We can look forward to many exciting future discoveries on how protein degradation helps sculpt synapses.

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4.27 Transcription Regulation of Memory: CREB, CaMKIV, Fos/Jun, CBP, and SRF

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4.27.1 Introduction

Memories can persist for dramatically different lengths of time, from seconds and minutes to a lifetime. These different forms of memory have distinct molecular requirements. Short-term memory (STM) persists for minutes to hours and is thought to be mediated by covalent modifications of existing synaptic molecules, such as phosphorylation or dephosphorylation of enzymes, receptors, or ion channels (Stork and Welzl, 1999). In contrast, long-term memory (LTM) persists for days or longer and is thought to be mediated by both the growth of new synapses and restructuring of existing synapses (Bailey and Chen, 1989). There is extensive evidence from a range of species demonstrating that, unlike STM, LTM requires the transcription and translation of new proteins. Therefore, regulatory mechanisms that direct transcription may be important in the formation of LTM (Davis and Squire, 1984; Matthies, 1989). Here we examine the role of several transcriptional regulators (including transcription factors, a coactivator, and an upstream protein kinase) in LTM formation in rodents.

Transcription factors are proteins that activate or suppress transcription of a particular gene by binding to specific sets of short conserved sequences contained in the gene's promoter region. In general, transcription factors are composed of two domains: a DNA-binding domain that interacts with these gene-specific regulatory sites and a domain that exhibits transcriptional activation potential (for review, see Schwabe and Rhodes, 1991; Brivanlou and Darnell, 2002). The DNA-binding domain consists of amino acids that recognize specific DNA bases near the start of transcription. The activator domain of a transcription factor typically interacts with regulatory proteins and components of the transcriptional apparatus (RNA polymerase), thereby affecting the efficiency of DNA binding and regulating transcription (Agalioti et al., 2000). There are three main types of transcription factors: inducible transcription factors that are expressed as immediate-early genes in response to a particular stimulus (such as c-fos, c-jun, and Krox), constitutive transcription factors that are always expressed and are regulated by posttranslational modifications (such as Ca^{2+}/cAMP (cyclic adenosine 3′,5′-monophosphate); cAMP response element binding protein (CREB); and serum response factor (SRF)), and ligand-activated transcription factors that are activated by binding to a particular ligand (such as an estrogen receptor).

Transcription factors do not function in isolation to control transcription but work in concert with other transcription factors, upstream signaling pathways, and transcriptional cofactors. In this way, LTM probably requires the coordinated action of many transcriptional regulators. Nevertheless, this chapter focuses on the role of six well-studied transcriptional regulators, namely, CREB, c-fos, c-jun, CaMKIV (Ca^{2+}-calmodulin kinase type IV), CBP (CREB binding protein), and SRF in LTM formation.

4.27.1.1 Techniques to Examine the Role of Transcriptional Regulators in Memory

4.27.1.1.1 Correlating transcriptional activation with memory: Imaging studies

The role of transcriptional mechanisms in memory may be studied in two complementary ways. The first method uses imaging techniques to visualize activation of the transcription factor or a reporter of transcription following various types of behavioral training that normally induce LTM (see following). Although the data from these studies are correlative in nature, they are invaluable because they provide a powerful combination of systems and molecular approaches. They can identify where and when activation of a transcription factor occurs following stimulation that induces memory.

4.27.1.1.2 Altering transcription mechanisms

The second approach manipulates the function of a transcriptional regulator and assesses the impact of this manipulation on LTM. Several techniques have been used to disrupt or enhance the function of a

given transcriptional regulator. For instance, the gene of interest can be deleted constitutively throughout the body (classical knockout mice) or in specific brain regions (conditional knockout mice), or the function of the gene may be antagonized by the expression of a dominant negative form of the gene (through a transgenic mouse) or disrupted using antisense or, more recently, RNAi. Alternatively, viral vectors may be used to express different constructs (a wild-type, dominant negative, or dominant active version of the gene of interest) in specific brain regions at specific times. Each method alters the function of the gene of interest in a distinct way, and there are advantages and disadvantages associated with each approach. These are briefly reviewed below.

In a classical knockout mouse, the gene of interest is constitutively deleted throughout the body throughout development into adulthood. Although the analysis of mice in which transcriptional regulators have been constitutively knocked out have provided important information regarding the role of transcriptional regulators in memory formation, these constitutive knockout mice may have developmental deficits that interfere with the interpretation of the results from behavioral tests. In addition, because of the chronic nature of the disruption, there may be a compensatory upregulation of related transcriptional regulators.

Conditional gene knockouts, in which a gene of interest may be knocked out in a time-dependent and tissue-specific manner, may alleviate some of these interpretive difficulties. At present, most conditional knockouts are generated using the Cre-loxP system. Cre recombinase is an enzyme that excises a gene that is flanked by two target loxP recombination sites. Mice with the 'floxed' gene of interest are bred with a transgenic mouse expressing Cre recombinase. In the offspring, the floxed gene is excised only in tissue expressing sufficient Cre recombinase. Tissue-specific expression of Cre recombinase may be achieved by using different promoters. Two commonly used promoters are αCaMKII (which directs gene expression in postnatal excitatory neurons of the forebrain) and nestin (which directs gene expression specifically in neural precursor cells, such that both glia and neurons throughout the central nervous system (CNS) will express Cre recombinase). It is important to note that the efficiency of the gene knockout depends on the efficiency of recombination, and this may vary among different mouse lines. In other words, a gene may not be deleted in all cells of a given region in some conditional knockouts lines.

To manipulate gene function in a temporally and region-specific manner, antisense, RNAi, or viral vectors may be microinjected into target brain regions at specific times. Although these techniques are powerful, there may be between-animal variability in the manipulation. Further, some of these manipulations may produce cytotoxicity or off-target effects (knockdown of genes other than the intended target).

Because each method of manipulating gene function is associated with potential interpretive limitations, it is important that converging data are obtained from multiple methods before firm conclusions regarding the importance of a particular transcriptional regulator in memory formation are drawn.

4.27.1.2 Paradigms Used to Examine Memory

Just as there are multiple methods to alter the function of transcriptional regulators, there are several techniques commonly used to assess memory in rodents.

4.27.1.2.1 Conditioned fear memory

In a typical conditioned fear experiment, mice or rats are placed in a conditioning chamber, and a footshock is delivered. In the discrete cue version of this task, a tone (or light) that coterminates with the footshock is presented. Memory is commonly assessed as the percentage of time rodents spend freezing (defined as the cessation of all movements except respiration) when placed back in the conditioning chamber (referred to as contextual fear conditioning) or when the tone is presented in a novel chamber (tone or discrete cue fear conditioning). One advantage of this paradigm is that the neural systems critical for mediating fear conditioning have been well characterized. The amygdala, and in particular the lateral nucleus, is crucial for both tone and contextual fear conditioning. Lesioning or inhibiting protein synthesis in the lateral nucleus of the amygdala disrupts the formation of LTM (Davis, 1992; LeDoux, 2000; Schafe and LeDoux, 2000; Fanselow and Gale, 2003). The role for the hippocampus in fear conditioning is not as straightforward (see following).

4.27.1.2.2 Conditioned taste aversion memory

In the conditioned taste aversion (CTA) paradigm, ingestion of a novel taste is paired with transient sickness (produced by injection of lithium chloride). Memory for this association is evident when the animal avoids that taste on subsequent presentations (Garcia et al., 1955). In addition to brainstem (nucleus of the solitary tract) and pontine (parabrachial nucleus (PBN)) regions, several forebrain areas have been shown to be important for conditioned taste aversion (Schafe and Bernstein, 1996; Tokita et al., 2004). Furthermore, LTM for conditioned taste aversion requires protein synthesis in the amygdala (Yamamoto and Fujimoto, 1991; Josselyn et al., 2004) and insular cortex (Rosenblum et al., 1993).

4.27.1.2.3 Recognition memory (object and social recognition)

Recognition memory tests rely on the natural exploratory behavior of rodents. In a typical object recognition task, training consists of exposing mice or rats to two objects for a short period of time (e.g., 15 min). In the test phase, the subject is presented with one of the objects used during training (familiar object) and a novel object. The time the subject spends interacting with each object is compared. Memory is shown by animals spending more time exploring the novel object rather than the familiar object. Testing may occur at any time after training, allowing for the evaluation of both STM and LTM. Object recognition memory has been linked to the hippocampus (Clark et al., 2000; Ainge et al., 2006) and cortex, and especially the perirhinal cortex (Brown and Aggleton, 2001).

The social recognition task is based on similar principles. During the training phase, the subject is presented with a conspecific mouse. During the test phase, the subject is allowed to interact with the mouse previously used in the training (familiar) and a novel mouse. Again, memory is shown by the subject interacting longer with the novel mouse.

4.27.1.2.4 Social transmission of food preference memory

In the social transmission of food preference task, rodents develop a preference for foods recently smelled on the breath of other rodents (Galef et al., 1983, 1988; Bunsey and Eichenbaum, 1995). When subsequently given a choice between this food and a novel food, the subject demonstrates its memory for the odor through a preference for the same-scented food (Galef, 1985). This task can be used to assess memory for the sampled food at various intervals such that both STM and LTM may be tested. Previous research shows that this memory depends on the hippocampus (Winocur, 1990; Winocur et al., 2001). Therefore, object and social recognition, as well as the social transmission of food preference task, are memory tests that critically rely on the hippocampus but that are not spatial in nature.

4.27.1.2.5 Spatial memory

Spatial memory is most commonly assessed using the Morris water maze. In the hidden platform version of the water maze, rodents learn to find a platform submerged in a pool of opaque water by using spatial cues in the experimental room (Morris et al., 1982). Typically, spatial memory is assessed during a probe trial in which the platform is removed and the percentage of time the animals spend searching in the spatial location where the platform was previously positioned (target quadrant) is measured. This form of spatial memory is sensitive to hippocampal lesions (Morris et al., 1982; Sutherland et al., 1982). The Barnes maze is a dry land task that also measures spatial memory. In this task, animals escape from a brightly lit open arena into a small recessed chamber located under the arena. Animals learn to navigate toward this target escape box by using spatial cues (Barnes, 1979). The hippocampus is similarly important for this form of spatial memory (Koopmans et al., 2003).

These behavioral tests tap into different aspects of memory and place unique performance demands on the subject. Although there is not strict one-to-one mapping, in general, these tasks rely to a lesser or greater degree on different brain regions. For instance, tone fear memory and conditioned taste aversion both depend on the amygdala, among other regions, while the recognition and spatial memory tasks heavily depend on intact hippocampal function. It is conceivable that different molecular mechanisms may mediate memory formation in different brain regions. For these reasons, it is also important that the results from multiple memory tests are compared.

4.27.2 CREB

CREB is perhaps the best-studied transcription regulator in terms of memory. CREB refers to a family of structurally related transcription factors that modulate the transcription of genes that contain cAMP responsive elements (CRE) in their promoter regions.

4.27.2.1 Structure

In mammals, at least three genes encode CREB-like proteins: CREB, CREM (cAMP response element modulator), and ATF-1 (activating transcription factor 1) (Hoeffler et al., 1988; Rehfuss et al., 1991; Foulkes and Sassone-Corsi, 1992). The mouse and human CREB gene is composed of 11 exons (Waeber et al., 1991; Cole et al., 1992; Hoeffler, 1992), and alternative splicing generates the three major activator isoforms of CREB: α, δ, and β (Gonzalez and Montminy, 1989; Yamamoto et al., 1990; Blendy et al., 1996). In addition to these transcriptional activators, the CREB family also includes transcriptional repressors. For example, the CREM gene codes several isoforms that repress CRE-dependent transcription, the CREM α, β, and γ isoforms, as well as the inducible cyclic-AMP early repressor (ICER) (Foulkes et al., 1991; Molina et al., 1993).

CREB, CREM, and ATF1 proteins share a conserved basic leucine zipper (bZip) domain in the C-terminus region that is responsible for dimerization between CREB family members and binding to the CRE site (Busch and Sassone-Corsi, 1990). The kinase-inducible transactivation domain (KID) is located in the N-terminal region and contains a key phosphorylation site that was initially characterized as being regulated by cAMP-dependent protein kinase A (PKA). CREB is constitutively expressed and is thought to be constitutively bound to DNA in the nucleus. Transactivation of CREB, however, largely depends on phosphorylation.

Although CREB may be phosphorylated at a number of residues (such as Ser129 or Ser142; see Sun et al., 1994; Giebler et al., 2000; Kornhauser et al., 2002), activation of transcription requires phosphorylation at Ser133 in the KID domain (for review, see Fimia et al., 1998). Mutation of this site (to a nonphosphorylatable alanine) abolishes the stimulus-induced transcriptional activation of CREB (Gonzalez and Montminy, 1989). The phosphorylation state of Ser133 is regulated by the balance between protein phosphatases and kinases, which remove or add phosphate groups, respectively. Numerous kinases transmit signals from the plasma membrane to the nucleus to phosphorylate CREB at Ser133 (Dash et al., 1991; Chen et al., 1992; Bacskai et al., 1993; Hagiwara et al., 1993; Bito et al., 1996; Xing et al., 1996; Finkbeiner et al., 1997; Hardingham et al., 2001; Wu et al., 2001). Perhaps the best-characterized of these kinases are PKA, p38 mitogen-activated protein kinase (MAPK), and CaMKIV. In addition, a potent CREB coactivator, transducer of regulated CREB (TORC2), has recently been identified (Conkright et al., 2003a). TORC2 may mediate the cooperativity between cAMP and calcium signals (Screaton et al., 2004).

Phosphorylation of CREB at Ser133 alters the affinity of the KID for the KIX domain of the coactivators CBP and p300, resulting in enhanced transcriptional activity (Chrivia et al., 1993). On the other hand, protein phosphatase 1 (PP1) and PP2A dephosphorylate Ser133 (Hagiwara et al., 1992; Wadzinski et al., 1993; Bito et al., 1996), which decreases transcriptional activity. In addition to this mechanism, recent evidence suggests that the transcriptional activity of CREB may also be regulated by mechanisms in the bZip domain (Katoh et al., 2006).

4.27.2.2 Correlating Transcriptional Activation with Memory: Results from Imaging Studies

CREB activation may be monitored by examining the levels of activated CREB (CREB phosphorylated at Ser133) or products of CRE-mediated transcription that are endogenous (such as C/EBP) (Taubenfeld et al., 2001a; Desmedt et al., 2003) or engineered (CRE-reporter mice) (Impey et al., 1996, 1998). For instance, training that induces LTM for conditioned fear was correlated with an increase in the levels of phosphorylated CREB (pCREB) (Impey et al., 1998; Stanciu et al., 2001) and CRE-reporter in the amygdala and hippocampus (Impey et al., 1998; Athos et al., 2002). Additional studies have shown that behavioral training that normally induces LTM in other tasks is also associated with increased levels of pCREB and CRE-regulated genes (such as C/EBP) (Impey et al., 1998; Bevilaqua et al., 1999; Taubenfeld et al., 1999; 2001b; Cammarota et al., 2000; Viola et al., 2000; Stanciu et al., 2001). The results of these studies converge to show that training that induces LTM in a variety of paradigms is associated with CREB activation, as measured by several techniques.

4.27.2.3 Effects of Manipulating CREB Function on Memory

The role of CREB in memory has been well studied. Indeed, the effects of a range of CREB manipulations have been assessed in a variety of memory paradigms.

4.27.2.3.1 Conditioned fear memory

There are a variety of ways to manipulate CREB function. The homozygous deletion of the three major CREB isoforms (α, β, and δ) produced by a deletion in the bZIP domain (exon 10) of the CREB gene results in fully formed mice ($CREB^{null}$) that die at birth because of atelectasis of the lung (Rudolph et al., 1998). However, mice that lack the two main isoforms of CREB ($CREB^{\alpha\delta}$ mice) develop into adults and were tested for memory.

$CREB^{\alpha\delta}$ mice generally showed disrupted LTM but intact STM for both contextual and cued fear conditioning (Bourtchuladze et al., 1994; Kogan et al., 1997; Graves et al., 2002; Frankland et al., 2004). This LTM deficit in the $CREB^{\alpha\delta}$ mice was replicated using fear-potentiated startle to measure conditioned fear (Falls et al., 2000). It is important to note that the LTM deficit in these CREB mutants may be overcome by additional spaced training trials, highlighting the importance of the intensity and temporal dynamics of the training protocol to the memory phenotype (Kogan et al., 1997).

Using $CREB^{\alpha\delta}$ mice from a different genetic background (FVB/N x C57BL\6), Gass and colleagues (1998) found normal STM and mild LTM deficits in both cued and contextual fear conditioning (Gass et al., 1998). However, as pointed out by Graves and colleagues (2002), mice from this genetic background are generally poorer learners, potentially masking a greater effect of the $CREB^{\alpha\delta}$ mutation. These results highlight the importance of genetic background in behavioral studies.

Of interest is the finding that $CREB^{comp}$ mice (with only one CREBβ allele, obtained by crossing a $CREB^{\alpha\delta}$ mouse with a mouse heterozygous for the $CREB^{null}$ mutation) showed severe LTM deficits in both context and cued fear conditioning (Gass et al., 1998). This finding indicates that gene dosage is another important variable in the LTM phenotype produced by disrupting CREB function. That is, mice with a greater disruption in CREB function ($CREB^{comp}$ vs. $CREB^{\alpha\delta}$ mice) show a greater impairment in LTM for conditioned fear.

Conditioned fear memory has also been probed using transgenic mice overexpressing a dominant negative form of CREB. Three lines of mice that constitutively express a dominant negative form of CREB (mCREB) in the amygdala and hippocampus were generated. Of these, only one line showed impaired LTM for cued fear conditioning as measured by conditioned suppression of ongoing behavioral activity (Rammes et al., 2000). However, as the CREB repressor is constitutively expressed in these transgenic mice, compensatory upregulation of other CREB family members may confound the interpretation of the role of CREB in memory.

To overcome this potential limitation, transgenic mice were developed that allowed precise temporal control over the expression of the dominant negative CREB repressor (Kida et al., 2002). Importantly, these CREB inducible repressor ($CREB^{IR}$) transgenic mice developed with normal CREB levels. CREB function was acutely and reversibly disrupted by administering the inducer (tamoxifen) 6 h before training for conditioned fear. Mice were tested 24 h later. In this way, mice were trained with impaired CREB function but tested when CREB function had presumably returned to normal. Acutely disrupting CREB during training impaired LTM, but not STM, for both cued and contextual fear conditioning (Kida et al., 2002). Consistent with these findings, acutely disrupting CREB function in the CA1 region of the hippocampus through the use of antisense oligonucleotides also disrupted LTM for contextual fear (Athos et al., 2002). As the disruptions of CREB function are temporary, these converging data also provide compelling evidence that the LTM phenotype observed in mutant mice with a chronic disruption of CREB ($CREB^{\alpha\delta}$ or $CREB^{comp}$ mice) cannot be solely attributed to developmental abnormalities.

Therefore, the finding of normal LTM for contextual fear conditioning found in three additional lines of CREB mutant mice may be initially surprising. Normal LTM for contextual fear conditioning was observed in dCA1-KCREB mice, which express a dominant negative form of CREB in the CA1 region of the dorsal hippocampus (Pittenger et al., 2002), and in two lines of conditional CREB knockout mice, in which a floxed CREB mouse was crossed with two lines of transgenic mice expressing Cre recombinase under different promoters ($CREB^{CaMKCre7}$ and $CREB^{NesCre}$) (Balschun et al., 2003). However, these findings may not be entirely unexpected in light of results showing that the hippocampus is not strictly required for contextual fear conditioning. Whereas posttraining lesions of dorsal hippocampus dramatically impaired contextual fear conditioning, pretraining lesions did not (Maren et al., 1997; Frankland et al., 1998). It has been hypothesized that rodents with pretraining lesions of the hippocampus use elemental, rather than contextual, strategies to produce normal LTM for contextual fear conditioning (Maren et al.,

1997; Frankland et al., 1998). Importantly, the lines of CREB mutant mice that show normal LTM for contextual fear have chronic disruptions in CREB that could be analogous to 'pretraining lesions' of CREB. Thus, unlike antisense that acutely disrupts CREB function, CREB function was chronically impaired in both the $CREB^{CaMKCre7}$ and $CREB^{NesCre}$ mice. Even in the inducible dCA1-KCREB mice, the dominant negative CREB transgene was expressed for a significant period of time before training. Taken together, these findings suggest that contextual fear conditioning (in which mice may acquire the task by using nonhippocampal, elemental-based strategies) may not be sufficiently sensitive to show potential LTM deficits in mice with chronic pretraining perturbations in hippocampal CREB function.

To address this potential shortcoming, a variation of the context fear conditioning task that critically relies on the hippocampus was used to evaluate the role of CREB in contextual LTM. In the context preexposure task, the acquisition of context information is temporally separated from its association with footshock (Wiltgen et al., 2001; Frankland et al., 2004; Matus-Amat et al., 2004). Unlike animals given a chance to explore a novel environment before footshock (as in the standard context fear conditioning paradigm), animals given a footshock immediately after placement in a novel environment showed low levels of conditioned fear. This phenomenon is sometimes referred to as the immediate shock deficit. Animals preexposed to the training context 24 h before the immediate shock, however, showed robust conditioned fear (Wiltgen et al., 2001; Frankland et al., 2004; Matus-Amat et al., 2004). Thus, context preexposure rescued the immediate shock deficit. Protein synthesis in the hippocampus is crucial for the rescue of the immediate shock deficit by context preexposure, as infusion of anisomycin into the dorsal hippocampus following context preexposure produced low levels of freezing following the immediate shock (Barrientos et al., 2002). Similarly, $CREB^{\alpha\delta}$ mice showed low levels of freezing following an immediate shock despite being preexposed to the training context the day before. Indeed, freezing levels in the $CREB^{\alpha\delta}$ mice were similar to those observed in wild-type (WT) mice that were not preexposed to the training context (Frankland et al., 2004). Therefore, by using a task that critically depends on protein synthesis in the hippocampus, CREB was shown to be important for contextual LTM.

Together, the majority of these findings indicate that decreasing CREB function disrupts LTM for conditioned fear. But what are the effects of increasing CREB function on LTM for conditioned fear? To answer this question, Josselyn and colleagues (2001) used viral vectors to increase CREB levels and function in a subpopulation of amygdala neurons (roughly 20–30% of neurons in the lateral/basolateral nucleus of the amygdala) (Josselyn et al., 2001). Rats were trained for cued fear conditioning using a massed training protocol that typically produces weak STM but no, or weak, LTM. However, rats infused with viral vectors encoding CREB in the amygdala showed robust LTM at levels similar to those produced by more intense, spaced training. The finding of enhanced cued fear memory following infusion of herpes simplex virus encoding CREB into the amygdala was replicated using freezing as a measure of conditioned fear (Wallace et al., 2004) and extended to include memory for social defeat in hamsters (Jasnow et al., 2005). These findings in rodents are consistent with results from *Drosophila* (Yin et al., 1995) and *Aplysia* (Bartsch et al., 1995), showing that increasing CREB function enhances LTM.

4.27.2.3.2 Conditioned taste aversion memory

Data from multiple studies have shown that CREB is important in LTM for conditioned taste aversion. Thus, $CREB^{\alpha\delta}$ (Josselyn et al., 2004), $CREB^{IR}$ (Josselyn et al., 2004), and $CREB^{NesCre}$ (Balschun et al., 2003) mice showed disrupted LTM for conditioned taste aversion. Other lines of CREB mutant mice ($CREB^{CaMKCre7}$ mice and $CREB^{comp}$ mice) have not been tested in this paradigm. Acutely disrupting CREB function in the amygdala through the use of antisense oligonucleotides similarly disrupted LTM, but not STM, for conditioned taste aversion (Lamprecht et al., 1997). Furthermore, training that produced conditioned taste aversion (pairing the novel taste with lithium chloride) also induced robust CREB activation (phosphorylation) in the lateral nucleus of the amygdala (Swank, 2000). Similar increases were not observed if rats were exposed to the taste or lithium chloride alone, indicating that activation of CREB is related to associative learning. Together, these data from rats and mice show that CREB is important in CTA memory. Unlike some other memory tasks, conditioned taste aversion places few performance demands on the subject. Therefore, conclusions regarding the role of CREB in memory may

be drawn independently of potential confounding deficits in other behavior, such as motor deficits.

4.27.2.3.3 Recognition memory (object and social recognition)

Several studies have shown that decreasing CREB function specifically disrupted LTM, but not STM, in both the object and social recognition tasks. Thus, dCA1-KCREB mice (Pittenger et al., 2002) and $CREB^{IR}$ mice (Bozon et al., 2003) have disrupted LTM for object recognition. In addition, viral-mediated transfer of a dominant negative form of CREB to the perirhinal cortex similarly impaired LTM for object recognition in rats (Warburton et al., 2005). CREB has also been shown to be important in memory for the social recognition task. LTM, but not STM, for social recognition was impaired in both the $CREB^{\alpha\delta}$ (Kogan et al., 2000) and $CREB^{IR}$ mice (Bozon et al., 2003).

Consistent with previous data using a variety of learning tasks and species, spaced training produces maximal LTM whereas massed training produces weak LTM for object recognition (Genoux et al., 2002). Furthermore, spaced but not massed training in the object recognition task has been associated with increased levels of phosphorylated CREB and CRE-mediated transcription in the hippocampus and cortex (Genoux et al., 2002). However, in transgenic mice with decreased protein phosphatase-1 (PP1) activity (PP1 dephosphorylates CREB at Ser133, thereby decreasing transactivation), massed training alone was sufficient to produce both an increase in CRE-mediated transcription and robust LTM (Genoux et al., 2002). A similar pattern of results was also observed in transgenic mice that overexpress type 1 adenylyl cylase (Wang et al., 2004). These transgenic mice, but not WT littermates, showed both elevated levels of pCREB and strong LTM following training that normally induces weak object recognition memory. Therefore, increasing CREB function through indirect manipulations also enhances LTM.

4.27.2.3.4 Social transmission of food preference memory

The role of CREB in memory has also been tested in another task that critically involves the hippocampus: social transmission of food preference. Training that produced LTM for social transmission of food preference was associated with increased levels of pCREB in the hippocampus (Countryman et al., 2005b). Furthermore, disrupting CREB function by expression of a dominant negative form of CREB (S133A) in the dorsal hippocampus through viral vectors blocked LTM, but not STM, for this task (Brightwell et al., 2005). Consistent with this, $CREB^{\alpha\delta}$ mice showed intact STM, but impaired LTM, in this task (Kogan et al., 1997). However, using a more intense training protocol (a longer 'training time' (10 min) that permitted an observer mouse to smell the breath of a demonstrator mouse for a longer period of time), Gass and colleagues (1998) found normal LTM in both $CREB^{\alpha\delta}$ and $CREB^{comp}$ lines of mutant mice. Together, these results indicate that the effects of disrupting CREB are dependent on the training conditions used to induce LTM for social transmission of food preference.

4.27.2.3.5 Olfactory memory

Memory may also be assessed using an olfactory conditioning task in neonatal rats. In this task, an odor is paired with an appetitive (such as a stroke of the back) or an aversive (footshock) stimulus. Both appetitive and aversive olfactory conditioning were associated with an increase in pCREB levels in the olfactory bulbs (McLean et al., 1999; Zhang et al., 2003). Moreover, disrupting CREB function (via infusion of CREB antisense oligonucleotides or viral vectors expressing mCREB) produced a specific LTM deficit for olfactory conditioning (Yuan et al., 2003; Zhang et al., 2003).

4.27.2.3.6 Spatial memory

In the first study to assess the role of CREB in spatial memory, $CREB^{\alpha\delta}$ mice were trained in the water maze using a low-intensity training protocol (one trial a day for 15 days) (Bourtchuladze et al., 1994). At the beginning of training, WT and $CREB^{\alpha\delta}$ mice showed long latencies to reach the platform. As training progressed, the time to reach the platform was reduced in WT mice (indicating learning), whereas the latencies of $CREB^{\alpha\delta}$ mice remained significantly longer. As predicted from these training latencies, WT mice searched selectively in the target quadrant during the probe test, while $CREB^{\alpha\delta}$ mice searched randomly (Bourtchuladze et al., 1994). Moreover, WT mice crossed the exact location of the platform more often than the $CREB^{\alpha\delta}$ mice. This initial spatial memory deficit in $CREB^{\alpha\delta}$ mice was replicated using similar, as well as more intense, training schedules (Bourtchuladze et al., 1994; Kogan et al., 1997). However, similar to other memory tests discussed above, the role of CREB in spatial memory is sensitive to the training parameters. Thus, increasing the time between trials to 10 min or 1 h (rather than

1 min) rescued the spatial memory deficit in CREB$^{\alpha\delta}$ mice (Kogan et al., 1997; Gass et al., 1998).

Spatial memory deficits have also been observed using two different techniques to significantly disrupt CREB function specifically in the hippocampus. Transgenic mice that express a dominant negative form of CREB in the dorsal CA1 region of the hippocampus (dCA1-KCREB) also showed impaired spatial memory as measured by several variables (percentage time spent in the target quadrant, proximity to the training location of the platform, and platform crossing) (Pittenger et al., 2002). A critical control experiment showed that dCA1-KCREB transgenic mice fed doxycycline (thereby turning off transgene expression) for 2 weeks before the start of water maze training showed normal spatial memory. This result indicated that the spatial memory phenotype in these mutant mice cannot be attributed to developmental deficits. In addition, infusion of CREB antisense into the dorsal hippocampus of adult rats (Guzowski and McGaugh, 1997) and mice (Florian et al., 2006) disrupted spatial memory in the water maze. Furthermore, disrupting CREB function in aged mice using a viral vector expressing the CREB inhibitor ICER disrupted spatial memory on a Barnes maze (Mouravlev et al., 2006). In accordance with these findings, prenatal administration of morphine, a treatment that significantly decreased the level of phosphorylated CREB in the hippocampus of adult mice, also produced a deficit in spatial memory (Yang et al., 2003). Collectively, these findings indicate that intact CREB function in the hippocampus is critical for spatial memory.

In an overall analysis of the impact of various CREB mutations on spatial memory, Balschun and colleagues (2003) compared the water maze performance of various lines of CREB mutant mice (using all CREB mutant mice lines studied by this group, including those of Gass et al., 1998). They found that spatial memory varied in the different lines of CREB mutant mice; the percentage time spent in the target quadrant during the probe trial for WT mice = 35.78%, CREB^{comp} = 26.39%, CREB$^{\alpha\delta}$ = 28.5%, CREB^{NesCre} = 31.11%, and CREB^{CaMKCre7} = 40.87%. These authors interpreted this pattern of results as evidence that CREB is dispensable for spatial memory. However, a more parsimonious explanation of these data is that the spatial memory phenotype is determined by the degree of CREB disruption. In support of this interpretation, the CREB^{comp} mice (with a loss of both CREBα and δ alleles as well as one CREBβ allele) showed poorer spatial memory than the CREB$^{\alpha\delta}$ mice (with a loss of both CREBα and δ alleles but two intact CREBβ alleles) (see also Gass et al., 1998). Similarly, the CREB^{NesCre} mice (with a virtually complete deletion of CREB throughout the CNS) showed poorer spatial memory than the CREB^{CaMKCre7} mice (with a loss of CREB in 70–80% of CA1 hippocampal neurons only). Together, these results suggest that transgenic overexpression of a dominant negative form of CREB (as in dCA1-KCREB transgenic mice) or CREB antisense may disrupt CREB function in a larger portion of hippocampal neurons than the 'mosaic' pattern of CREB deletion observed in the CREB^{CaMKCre7} mice.

4.27.2.4 Conclusion

The majority of evidence suggests that CREB is critically involved in consolidation of LTM. In tasks ranging from conditioned fear to conditioned taste aversion, various recognition tasks, and spatial memory, most manipulations that disrupt CREB function similarly disrupt LTM. On the other hand, treatments that enhance CREB function generally enhance LTM. This loss- and gain-of-function pattern of results is also produced by CREB manipulations in invertebrate species (Dash et al., 1990; Yin et al., 1994, 1995; Bartsch et al., 1995).

This conclusion, however, has two important caveats. First, the memory phenotype is sensitive to the training parameters (specifically the spacing between successive trials). The sensitivity was observed across several paradigms and may be related to the fundamental differences between the types of memory produced by massed and spaced training. In many instances, multiple spaced trials rescued the LTM deficit resulting from decreased CREB function, suggesting that spaced training protocols may recruit additional molecular mechanisms, including other transcription regulators.

The second important caveat is that the extent of CREB disruption determines the presence and degree of LTM impairment. In general, greater disruptions in CREB function produced greater disruptions in LTM. For instance, the CREB^{comp} (with only one CREB β allele) mice showed a larger impairment than the CREB$^{\alpha\delta}$ mice (with two CREB β alleles) in many memory tasks (Gass et al., 1998). Furthermore, CREB^{NesCre} mice (with a brainwide deficit in CREB) showed larger spatial memory impairments than CREB^{CaMKCre7} mice (with a

deletion of CREB in only 70–80% of CA1 neurons) (Balschun et al., 2003). Indeed, these results show that the remaining 20–30% of CA1 neurons with normal CREB levels in the $CREB^{CaMKCre7}$ mice are sufficient to support normal spatial memory. This result is consistent with the observation that increasing CREB function in a small proportion of neurons in the lateral amygdala was sufficient to enhance LTM for conditioning fear (Josselyn et al., 2001; Wallace et al., 2004) and social defeat (Jasnow et al., 2005).

4.27.3 c-Fos

4.27.3.1 Structure

Inducible transcription factors regulate the delayed onset of effector genes that may code for structural, growth-related, and synaptic proteins, and as such, they have been implicated in LTM (Herdegen and Leah, 1998). Two of the best-studied inducible transcription factors are Fos and Jun. c-fos codes for a 380 amino acid protein that belongs to a family of transcription factors that also includes FosB and the Fos-related antigens 1 and 2 (Fra-1 and Fra-2) (Karin et al., 1997; Herdegen and Leah, 1998). The transcriptional regulatory region of c-fos contains recognition sequences for CRE, SRE, c-Sis inducible element (SIE), TCF (ternary complex factor), and AP-1 (Treisman, 1992).

Fos proteins activate late-effector genes by forming heterodimeric complexes with Jun (c-Jun, JunB, JunD) proteins and the related activating transcription factor (ATF2, ATF3/LRF1, B-ATF) subfamilies to form the activating protein 1 (AP-1) transcription factor (Morgan and Curran, 1991). The AP-1 complex binds to a palindromic DNA binding sequence (5′-TGACGTCA-3′), referred to as the TRE (TPA response element) site, in the regulatory region of several target genes, including nerve growth factor, proenkephalin, prodynorphin, endorphin, neurotensin, tyrosine hydroxylase, and neuropeptide Y (Greenberg and Ziff, 1984; Karin et al., 1997; Herdegen and Leah, 1998).

4.27.3.2 Correlating Transcriptional Activation with Memory: Results from Imaging Studies

The first hint that c-fos played an important role in memory came from expression studies in which Fos induction was observed following behavioral training that induced LTM (Tischmeyer and Grimm, 1999; Guzowski, 2002). Fos protein was upregulated in various brain regions following training in many behavioral tasks, including conditioned taste aversion (Schafe and Bernstein, 1996), Morris water maze (Guzowski et al., 2001), socially transmitted food preference (Countryman et al., 2005a), and conditioned fear (Stanciu et al., 2001). However, because c-fos is induced by a variety of physiological and pathological stimuli (Morgan et al., 1987), the functional relevance of the increase in Fos protein was equivocal without corresponding studies examining the effects of manipulating c-fos function on memory.

4.27.3.3 Effects of Manipulating c-fos Function on Memory

4.27.3.3.1 Spatial memory

To manipulate c-fos function, several techniques have been used. One of the first studies to examine the impact of c-fos manipulations on memory took advantage of a global c-fos knockout mouse in which the c-fos gene was deleted throughout the body throughout the lifetime of the mouse. Although these c-fos knockouts were viable, they lacked tooth eruption, showed altered hematopoiesis, and were smaller than their wild-type littermates (Wang et al., 1992). These mice showed severe impairments in the hidden version of the water maze (Paylor et al., 1994). However, they also showed significant deficits in the visible (nonhippocampal) version of the water maze, suggesting that the behavioral deficit may be a result of nonspecific sensorimotor and/or motivational dysfunction. Therefore, the strong developmental abnormalities in the global c-fos knockout mouse make it difficult to discriminate putative mnemonic disruption from confounding nonspecific impairments.

To overcome the limitations of the c-fos global knockout, two lines of conditional c-fos knockout mice in which c-fos function was disrupted in the brain only have been developed. In this way, the impact of disrupting c-fos function in the brain could be assessed free from gross developmental deficits. Mice with a floxed c-fos gene were crossed with transgenic mice expressing Cre recombinase under the control of two different promoters. Crossing the floxed c-fos mice with transgenic mice in which Cre recombinase was driven by the αCaMKII promoter (Tsien et al., 1996) produced a mouse line in which the disruption of c-fos was largely limited to the hippocampus (Zhang et al., 2002a). Using an intense

training protocol (6 days, four trials a day, with a 1-h intertrial interval), these mice showed normal spatial memory as assessed in the water maze (Zhang et al., 2002a). In addition, these mice showed normal memory on the Barnes maze (Zhang et al., 2002a). However, a different result was obtained when a less intense training protocol was used to train mice with a greater disruption of c-fos.

Deleting c-fos throughout neurons and glia of the central nervous system using transgenic mice in which Cre recombinase was driven by the nestin promoter produced c-fos^{NesCre} or c-fos$^{\Delta CNS}$ mice. These c-fos^{NesCre} mice showed spatial memory deficits using a less intense training protocol in the water maze (one trial per day for 11 days; Fleischmann et al., 2003). Consistent with this observation, Guzowski and colleagues also showed that disrupting c-fos function in the hippocampus impaired spatial memory in the water maze. Injection of c-fos antisense oligonucleotides into the hippocampus had no effect on short-term spatial memory but did disrupt LTM (Guzowski, 2002). It is important to note that the c-fos^{NesCre} mice also showed deficits in LTM for contextual fear conditioning (Fleischmann et al., 2003).

4.27.3.3.2 Social transmission of food preference memory

In addition to spatial memory tests, the role of hippocampal c-fos has also been examined in the social transmission of food preference memory task. Training that produced robust LTM for this task was also associated with increased levels of Fos protein in the hippocampus (Countryman et al., 2005a). Moreover, disrupting c-fos function in the hippocampus using antisense impaired LTM but not STM for socially transmitted food preferences (Countryman et al., 2005a).

On balance, these data suggest that disrupting c-fos function disrupts LTM for two different hippocampal-dependent tasks. However, memory disruption was not observed in all lines of mice under all training conditions. Specifically, the mice with a disruption of c-fos function only in the hippocampus (c-fos^{CaMKCre}) showed intact spatial memory following intensive training. The levels of Fos protein in these mice were reduced by roughly 95% in CA1, CA2, and CA3 neurons but by only 70% in dentate granule cells following kainic acid administration (a potent inducer of c-fos) (Zhang et al., 2002b). It is possible that the remaining unaffected neurons with intact c-fos function in the dentate gyrus are sufficient to support normal spatial memory following intense training. In contrast, the deletion of c-fos was nearly complete in the CNS-specific (c-fos^{NesCre}) mice in that kainic acid administration produced no observable Fos. These mice also showed a more pronounced memory deficit. In order to truly compare the results of different levels of c-fos disruption on memory, these different lines of mutant mice should be trained using the same training protocol.

4.27.3.3.3 Conditioned taste aversion memory

The role of c-fos in memory has also been assessed using the conditioned taste aversion paradigm. Training that induced conditioned taste aversion was associated with increased Fos levels in the amygdala (basolateral and central nuclei), insular cortex, and intermediate nucleus of the solitary tract (Wilkins and Bernstein, 2006). Whether Fos is increased in the PBN is unclear (Wilkins and Bernstein, 2006; Yamamoto and Sawa, 2000). Indeed, the type and strength of conditioned taste aversion training may be an important factor in determining the precise brain regions that express Fos (Yamamoto and Sawa, 2000). Overall, however, these data indicate that training in this task reliably increases Fos expression in the brain.

Several studies have examined the impact of disrupting c-fos function on memory for conditioned taste aversion. Recently, Yasoshima and colleagues (2006) examined the effects of microinjecting c-fos antisense oligonucleotide into various brain regions, including the amygdala (targeted to the border between the basolateral amygdala and central amygdala), insular cortex, and PBN. They found that infusions of c-fos antisense into the PBN (but not insular cortex or amygdala) blocked LTM for conditioned taste aversion. Surprisingly, these researchers also found normal taste aversion memory in global c-fos knockout mice. In addition, infusion of c-fos antisense into the PBN, amygdala, and insular cortex disrupted the retrieval of conditioned taste aversion when injected before testing (Yasoshima et al., 2006). These findings are not consistent with a previous report from Lamprecht and Dudai (1996), who found that injection of c-fos antisense into the central amygdala impaired LTM but not retrieval of conditioned taste aversion memory. These discrepancies could be a result of the different efficiencies of the c-fos antisense or the precise brain regions targeted, as well as procedural differences in the conditioned taste aversion task (one bottle vs. multiple bottles).

4.27.4 c-Jun

4.27.4.1 Structure

c-jun is a related immediate early gene that encodes a protein of 334 amino acids in the rat (Herdegen and Leah, 1998). The c-jun promoter contains binding sites for the transcription factors CREB, ATF-1, TFIID, Sp1, CTF, AP-1, NF, SRF, and ATF-2 (Angel et al., 1988; de Groot et al., 1991; Rozek and Pfeifer, 1993). c-jun is induced by a diverse array of stimuli, including serum, TPA, and various growth factors, cytokines, and second messengers (Angel et al., 1988; de Groot et al., 1991; Rozek and Pfeifer, 1993).

Transactivation of Jun is tightly regulated by phosphorylation in two distinct regions. C-terminal phosphorylation sites lie proximal to the DNA binding domain and prevent DNA binding. These sites are dephosphorylated in response to growth stimulation and likely function to repress the activity of the transcription factor under resting conditions (de Groot et al., 1991; Rozek and Pfeifer, 1993). Since Jun protein is present in many types of resting cells, this may provide a mechanism for rapid induction of Jun activity. Jun is also phosphorylated at two residues proximal to the major transactivation domain, and phosphorylation of Ser 63 and 73 is required for efficient transactivation. The kinases primarily responsible for this modification in vivo are the Jun N-terminal kinases (JNKs) (Smeal et al., 1992).

4.27.4.2 Correlating Transcriptional Activation with Memory: Results from Imaging Studies

Similar to c-fos, several studies have shown that training that normally induced LTM also induced c-jun expression. In fact, in most cases enhanced expression of Fos is not a solitary event but is often accompanied by an induction of Jun. For instance, neurons in the amygdala show increased levels of both Fos and Jun proteins following conditioned taste aversion training (Swank, 1999). This corresponding increase in both Fos and Jun would allow for the formation of AP-1.

4.27.4.3 Effects of Manipulating c-jun Function on Memory

Although the role of c-fos in LTM has been widely studied, fewer studies have examined the impact of disrupting c-jun function on LTM. One of the few studies showed that training that produces memory in a shock-motivated brightness discrimination paradigm increased the levels of hippocampal c-jun mRNA (Grimm et al., 1997). Consistent with this, intrahippocampal infusion of c-jun antisense oligodeoxynucleotides blocked LTM for this task (Tischmeyer et al., 1994). However, another study reported no effects of c-jun disruption on memory. Although training in the hidden platform version of the Morris water maze increased levels of Jun protein in the hippocampus (Teather et al., 2005), mice with a CNS-wide disruption of c-jun (produced by crossing a floxed c-jun mouse with a nestin-Cre recombinase transgenic mouse) showed normal spatial memory following intense training (six trials per day) in the water maze (Raivich et al., 2004). It is possible that memory impairment would be observed if these mice were trained with a less intense protocol. Overall, the results from the studies that manipulate c-jun function are inconclusive as to the importance of c-jun function in memory formation.

4.27.4.4 Conclusion

c-fos and c-jun are closely related immediate early genes. The results from these studies clearly suggest that the c-fos is important in memory. However, the precise role of c-jun is unclear, and additional research is necessary before firm conclusions may be reached.

4.27.5 CaMKIV

4.27.5.1 Structure

CaMKIV is a multifunctional serine/threonine kinase present throughout the forebrain (Ohmstede et al., 1989; Jensen et al., 1991a,b; Means et al., 1991; Anderson et al., 2004). The CaMKIV gene is composed of 13 exons that extend over roughly 42 kb of DNA (Sun et al., 1995). Through alternative transcription initiation and splicing, three isoforms are generated, the α and β isoforms of CaMKIV and calspermin (Ohmstede et al., 1991; Sun et al., 1995). Similar to other CaM kinases, the active site of CaMKIV is sterically blocked by an autoinhibitory domain that prevents substrate binding to the enzyme. Upon a transient rise in intracellular calcium, calcium-bound calmodulin (Ca^{2+}/CaM) binds to the autoregulatory domain of CaMKIV, which relieves this intrasteric inhibition and allows intramolecular

phosphorylation of multiple serine residues (Cruzalegui and Means, 1993; McDonald et al., 1993; Okuno et al., 1995). Unlike other CaM kinases, however, CaMKIV may be localized in the cytoplasm or nucleus (Jensen et al., 1991a; Gao et al., 2004). CaMKIV is imported into the nucleus by importin α (Kotera et al., 2005), where it directly activates several transcription factors, including CREB, ATF-1, the MADS-box family members SRF and MEF2D, and the transcriptional coactivators CBP and p300 (Sun et al., 1994; Enslen et al., 1995; Impey et al., 2002). CaMKIV has been implicated in regulation of transcription of a number of genes including those encoding interleukin 2, c-fos, c-jun, BDNF, and orphan members of the steroid receptor superfamily such as ROR and COUP-TF (Sun et al., 1994; Enslen et al., 1995; Impey et al., 2002).

4.27.5.2 Effects of Manipulating CaMKIV Function on Memory

The role of CaMKIV in LTM has been examined using two lines of mutant mice that employ different strategies to disrupt CaMKIV function. The first line of mice has a targeted deletion of the α and β isoforms of CaMKIV (Ho et al., 2000). CaMKIV$^{\alpha\beta}$ mice showed normal spatial memory in the water maze (four trials a day with an intertrial interval of roughly 35 s) and radial arm maze tasks (Ho et al., 2000). However, these mice showed disrupted LTM (measured 1 and 7 days following training) in both the contextual and cued fear conditioning tasks (Wei et al., 2002). It is interesting to note that a decrease in pCREB levels in the amygdala was observed following fear conditioning, suggesting that the observed memory deficit may be partly attributed to decreased CREB function in this region.

A different pattern of results was found using a line of transgenic mice expressing a dominant negative form of CaMKIV in postnatal forebrain regions (Kang et al., 2001). These mice showed spatial memory deficits in the water maze that were partially overcome with extensive training and LTM deficits in contextual fear conditioning (measured 7 days but not 1 day following training) (Kang et al., 2001). However, normal memory for cued fear conditioning was observed, perhaps due to the differential pattern of transgene expression (highest expression in the hippocampus and lower levels in the amygdala).

Although the majority of findings suggest that CaMKIV is important in memory, perhaps through a CREB-related process, more research is required. It would be ideal if the two lines of CaMKIV mutant mice could be tested side-by-side in the same behavioral task. In addition, the effects of other techniques that disrupt CaMKIV function on memory would be informative.

4.27.6 CBP

4.27.6.1 Structure

CBP codes for a 2442–amino acid protein that was first identified by a physical interaction with CREB (Chrivia et al., 1993). In addition to CREB, CBP is an essential coactivator protein for many transcription factors, including c-jun, c-fos, SRF, and many others (Chrivia et al., 1993). CBP is composed of different domains, including three cystidine-histidine rich domains, a bromodomain, a PKA phosphorylation site, two zinc finger motifs, an N-terminal nuclear receptor binding domain, and a histone acetyltransferase (HAT) domain (Kalkhoven, 2004). CBP facilitates transcription by coupling transcription factor recognition to chromatic remodeling. Specifically, CBP serves as a physical link between DNA-binding transcription factors and the basal transcriptional machinery (McManus and Hendzel, 2001). Through intrinsic HAT activity, CBP also facilitates transcription by opening up condensed DNA, thereby allowing greater access by the transcriptional machinery (McManus and Hendzel, 2001).

4.27.6.2 Effects of Manipulating CBP Function on Memory

Several mouse models have been generated in which CBP function is compromised. The first is a conventional knockout of CBP. Although the homozygous mutation is lethal, heterozygous mice with a null mutation of CBP (CBP$^{+/-}$) develop into adults (Tanaka et al., 1997). Despite showing deficits in skeletal patterning, growth retardation, and motor coordination, these mice show normal activity, working memory, motivation, and anxiety levels (Alarcon et al., 2004; Tanaka et al., 1997). Furthermore, CBP$^{+/-}$ mice showed normal STM but impaired LTM for conditioned fear (both cued and context), as well as object recognition (Alarcon et al., 2004). In contrast, spatial memory was intact following spaced training in the water maze (four trials a day with a 15-min intertrial interval) (Alarcon et al., 2004).

Second, a line of mice that have an insertional mutation in the cbp allele that produces a truncated form of CBP protein ($CBP^{+/-C\text{-}term}$) was developed (Oike et al., 1999). This truncated protein functions as a dominant negative (Petrij et al., 1995). Mice homozygous for this mutation die prenatally, but heterozygous mice survive into adulthood. These mice showed abnormal growth, osseous maturation retardation, hypoplastic maxilla, and changes in locomotor activity (Oike et al., 1999). Spatial memory in the water maze and memory for contextual fear conditioning were intact in these mice (Oike et al., 1999). However, $CBP^{+/-C\text{-}term}$ mice showed impaired LTM but normal STM in passive avoidance, cued fear conditioning (Oike et al., 1999), and object recognition tests (Bourtchouladze et al., 2003).

Transgenic mice expressing a dominant negative form of CBP (CBPΔ1) in postnatal forebrain neurons were also tested for memory (Wood et al., 2005). These CBPΔ1 transgenic mice showed severe deficits in spatial memory (using a low-intensity training protocol in which they were given two training trials per day). Importantly, normal performance was observed in the visible version of the water maze, indicating that the motor abilities and motivation aspects required to perform the task were intact (Wood et al., 2005). Furthermore, CBPΔ1 transgenic mice showed a specific deficit in LTM for contextual fear conditioning. Perhaps surprisingly, no impairment was observed in cued fear conditioning. This may be a result of low expression levels of the truncated CBP protein in the amygdala.

Finally, to gain spatial and temporal control over CBP function, transgenic mice were developed that inducibly express CBP with a point mutation in HAT domain (Korzus et al., 2004). Expression of this dominant negative form of CBP was observed in the hippocampus, caudate, and cortex. These transgenic mice showed intact STM but impaired LTM for object recognition (Korzus et al., 2004). In addition, mice receiving moderate (two trials per day) training in the water maze showed a spatial memory deficit, whereas more intense training rescued the spatial memory deficit.

4.27.6.3 Conclusions

In general, most of the behavioral results from the various lines of CBP mutant mice converge to indicate that CBP is critical for LTM. Moreover, the finding that postnatal disruption of CBP function impaired memory suggests that the LTM deficits observed in mice with more chronic disruptions of CBP cannot be solely attributed to developmental deficits. Similar to the memory phenotypes in CREB and c-fos mutant mice, the amount and type of training is an important factor in determining the presence or extent of memory impairment in the lines of CBP mutant mice.

4.27.7 SRF

4.27.7.1 Structure

SRF is a 67-kDa protein that belongs to the MADS box family of transcription factors (Treisman, 1986). These proteins, which include the MEF2 family of transcription factors, all share a MADS box at their N terminus that is composed of a basic DNA-binding domain, a dimerization domain, and an interface for protein–protein interactions (Norman et al., 1988). SRF is a constitutive transcription factor that binds to the 10-bp sequence CC(A/T)6GG (called CArG box) found in the regulatory regions of dozens of genes, including c-fos, c-jun, and egr-1 (Chai and Tarnawski, 2002).

4.27.7.2 Effects of Manipulating SRF Function on Memory

SRF is important during development; mice with complete deletions of SRF die 3 weeks postnatally (Arsenian et al., 1998). Therefore, to examine the effects of SRF manipulations on memory, strategies must be employed that overcome the developmental necessity of SRF. To date, two such strategies have been used. First, conditional SRF knockout mice in which SRF was excised in postnatal forebrain regions (obtained by crossing a mouse with floxed srf alleles with a mouse expressing Cre recombinase driven by an αCaMKII promoter) were generated (Etkin et al., 2006). The resulting conditional SRF knockout mice had a near total elimination of SRF in the CA1 and dentate gyrus of the hippocampus and decreased SRF levels in CA3 of the hippocampus, cortex, and striatum. These mice showed abnormal habituation in a novel environment and impaired spatial memory in the water maze. A second strategy used infusions of SRF antisense to disrupt SRF function in a temporally and spatially restricted manner. Infusion of SRF antisense oligonucleuotides impaired spatial memory in the water maze (Dash et al., 2005). Although there are only two experiments that examined the role of SRF in memory, the available data support the

conclusion that SRF is important in LTM formation. The finding that two different methods of disrupting SRF produced similar memory impairments bolsters this conclusion.

4.27.8 Overall Conclusions

Long-term changes in synaptic strength and structural remodeling are thought to underlie the formation of LTM. It is widely accepted that changes in gene expression mediate some aspects of this process. Here we examined the role of six transcriptional regulators in memory. The preponderance of evidence indicates that CREB, CaMKIV, c-fos, c-jun, CBP, and SRF are important in mammalian LTM. In many cases, a variety of techniques were used to disrupt the function of the transcriptional regulator, and the impact of this disruption was measured using multiple tests. It is important to point out that few studies have reported effects of these manipulations on learning or STM. Therefore, the effects of disrupting these transcriptional regulators seem to be specific for LTM. These findings, however, do not preclude the importance of other transcriptional regulators in memory formation.

It is perhaps not surprising that similar memory disruptions were produced by manipulations of the transcriptional regulators reviewed here, because they are closely linked. For instance, although CBP was initially identified based on its physical interaction with CREB, it also serves as an essential transcriptional cofactor for c-fos, c-jun, and SRF. CaMKIV phosphorylates both CREB and CBP, thereby enhancing CBP-mediated transcription (Impey et al., 2002). Finally, both CRE and SRE recognition sites are found in the promoter regions of c-fos and c-jun. Indeed, these transcriptional regulators likely work in concert to coordinate gene expression necessary for memory. This close relationship between these transcriptional regulators also suggests that there could be functional redundancy. This could account for the subtle phenotypes observed in some instances where a single transcriptional regulator is disrupted.

Two interesting themes emerged from the overall pattern of the data examined in this chapter. The first is that the memory phenotype is sensitive to the training parameters used to induce LTM (particularly the spacing between successive trials). This sensitivity was observed in mice with mutations in CREB, c-fos, c-jun, CaMKIV, and CBP and across several memory paradigms. More intense training (typically spaced training trials) generally produces stronger memory. Moreover, some of the memory deficits in the mutant lines of mice are overcome with intense training protocols. This may be because a stronger memory is less likely to be disrupted, or it could be that intense training may recruit additional molecular mechanisms.

The second theme is that the extent of gene disruption determines the memory phenotype. This pattern was observed in both CREB mutants and in conditional c-fos knockouts. In general, the greater the gene disruption, the greater the memory impairment.

The ultimate products of transcription mediated by these transcriptional regulators may support the structural and functional processes necessary for LTM in species ranging from snails to flies to rodents and, perhaps, humans. The identity of these genes, however, is currently unknown, and the list of candidate genes is long. For instance, a genome-wide analysis of CREB binding motifs resulted in 1349 sites in the mouse genome and 1663 'hits' in the human genome (Conkright et al., 2003b). Application of proteomic strategies may assist in confirming important targets.

4.27.8.1 Transcriptional Regulators and Memory Disorders in Humans

The importance of transcriptional regulation in memory formation has been highlighted by recent findings showing that some cognitive disorders in humans are associated with molecular lesions in several transcriptional regulators, including some reviewed earlier (see Weeber and Sweatt, 2002; Hong et al., 2005). For instance, mutations in the gene encoding RSK2, a protein kinase that phosphorylates CREB, is thought to be responsible for Coffin-Lowry Syndrome, a syndrome that produces cognitive deficits (Trivier et al., 1996). Rett syndrome, an X-linked disorder that is characterized by arrested neurological development and cognitive decline, is associated with a disruption in the transcription factor methyl-CpG-bindign protein 2 (MeCP2) (Amir et al., 1999). Mutations in CBP are thought to be responsible for Rubinstein-Taybi Syndrome (Petrij et al., 1995). Rubinstein-Taybi Syndrome is an autosomal dominant disorder that produces, among other deficits, mental retardation. Finally, dysregulation of CREB function (Asanuma et al., 1996; Foster et al., 2001; Brightwell et al., 2004),

PKA (Karege et al., 2001), and CBP (Chung et al., 2002) has also been implicated in age-related cognitive decline. Together, these examples highlight the importance of transcriptional regulation in human memory and cognition. They also raise the intriguing possibility that some forms of cognitive dysfunction in humans may be caused by deficits in the transcription regulation necessary for memory formation. Therefore, targeting the transcriptional regulators, including those reviewed in this chapter, may provide novel therapeutic targets for cognitive and memory disorders in humans.

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4.28 The NF-κB Family in Learning and Memory

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4.28.1 Overview of NFκB Signaling

The nuclear factor kappa B (NFκB) family of transcription factors was first discovered as transcriptional regulators of the immunoglobulin loci (Sen and Baltimore, 1986) and has received widespread attention in many fields for roles in inflammation, host defense, and programmed cell death. NFκB transcription factors function as dimers that regulate gene expression by binding to consensus κB-binding sites of target genes. The mammalian NFκB family (also known as the Rel family) consists of five members which can hetero- and homodimerize: p50 (product of the NFκB1 gene), p52 (product of the NFκB2 gene), p65 (also known as Rel A), c-Rel, and Rel B. All Rel family members contain a conserved Rel homology domain (RHD), a 300-amino-acid amino-terminal region containing an immunoglobulin fold DNA-binding domain, a dimerization domain, and a nuclear localization sequence (NLS). The carboxyl-terminal domains of Rel family members differ, with only p65, c-Rel, and RelB containing transactivation domains (TADs). Although p50 and p52 do not contain TADs and therefore do not function as transcriptional activators by themselves, they may activate gene transcription through binding interactions with other transcription factors. In addition, homo- or heterodimers of p50 or p52 may repressively regulate transcription by competing for DNA binding sites with NFκB dimers that do contain TADs.

Dimers of NF-κB are held latent in the cytoplasm by noncovalent interactions with a class of inhibitors called inhibitor of NFκB (IκB) proteins. Activation of NFκB does not require *de novo* synthesis of the transcription factor and is mediated by the IκB kinase complex (IKK) containing IKKα, IKKβ, and IKKγ subunits. The IKK complex is activated by a plethora of upstream signals including cytokines, neurotransmitters, and growth factors, as well as numerous cytotoxic stimuli (Scheidereit, 2006). The canonical pathway of NFκB activation proceeds through phosphorylation of IκB mediated by IKK (**Figure 1**). Phosphorylated IκB is targeted for ubiquitination and subsequent degradation by the 26S proteasomal complex. This removes IκB from the NFκB dimer, exposes both the NLS and DNA-binding domains of NFκB, and permits stable nuclear translocation and DNA binding by the NFκB dimer.

The p50 and p52 members of the NFκB family are initially synthesized as the precursor molecules p105 and p100, respectively. The carboxyl-terminal domains of these precursor molecules contain ankyrin repeats which resemble IκB. Like IκB molecules, the carboxyl-termini of p105 and p100 are capable of binding and inhibiting the NFκB dimer and causing the cytoplasmic localization of dimers containing p105 or p100. Activation of RelB:p100 dimers occurs through what is known as the noncanonical or alternative NFκB activation pathway. Phosphorylation of p100 by a homodimer of IKKα is essential in this activation pathway and serves to target p100 for subsequent ubiquitination. Ubiquitination is followed by an unusual process of limited proteolytic degradation mediated by the 26S proteasomal complex that removes the C-terminus of p100 to generate active p52 proteins (**Figure 1**).

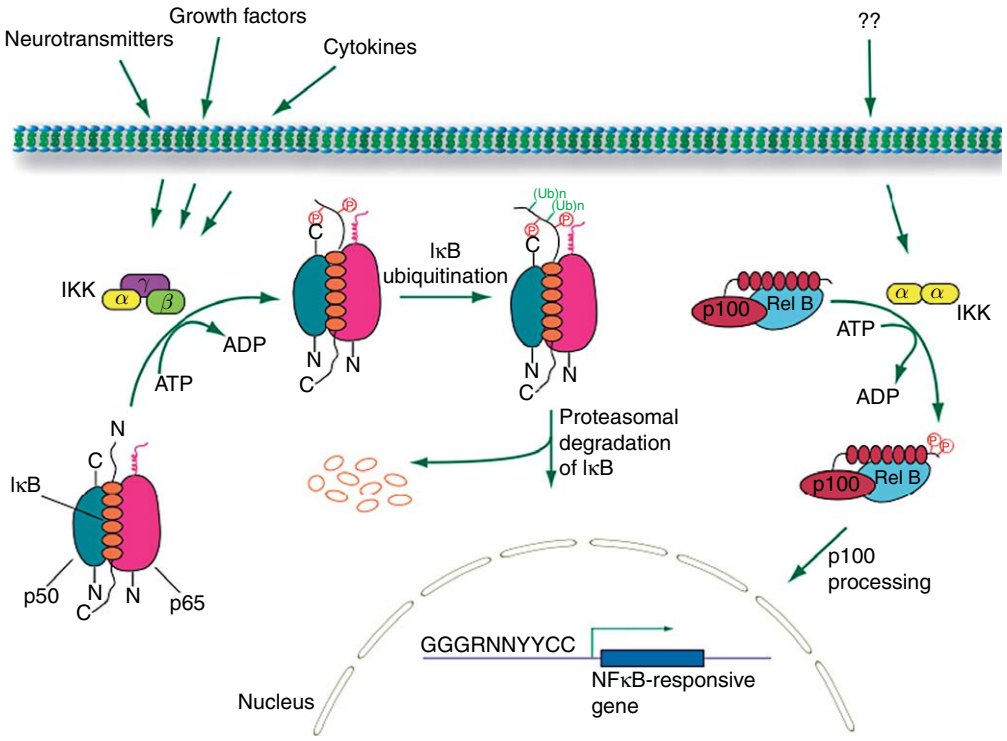


Figure 1 Activation pathways of nuclear factor kappa B (NFκB). Dimers of NFκB family members (shown as p50:p65) can be activated by the canonical pathway within the central nervous system. This pathway depends upon the ubiquitination and degradation of inhibitor of NFκB (IκB) by the 26S proteasome to permit the stable nuclear translocation of activated NFκB. The alternative pathway of NFκB activation, in which ankyrin repeats of p100 are removed by proteasomal processing to generate an active p52:RelB dimer, has not yet been clearly demonstrated in neurons. Activated NFκB can bind to exposed consensus sequences in the regulatory regions of target genes to alter transcription. IKK, IκB kinase complex.

Activated nuclear NFκB regulates transcription at many promoters containing variations in a highly divergent consensus DNA-binding sequence (the κB site). The general loose consensus sequence for the decameric κB site is: GGGRNNYYCC (R = purine, Y = pyrimidine). Variations in the κB site appear to confer regulatory specificity for dimers of different NFκB family members by two general mechanisms. The exact sequence of the κB site can determine which coactivators are capable of forming productive interactions with the bound NFκB dimer (Leung et al., 2004). This mode of regulatory specificity exists independently of any inherent differences in binding affinities of individual κB sites for discrete NFκB dimers. Specificity in transcriptional regulation can also be conferred by preferential binding of distinct NFκB dimer combinations to divergent κB sites. The finding of a variation in the κB site which preferentially binds RelB:p52 heterodimers provides one example of this type of regulatory specificity (Bonizzi et al., 2004).

4.28.2 Activation Pathways in the CNS

Expression of multiple Rel family members has been documented in the central nervous system (CNS), including p50, p65, and c-Rel. The most commonly reported neuronal species is the p65:p50 heterodimer, but dimers containing c-Rel have also been described. Neuronal NFκB can be activated by a wide variety of stimuli occurring in both physiological settings, such as glutamate and nerve growth factor (NGF), and pathological settings, such as β-amyloid and viral infection. Inducers of NFκB with particular relevance to normal brain function are listed in **Table 1**. Many stimuli capable of activating NFκB were first discovered and characterized in cells of the immune system. Interestingly, some of these immune modulators, including cytokines, chemokines, and their receptors, are now known to have additional functions in regulating neuronal development and

Table 1 Activators of NFκB with potential relevance to plasticity and cognition

Gene	*Tissue/cell type*	*Methods used*	*Reference*
Neurotransmitters			
Adenosine	Rat basal forebrain	1. EMSA (electrophoretic mobility shift assay) – NFκB activation inhibited by an A1 adenosine receptor antagonist 2. IκB degradation	Basheer et al., 2001
Glutamate	Murine cerebellar granule neurons and astrocytes	EMSA, supershift – NFκB activation in neurons but not astrocytes; inhibited by NMDA or AMPA glutamate receptor antagonists	Guerrini et al., 1995
	Rat cerebellar granule neurons	IHC – diminished staining with AMPA glutamate receptor antagonist	Kaltschmidt et al., 1995
	Rat cerebellar granule neurons	EMSA	Grilli et al., 1996
	Rat cerebellar granule neurons	1. EMSA, supershift – NMDA activation of p50:p65 2. Immunoblot – cytoplasmic levels of IκBα and phospho-IκBα	Lipsky et al., 2001
	Murine hippocampal cultures	1. EMSA, supershift – activation of p65:p50 and p50:p50 in neurons but not astrocytes through NMDA receptors, L-type Ca^{2+} channels, and Ca^{2+} ionophore. 2. Reporter assay – tests specificity with mutated NFκB consensus site	Meffert et al., 2003
	Murine cerebellar granule neurons	1. Reporter assay 2. EMSA – Ca^{2+}-dependent basal activation of p50:p65 and p50:p50	Lilienbaum and Israel, 2003
	Murine cortical neurons and neuronal SK-N-SH cells	1. EMSA – NFκB activation with Group I metabotropic receptor agonist 2. Increased DNA-binding activity of dimers containing p65, p50, and c-rel through mGlu5 receptor using NFκB ELISA-based assay kit	Pizzi et al., 2005
	Rat piriform cortex and hippocampus	EMSA – following *in vivo* kainate injection	Rong and Baudry, 1996
	Murine hippocampal slices	Increased DNA binding activity of p65, p50, and c-rel via group I metabotropic glutamate receptors using NFκB ELISA-based assay kit	O'Riordan et al., 2006
Nitric oxide	Rat striatal neuronal culture	EMSA	Simpson and Morris, 1999
Serotonin	Chinese hamster ovary cells	1. Immunoblot – cytoplasmic levels of IκBα 2. Reporter assay – serotonin 1A agonist, uses HIV-LTR which also contains an SP1 site	Cowen et al., 1997
	Chinese hamster ovary cells	1. NFκB activation through serotonin 1A receptor using NFκB ELISA-based assay kit 2. Immunoblot – cytoplasmic levels of IκBα and phospho-p65	Hsiung et al., 2005
Cytokines/chemokines			
IL-1α	Rat glioma C6 cell line	EMSA – NFκB activation inhibited by IL-1 receptor antagonist	Moynagh et al., 1993
	Astrocytes	EMSA	Guerrini et al., 1995
IL-1β	Rat glioma C6 cell line	1. EMSA – NFκB activation inhibited by IL-1 receptor antagonist, specificity tested by competitor oligonucleotides 2. Reporter assay – NFκB activation inhibited by IL-1 receptor antagonist	Moynagh et al., 1993

(*Continued*)

Table 1 (Continued)

Gene	*Tissue/cell type*	*Methods used*	*Reference*
	Rat astrocytes and cerebellar granule neurons	Reporter assay	Krushel et al., 1999
	Rat cerebellar granule neurons and SH-SY5Y neuroblastoma cells line	1. EMSA 2. Reporter assay	Grilli et al., 1996
	Rat cerebellar granule neurons	1. EMSA, supershift 2. Immunoblot – cytoplasmic levels of IκBα and IκBβ	Pizzi et al., 2002
	Murine hippocampal slices	IHC – increased p65 and c-rel nuclear localization	
SDF-1α	Murine cortical astrocytes	1. EMSA, supershift – activation of p65:p50 dimer; specificity tested by competitor wild-type and mutant κB oligonucleotides 2. Reporter assay	Han et al., 2001
TNFα	Neuroblastoma cell line	EMSA, supershift	Drew et al., 1993
	Hippocampal cultures	EMSA	Barger et al., 1995
	Astrocytes	EMSA	Guerrini et al., 1995
	Astrocytes and cerebellar granule neurons	Reporter assay	Krushel et al., 1999
	Cerebellar granule neurons	Reporter assay	Lilienbaum and Israel, 2003
TNFβ	Hippocampal cultures	EMSA	Barger et al., 1995
	SH-SY5Y neuroblastoma cell line	CAT reporter assay	Barger and Mattson, 1996
Neurotrophins/growth factors			
ADNF	Hippocampal cultures	1. EMSA, supershift – shows p65 and p50 activation 2. IHC – increased p65 nuclear localization	Glazner et al., 2000
BDNF	Cerebellar granule neurons	EMSA	Lipsky et al., 2001
	Murine P19 neurons	EMSA, supershift	Burke and Bothwell, 2003
IGF-1	Rat cerebellar granule neurons and GT1-7 neuronal cell line	1. EMSA, supershift – activation of p65:p50 dimers, specificity tested by competitor oligonucleotides 2. Reporter assay 3. Immunoblot – cytoplasmic levels of IκBα and nuclear levels of p65	Heck et al., 1999
NGF	Rat and murine Schwann cells	EMSA, supershift – activation of p65, p50, c-Rel	Carter et al., 1996
	Sympathetic neuronal cultures	EMSA, supershift – activation of p65, p50, and c-Rel	Maggirwar et al., 1998
	Rat cortical oligodendrocyte cultures	1. EMSA 2. IHC – increased p65 nuclear localization	Yoon et al., 1998
	Murine P19 neurons	EMSA, supershift	Burke and Bothwell, 2003
NT-3	Murine P19 neurons	EMSA, supershift	Burke and Bothwell, 2003

(*Continued*)

Table 1 (Continued)

Gene	*Tissue/cell type*	*Methods used*	*Reference*
NT-4	Murine P19 neurons	EMSA, supershift	Burke and Bothwell, 2003
PEDF	Rat cerebellar granule cell culture	1. EMSA, supershift – activation of p65:p50 and p50:50 dimers, specificity tested by competitor oligonucleotides 2. Immunoblot – cytoplasmic levels of IκBα and phospho-IκBα 3. IHC – increased p65 nuclear localization	Yabe et al., 2001
TGF-β1	Murine hippocampal cultures	1. Reporter assay 2. Immunoblot – cytoplasmic levels of IκBα, IκBβ and phospho-IκBα 3. IHC – increased p65 nuclear localization	Zhu et al., 2004
VEGF	Murine HN33 neuronal cell line	Immunoblot – cytoplasmic levels of phospho-IκBα and nuclear levels of p65	Jin et al., 2000
Cell adhesion molecules			
N-CAM	Astrocytes and cerebellar granule neurons	1. EMSA and supershift – shows p65 and p50 activation 2. Reporter assay 3. Immunoblot of cytoplasmic IκBα levels	Krushel et al., 1999
Miscellaneous			
Apotransferrin (aTf)	Oligodendroglial cell lines	1. EMSA 2. Immunoblot of cytoplasmic IκBα levels	Paez et al., 2006
Bradykinin	Murine astrocytes	1. EMSA, supershift – activation of p65:p50 and p50:p50, specificity tested by competitor mutant κB oligonucleotides 2. Reporter assay – activation inhibited by DNIκBα	Schwaninger et al., 1999

Abbreviations: ADNF, activity-dependent neurotrophic factor; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; BDNF, brain-derived neurotrophic factor; CAT assay, chloramphenicol acetyltransferase reporter assay; ELISA, enzyme-linked immunosorbent assay; HIV-LTR, long terminal repeat from HIV; IGF-1, insulin-like growth factor; IHC, immunohistochemistry; N-CAM, neural cell adhesion molecule; NMDA, *N*-methyl-D-aspartate; NT-3, NT-4, neurotrophins 3 and 4; PEDF, pigment epithelium-derived factor; SDF-1α, stromal-derived factor 1α; TGF, tumor growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

synaptic plasticity. For example, the CD40 receptor, which activates NFκB during B cell development, is also expressed in neurons of the CNS, where it can regulate neuronal survival and may also promote neuronal differentiation (Tan et al., 2002). Likewise, tumor necrosis factor (TNF) and its receptors are well-characterized activators of NFκB in the immune system and are also expressed in the CNS. The functions of TNFα in the brain remain incompletely characterized, but one role appears to be the regulation of neuronal responses to the excitatory neurotransmitter glutamate (Albensi and Mattson, 2000; Beattie et al., 2002; Stellwagen et al., 2005; Stellwagen and Malenka, 2006). In most cases, the role that NFκB activation might play in the effects of these immune modulators in neurons is as yet unknown. In addition, some activators of NFκB, such as glutamate, depolarization, NGF, and activity-dependent neurotrophic factor (ADNF) (Glazner et al., 2000), have been most completely characterized in cells of the nervous system or neuronal cell lines and may play unique roles in the neuronal regulation of this transcription factor.

The canonical pathway of NFκB activation involving phosphorylation of the IKK complex and proteasomal degradation of the IκB inhibitor is well documented in neurons of the CNS (**Figure 1**). The upstream signaling pathways leading to IKK activation in the brain appear to be as diverse as they are outside the CNS. For example, the major excitatory neurotransmitter of the hippocampus, glutamate, can activate NFκB in hippocampal neurons through both an *N*-methyl-D-asprate (NMDA) receptor–mediated pathway dependent upon Ca^{2+} elevation (Lilienbaum and Israel, 2003; Meffert et al., 2003), and a metabotropic receptor-mediated pathway, which is likely to be independent of Ca^{2+} elevation and

involves the activities of phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase/extracellular signal-related kinase (MEK), and p38-mitogen-activated protein kinase (p38-MAPK) (Lubin et al., 2005; O'Riordan et al., 2006). Potential functions of NFκB activation by the alternative pathway (IKKα-regulated cleavage of the p100 precursor) have not been clearly determined in the brain. Although both the p100 and p105 precursors are present in brain tissue, the expression pattern of essential components of the alternative activation pathway, including the NFκB-inducing kinase (NIK), await characterization. NIK-deficient mice are reported to display no obvious defects in brain function and are capable of normal growth, behavior, reproduction, and nursing (Yin et al., 2001); however, a detailed analysis of the CNS in NIK-deficient mice has not been published. In a neuron-like cell line (PC12), overexpression of wild-type NIK but not kinase-dead NIK enhances the formation of neurites (Foehr et al., 2000a). Whether the alternative and canonical pathways of activation subserve separate or overlapping functions in the CNS remains to be determined.

Neurotrophins are essential for many aspects of cognition and participate in regulating differentiation and synaptogenesis during brain development as well as influencing growth and plasticity in the mature brain. NGF is the most well-characterized neurotrophic activator of neuronal NFκB. NGF can activate NFκB through signaling pathways using either the tyrosine kinase A (TrkA) receptor or the $p75^{NTR}$, both of which converge to activate IKK. Stimulation of chimeric NGF receptors has revealed that the $p75^{NTR}$ activates dimers containing p65, p50, or p52 subunits of NFκB, while trkA activates only dimers containing p50 or p65. This selective NFκB dimer activation may also have functional consequences, as certain genes are specifically induced by stimulation through one receptor or the other, in addition to a significant overlap in gene regulation (Carter et al., 1996; Foehr et al., 2000b). The $p75^{NTR}$ NGF receptor is a member of the TNF superfamily of receptors and, like other family members, is coupled to both Jun N-terminal kinase (JNK) and NFκB activation through the tumor necrosis factor receptor associated factor-6 (TRAF6) protein. A dominant negative TRAF6 eliminates NFκB activation through the $p75^{NTR}$ while sparing activation through TrkA (Foehr et al., 2000b). The loss of neurotrophin-induced apoptosis in sympathetic neurons from TRAF6-null mice suggests that TrkA stimulation alone could mediate prosurvival functions of neurotrophins (Yeiser et al., 2004). These potential roles for neurotrophin-induced NFκB activation have yet to be validated in neurons of the CNS, however.

Among the numerous stimuli capable of activating NFκB, one of the most interesting from the standpoint of plasticity and cognition is the regulation of this transcription factor by synaptic transmission. The application of exogenous glutamate and analogs such as NMDA and kainate were first shown to activate NFκB in cerebellar granular neurons (Guerrini et al, 1995; Kaltschmidt et al., 1995). The finding that endogenous synaptic activity could also activate NFκB made it clear that this pathway could have physiological relevance apart from a role in the response to excitotoxicity. Ca^{2+} influx through NMDA receptors and L-type Ca^{2+} channels appears to play an important role in glutamate-mediated NFκB activation in both the hippocampus and cerebellum (Lilienbaum and Israel, 2003; Meffert et al., 2003; Scholzke et al., 2003). Blockade of these receptors significantly depresses NFκB activity in cultured cells and *in vivo,* indicating that basal levels of synaptic transmission effectively regulate this transcription factor (Meffert et al., 2003). A study using cerebellar neurons in culture for 4 days (likely prior to synapse formation) has demonstrated that release of intracellular Ca^{2+} stores following glutamate stimulation could also contribute to NFκB activation (Lilienbaum and Israel, 2003).

Multiple neurotransmitters including glutamate, dopamine, adenosine (Basheer et al., 2001), and serotonin (Hsiung et al., 2005) have now been reported to activate NFκB. Stimulation of dopamine D2-like receptors in a neuroblastoma cell line (NG108) (Takeuchi and Fukunaga, 2004a) as well as intrastriatal injection of dopamine have been reported to activate NFκB (Luo et al., 1999). A deletion mutation analysis of the D2 receptor and pharmacological manipulation indicate that D2 receptor activation of NFκB proceeds through members of the MAPK family, including MAPK and the extracellular signal-regulated kinase (ERK) (Takeuchi and Fukunaga, 2004b). Adenosine-stimulated NFκB activation is mediated by the A1 receptors in basal forebrain neurons and may participate in regulating wakefulness and the response to sleep deprivation. Regulation by more than one neurotransmitter suggests that NFκB has the potential to participate in a variety of cognitive functions in distinct brain regions.

4.28.3 NFκB at Synapses

An unusual subcellular localization was one of the first intriguing aspects reported for neuronal NFκB (Kaltschmidt et al., 1993; Guerrini et al., 1995; Suzuki et al., 1997). The presence of NFκB in dendritic processes and at synapses was suggested by studies using immunohistochemistry and electron microscopy of brain regions containing synapses in discrete cell layers. These observations were strengthened by the finding of NFκB in isolated preparations of synapses termed synaptosomes (Kaltschmidt et al., 1993; Meberg et al., 1996). Interestingly, not all dimers of NFκB have been found to be equally represented at synapses. Isolated synapses from the hippocampus and cortex of wild-type mice contain primarily p65:p50 dimers, while synapses from p65-deficient mice are devoid of NFκB (Meffert et al., 2003). It is possible that neuronal stimulation could alter the complement of NFκB dimers that is targeted to synapses, although this type of regulation has not yet been reported. NFκB appears to be present at both the postsynaptic side of the synapse (in preparations of the postsynaptic density, PSD) as well as in the presynaptic compartment.

After isolation by subcellular fractionation, synaptosomes can reseal to form biochemically functional synaptic compartments. As reported for intact neurons, the latent NFκB found within these isolated synapses can be activated by glutamate or by Ca^{2+} elevation alone (Meffert et al., 2003). This finding implies that all of the components required for the NFκB activation pathway are located at synapses or in adjacent dendritic regions. Neuronal studies of the ubiquitin proteasomal degradation pathway, which is critical for activation of NFκB by IκB degradation, support this implication. Like NFκB activation, ubiquitination and activity of the 26S proteasome are also regulated by excitatory neurotransmission (Ehlers, 2003; Patrick et al., 2003). A recent analysis of the 26S proteasome in hippocampal dendrites has revealed that this complex uses the actin cytoskeleton to move into dendritic spines in response to synaptic input. In addition, the level of local proteasomal degradation is enhanced by neuronal depolarization in an NMDA receptor–dependent manner (Bingol and Schuman, 2006). These studies provide an upstream correlate for the finding that synaptic activity and localized Ca^{2+} elevations lead to neuronal NFκB activation. At hippocampal synapses, the Ca^{2+}-dependent pathway of NFκB activation is also inhibited by NMDA receptor blockade and appears to require the activity of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), which is concentrated in PSDs (Meffert et al., 2003).

4.28.4 Translocation from Cytoplasm to Nucleus

Imaging of neurons expressing a green fluorescent protein–tagged p65 (GFPp65) subunit of NFκB has confirmed the localization of NFκB in neuronal processes. The architectural complexity of neurons means that NFκB activated at synapses can be distant from the site of transcriptional regulation in the nucleus. The potential exists that synaptic NFκB might not effectively translocate long cytoplasmic distances following stimulation and might instead have local nontranscriptional functions. To date, however, nontranscriptional functions for NFκB have not been demonstrated, and studies in living neurons have revealed that NFκB in distal neuronal processes is capable of translocating to the nucleus following stimulation (Wellmann et al., 2001; Meffert et al., 2003). Little is known about the molecular machinery involved in the cytoplasmic phase of NFκB translocation to the nucleus. However, upon reaching the nucleus, an NFκB interaction with the importin/karyopherin class of chaperone molecules has been found to mediate its translocation across the nuclear pore (Fagerlund et al., 2005), most likely through a direct binding interaction (Cunningham et al., 2003). While importin molecules are found in the dendrites and axons of mature neurons (Hanz et al., 2003; Thompson et al., 2004), a requirement of importins for cytoplasmic transport of neuronal NFκB has not been reported to date. Involvement of the NLS of p65 in the redistribution of NFκB from neuronal cytoplasm to nucleus has been suggested by imaging studies evaluating the distribution of fluorophore-tagged wild-type and NLS mutant p65 (Wellmann et al., 2001). Interestingly, synthetic NLS peptides undergo active retrograde transport mediated through interactions with importins in *Aplysia* axons subjected to injury (Ambron et al., 1992; Schmied and Ambron, 1997; Hanz et al., 2003). Loss of an active *Aplysia* NFκB-like molecule along the axons near sites of axonal crush injury has also been reported (Povelones et al., 1997). Since levels of activated nuclear NFκB were not assessed

in this study, it remains uncertain whether the activated NFκB might have undergone nuclear translocation or whether it may have been degraded in the crushed axon.

4.28.5 NFκB in *In Vitro* Assays of Plasticity

The activation of NFκB by neurotransmission and, in particular, its regulation by the excitatory neurotransmitter glutamate suggest the potential participation of NFκB in plasticity requiring activity-dependent transcriptional regulation. Several investigators have examined the role of NFκB in *in vitro* paradigms of plasticity in which changes in gene expression are essential for long-lasting alterations of synaptic function. Long-term potentiation (LTP) and long-term depression (LTD) occur in many brain regions and are commonly studied in the Schaffer collateral pathway between CA1 and CA3 neurons in hippocampal slices. LTP is the use-dependent lasting enhancement of synaptic connections, while hippocampal LTD is the long-term weakening of synaptic connections in response to persistent low-frequency stimulation. Recent work has firmly established the often-proposed link between LTP and the cognitive functions of learning and memory (Whitlock et al., 2006). Initial studies in which NFκB was inhibited by bath application or microinjection of DNA oligos containing consensus binding sites for NFκB (called 'decoy' DNA) reported a deficit in LTP both in the hippocampus (Albensi and Mattson, 2000) and in the amygdala (Yeh et al., 2002).

These early studies have subsequently been confirmed by genetically manipulating the NFκB pathway using either transgenic expression of a dominant inhibitor of NFκB or by knockout of Rel family members. Expression of a dominant negative nondegradable mutant of IκBα (DNIκBα) is believed to result in broad spectrum inhibition of all Rel family members. Specific transgenic expression of the DNIκBα in neurons of the forebrain produced a modest but significant reduction in the late (≥100 min post potentiating stimulus), transcription-dependent phase of hippocampal LTP (Kaltschmidt et al., 2006). In addition, forebrain neuronal expression of the DNIκBα also appeared to impair the induction of hippocampal LTD (Kaltschmidt et al., 2006). A form of hippocampal LTD mediated by activation of metabotropic glutamate receptors (mGluRs) has also been examined in knockout mice selectively deficient in the c-Rel subunit of NFκB. Early LTD was found to be normal in c-Rel-deficient mice, however, a late (≥70 min post mGluR stimulus) transcription-dependent phase of LTD was disrupted with synaptic efficacy returning to predepression levels by 180 min after LTD simulation in c-Rel-deficient mice (O'Riordan et al., 2006). An analysis of frequency and amplitude of miniature synaptic currents in the same mice revealed no significant difference compared to c-Rel wild-type littermates.

Consistent with the inhibition of LTP and LTD produced by disrupting NFκB function, the generation of LTP by itself may also enhance the function of the NFκB pathway through two mechanisms. The high-frequency stimulation used to generate LTP is reported to activate latent NFκB (Freudenthal et al., 2004) and may also mediate increases in the transcription of the p65 subunit of NFκB (Meberg et al., 1996). A gel shift analysis of the hippocampus following *in vivo* perforant path LTP or LTD indicated that heterodimers of p65:p50 as well as p50:p50 homodimers were activated by high-frequency stimulation, while the low-frequency stimulation associated with LTD had no significant effect on NFκB activity (Freudenthal et al., 2004). The enhancement of p65 and p50 mRNA production was reported 60–120 min following LTP induction in hippocampal pyramidal and granule neurons. While increased production of p65 and p50 subunits might enhance the function of the NFκB pathway, a caveat to this interpretation is that NFκB is not typically regulated by transcription, and as the authors point out, a similar increase in the mRNA level of a classical NFκB target gene, IκBα, was not observed.

4.28.6 NFκB in Behavioral Assays of Learning and Memory

4.28.6.1 Crustaceae

The excitement of finding NFκB activation by synaptic activity and its role in *in vitro* assays of plasticity has led to the investigation of this transcription factor's potential involvement in cognition. Some of the first reports suggesting a potential cognitive role for NFκB came from habituation studies in the marine crab, *Chasmagnathus,* which contains a homolog of mammalian NFκB. Repeated presentation of a visual danger signal simulating an overhead predator leads to eventual habituation of the crab's escape response. This predator avoidance paradigm

has been characterized to function as a model of long-term memory (LTM) mediated by a conditioned association between contextual cues and the predator-eliciting stimulus. A growing body of work suggests that the crab NFκB homolog is critical both to the formation of this LTM and to the process of memory reconsolidation.

Initial reports demonstrated that spaced training resulting in habituation and LTM increased the levels of activated NFκB present in crab neuronal synapses and nuclei, while training which did not result in LTM also did not activate NFκB (Freudenthal et al., 1998; Freudenthal and Romano, 2000). In subsequent studies, injection of a nonspecific inhibitor of IKK, sulfasalazine, was reported to block NFκB activation and also inhibit LTM formation (Merlo et al., 2002). Sulfasalazine contains 5-aminosalicylic acid (the main ingredient in aspirin) in combination with a sulfa antibiotic and can also inhibit cyclooxygenase. However, control injection of alternative cyclooxygenase inhibitors did not block LTM. The process of memory retrieval is thought to initiate a second process of memory consolidation, termed reconsolidation, which invokes some of the same cellular mechanisms used in initial memory formation. A study of reconsolidation using the same predator avoidance paradigm in *Chasmagnathus* suggests that memory retrieval, activated by reexposure to the training context, also activates NFκB. IKK inhibition by sulfasalazine impaired reconsolidation when administered prior to memory retrieval, but had no apparent effect on the LTM when preceding exposure to an alternative training context not expected to activate memory retrieval (Merlo et al., 2005). Work by other researchers has implicated another activity-dependent transcription factor, cyclic adenosine monophosphate (cAMP) response element binding protein (CREB), as well as the immediate early gene and transcription factor Zif268, in reconsolidation (Kida et al., 2002; Bozon et al., 2003). Collectively, these findings provide evidence for an emerging consensus that transcription is required for memory reconsolidation in addition to the previously established role for new protein synthesis in this process.

4.28.6.2 Vertebrates

Evaluation of the function of NFκB in vertebrate cognitive functions has focused largely on behavioral paradigms testing either fear conditioning or spatial memory. A role for NFκB in these types of learning appears to be consistent in both mammalian and invertebrate systems. Studies using knockout mice indicate that deletion of the p65, p50, and c-Rel subunits can affect the processes of learning and memory. Deficits in contextual fear memory and a long-term passive avoidance task utilizing both the hippocampus and amygdala occur in mice lacking the c-Rel subunit of NFκB (Levenson et al., 2004; O'Riordan et al., 2006). Mice deficient in p50 are impaired in short-term active avoidance paradigms and may show reduced anxiety responses (Kassed et al., 2002). In addition, mice lacking p50 are reported to have increased exploratory activity and less apparent anxiety in open field and novel object tests (Kassed and Herkenham, 2004). Mice lacking the p65 subunit of NFκB are rescued from embryonic lethality if pro-apoptotic stimulation through the tumor necrosis factor receptor (TNFR) is abrogated. Behavioral assays comparing double knockout mice ($TNFR^{-/-}$,$p65^{-/-}$) to single knockout ($TNFR^{-/-}$,$p65^{+/+}$) littermates revealed that p65 deficiency results in a spatial learning deficit. Mice lacking p65 took approximately twice as long to reach peak performance in a spatial version of the radial arm maze compared to p65 wild-type littermates; no differences in performance were observed in a cued version of the radial arm maze which did not require the use of spatial information (Meffert et al., 2003). While differences in the behavioral phenotypes of NFκB knockout mice are intriguing, it remains too early to assign any subunit-specific functions in cognition.

Inhibition of the NFκB pathway using either pharmacological approaches or transgenic overexpression of a mutant nonphosphorylatable form of IκBα (DNIκB) which functions as a dominant negative can also disrupt the processes of learning and memory. Several studies in rats have used intra-amygdalar or intrahippocampal infusion of small double-stranded decoy oligonucleotides for NFκB to impair either long-term memory in paradigms involving fear conditioning (Yeh et al., 2002; Freudenthal et al., 2005) or long-term spatial memory in the Morris water maze (Dash et al., 2005), respectively. Decoy oligonucleotides resemble the consensus DNA binding site for NFκB and are hypothesized to function by competitively interfering with the ability of NFκB to bind to its target sequences upstream of regulated genes. In both cases, oligonucleotides containing a scrambled NFκB sequence were ineffective, but ruling out any

potential side effects of an NFκB DNA-binding sequence present in the cytoplasm is not possible.

Two lines of transgenic mice expressing the DNIκB either through the CaMKIIα promoter (Fridmacher et al., 2003; Kaltschmidt et al., 2006) or the prion promoter (O'Mahony et al., 2006) have been reported. While studies using knockout mice have permitted an analysis of subunit specificity, these are the first studies allowing an assessment of tissue or cell type–specific effects of NFκB deficiency. In the first study, tetracycline-regulated DNIκB was predominantly expressed in forebrain excitatory neurons in a double transgenic mouse with the tetracycline transactivator under control of the CaMKIIα promoter (TetOFF) (Fridmacher et al., 2003). Inhibition of NFκB by the DNIκB compromised spatial learning as assessed by the Morris water maze (Kaltschmidt et al., 2006). The effects of DNIκB expression were modest, however, and the authors also found that doxycycline itself compromised memory formation or retention and may have contributed to the lack of significant differences between double transgenics in the absence or presence of doxycycline. Single transgenics, harboring nonexpressed DNIκB, did perform significantly better in the spatial learning task than double transgenic mice expressing DNIκB (in the absence of doxycycline). Experiments by a separate team of investigators using a transgenic line expressing DNIκB under control of the prion promoter yielded disparate results. In this study, NFκB inhibition by the DNIκB actually enhanced spatial memory formation using both Morris water maze and radial arm maze tasks (O'Mahony et al., 2006). Opposing results in an assay of *in vitro* plasticity were also observed, with NFκB inhibition generating a modest enhancement of hippocampal LTP. The mechanisms underlying the opposing results found in this study may be complicated, but could potentially shed light on the function of NFκB in the CNS. The authors suggest that the prion promoter directs expression of the DNIκB to inhibitory GABAergic neurons and that this might enhance gamma-aminobutyric acid (GABA) release by upregulating the GAD65 GABA synthetic enzyme (see the section titled 'Transcriptional regulation by NFκB'). Differences in CNS GABA levels were not demonstrated, however. Evidence is presented that the prion promoter in this transgenic line does not drive glial expression but does direct DNIκB expression to both excitatory and inhibitory neurons. This is unlike CaMKIIα promoter-driven gene expression, which occurs predominantly in excitatory neurons. Spatial and temporal differences also exist in the brain gene expression patterns directed by these two promoters. The prion promoter is active during embryonic development and adulthood (Miele et al., 2003), while the CaMKIIα promoter becomes active only postnatally. In addition the prion promoter generates broad expression in neurons (and sometimes other brain cells such as endothelial cells, epithelial cells, glia, and microglia (Moser et al., 1995)) throughout the cortex, cerebellum, and brainstem, while the CaMKIIα promoter is active only in forebrain neurons. It remains to be determined whether any of these differences in gene expression patterns driven by the prion or CaMKIIα promoters account for the behavioral discrepancies between the two studies.

4.28.7 Transcriptional Regulation by NFκB

DNA microarrays have been successfully used to identify genes that are differentially regulated during events requiring plasticity, such as learning and memory. Recently, a bioinformatics analysis used microarray data to identify transcription factors involved in LTM by scanning for potential regulatory elements in the upstream regions (1 kb) of identified target genes associated with memory consolidation (Levenson et al., 2004). This open-ended approach used an NMDA receptor–dependent contextual fear conditioning paradigm to screen a significant fraction of the murine genome for transcripts with altered expression levels in area CA1 of the hippocampus. Analysis of these transcripts revealed that binding sites for NFκB were selectively overrepresented in the subset of genes regulated by LTM formation. Interestingly, while binding sites for other activity-regulated transcription factors such as CREB and serum response factor (SRF) were present in genes regulated by LTM, this study did not find them to be enriched in this population. NFκB-regulated gene expression has also been explicitly linked to learning in eyeblink conditioning, a form of associative learning that is well conserved across species (Park et al., 2006). In this learning paradigm the conditioned response (CR, eyeblink) is evaluated following the pairing of a conditioned stimulus (CS, tone) with an unconditioned stimulus (US, airpuff). A CS–US associative memory results in the generation of the CR in response to the CS. A recent study found that the prototypical NFκB-dependent gene,

IκBα, was differentially upregulated by microarray and *in situ* hybridization following paired training in the murine eyeblink response.

NFκB is a potent transcription factor, which can robustly regulate the expression of a broad range of target genes including growth factors, adhesion molecules, chemokines and cytokines, and components of inflammatory pathways. This review focuses primarily on genes regulated by NFκB with potential relevance to processes of cognition and neuronal plasticity (see **Table 2**). Many NFκB targets identified outside of the nervous system are likely to also be

Table 2 NFκB gene targets with potential relevance to plasticity and cognition

Gene	*Tissue/cell type*	*Methods used*	*Reference*
Cytokines/chemokines			
Interleukin-6	Murine astrocytes	Reporter assay – effects of DNIκB expression on promoter activity	Schwaninger et al., 1999
	Rat retinal cells	siRNA knockdown of p65 reduces mRNA levels by RT-PCR	Wang et al., 2006
Interleukin-1α	Hippocampus	κB decoy DNA downregulates mRNA levels by RPA	Qiu et al., 2004
TNFα	Hippocampus	κB decoy DNA downregulates mRNA levels by RPA	Qiu et al., 2004
TNFβ	Hippocampus	κB decoy DNA downregulates mRNA levels by RPA	Qiu et al., 2004
Cell adhesion molecules			
NCAM	Rat cultured neurons	1. NFκB binding site present in promoter region 2. EMSA 3. p50 antisense decreases protein levels by IHC and immunoblot	Simpson and Morris, 2000
Receptors			
Metabotropic receptor – mGlu2		NFκB binding site present in promoter region	Chiechio et al., 2006
Metabotropic receptor – mGlu3		NFκB binding site present in promoter region	Chiechio et al., 2006
Metabotropic receptor – mGlu5	Neuroblastoma and glioblastoma cell lines	1. NFκB binding site present in promoter region 2. EMSA – data not shown	Corti et al., 2003
NMDA receptor – NR2A	Rat, Human	Conserved NFκB binding site present in promoter region	Richter et al., 2002
NMDA receptor – NR1	Human	Polymorphism of NFκB binding site present in promoter region	Begni et al., 2003
	Murine P19 cell line	Reporter assay – modest effect of DNIκB or κB decoy DNA; SP1 factors show cooperativity and activation at this promoter[a]	Liu et al., 2004
AMPA receptor – GluR1	Human NT2-N cell line, murine brain	NFκB binding sites present in promoter region	Borges and Dingledine, 2001; Yu et al., 2002
		1. Diminished protein expression in hippocampus and cortex of p50 knockout mice by immunoblot 2. Decreased protein expression in NT2-N cells by immunoblot after p50 anti-sense treatment 3. Overexpression of p50 increases expression in NT-2 by IHC	
μ-Opioid receptor	Human Rajii, U937 immune cell lines	1. NFκB binding site present in promoter region 2. EMSA, supershift	Kraus et al., 2003

(*Continued*)

Table 2 (Continued)

Gene	*Tissue/cell type*	*Methods used*	*Reference*
δ-Opioid receptor	Rat PC12 cell line	1. Reporter assay – effects of p65 and DNIκB overexpression on promoter activity 2. NLS peptide reduces mRNA levels by RT-PCR 3. EMSA, supershift 4. siRNA knockdown of p65 reduces mRNA levels by RT-PCR	Chen et al., 2006
Dopamine D_2 receptor	Human prolactinoma, COS-7 cell lines	1. NLS peptide reduces mRNA levels by RT-PCR 2. Reporter assay – effects of p50 and/or c-rel overexpression on promoter activity	Fiorentini et al., 2002
Serotonin (5-HT) 1A receptor	Murine P19, monkey COS-1 and CV1-β cell lines	1. NFκB binding sites present in promoter region 2. Reporter assay – effects of p50 and p65 expression on promoter activity; deletions of NFκB sites in promoter 3. EMSA with p50, p65 overexpression; deletions of NFκB sites	Wissink et al., 2000
Adenosine A1 receptor	Rat PC12 cell line	Receptor upregulation inhibited by DNIκB expression using radioligand binding assay	Jhaveri et al., 2006
CD40–TNFR super family	Murine microglia and macrophage cell line	1. Reporter assay – effects of wtIKKα, dnIKKα, wtIKKβ, or dnIKKβ expression on promoter activity; deletion and mutations of NFκB site in promoter 2. Chromatin immunoprecipitation assay shows bound p65, p50	Qin et al., 2005
Growth factors			
NGF	Rat astrocyte cultures	κB decoy DNA and IκB overexpression decrease mRNA levels by RT-PCR	Zaheer et al., 2001
BDNF	Rat astrocyte cultures	1. NFκB binding site present in promoter region 2. κB decoy DNA and IκB overexpression decrease mRNA levels by RT-PCR	Zaheer et al., 2001
	Rat astrocyte cultures	1. NFkB binding site present in promoter region 2. EMSA, supershift 3. κB decoy DNA downregulates mRNA induction by RT-PCR	Lipsky et al., 2001
Inflammatory response genes			
Cox-2	Neuroblastoma, HeLa cell lines	1. NFκB binding site present in promoter region 2. EMSA 3. Reporter assay – test effects of IκBα overexpression and deletion of NFκB site on promoter activity	Kaltschmidt et al., 2002
	Neuroblastoma cell line	Reporter assay – test effects of p65 or IκBα overexpression, and deletion of NFκB sites on promoter activity	Alvarez et al., 2005
iNOS	Rat neuronal culture	p50 antisense decreases protein levels	Simpson and Morris, 2000
	Rat cerebral cortex	EMSA	Madrigal et al., 2001
nNOS		NFκB binding site present in promoter region	Boissel et al., 1998

(*Continued*)

Table 2 (Continued)

Gene	*Tissue/cell type*	*Methods used*	*Reference*
Enzymes			
PKA catalytic subunit α	Neuro2a cells, murine brain sections	1. NFκB binding site present in promoter region 2. Downregulation in hippocampus of DNIκB expressing mice by microarray analysis 3. Downregulation in hippocampus of DNIκB expressing mice by *in situ* hybridization 4. Decreased mRNA levels in Neuro2a expressing DNIκB by Northern blot 5. EMSA, supershift in Neuro2a 6. Reporter assay – effects of DNIκB expression on promoter activity in Neuro2a	Kaltschmidt et al., 2006
Na+ K+ ATPase γ subunit	Murine hippocampus	Analysis in p50 knockout mice by microarray and RT-PCR	Kassed et al., 2004
CaMKII δ	Murine hippocampus	Analysis in p50 knockout mice by microarray and RT-PCR	Kassed et al., 2004
Transcription factors			
Oct-6	DRG neuron and Schwann cell cocultures	Decreased activation of Oct-6 activation with DNIκB expression by EMSA, may or may not be transcriptionally mediated	Nickols et al., 2003
Hormones			
Human prolactin	Pituitary GH3 cells	Reporter assay – effects of wtIKKβ, dnIKKβ, dnNEMO, truncated IkBα, or dnIKKα expression on promoter activity	Friedrichsen et al., 2006
Pituitary proopiomelanocortin (POMC)	Pituitary corticotroph cell line (AtT20)	Upregulation of mRNA levels by IκBα overexpression using Northern blot	Karalis et al., 2004
Miscellaneous			
Amyloid precursor protein	Rate cerebellum extracts, H9 and HeLA cell lines, embryonal carcinoma F9 cells	1. NFκB binding site present in promoter region 2. EMSA, supershift 3. Reporter assay – effects of p65 and p50 overexpression on promoter activity	Grilli et al., 1995
Tissue-type plasminogen activator (t-PA)	Neuroblastoma and glioblastoma cell lines	1. NFκ B binding site present in promoter region 2. EMSA, supershift 3. Reporter assay – test wt and NFκB site deletion in promoter	Lux et al., 2005
Myelin basic protein	Human oligodendroglioma cell line	1. NFκB binding site present in promoter region 2. EMSA, supershift (with HeLa cell extracts) 3. Reporter assay – test wt and mutated NFκB site in promoter	Huang et al., 2002
	DRG neuron and Schwann cell co-cultures	1. DNIκB expression decreases protein levels by immunoblot 2. Blocking NFκB activity or using murine cells lacking p65 attenuates myelination	Nickols et al., 2003
SNAP-25A	Rat brain	κB decoy DNA downregulates mRNA levels by microarray analysis	Qiu et al., 2004

[a]A precedence for direct binding interactions and cooperative transcriptional activation between NFκB and SP1 factors bound to closely adjacent sites (within 10 bp) exists at the HIV and IL-2R promoters (Pomerantz et al., 1989; Perkins et al., 1993).
Abbreviations: AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; BDNF, brain-derived neurotrophic factor; Cox-2, cyclooxygenase-2; DRG, dorsal root ganglion; EMSA, electrophoretic mobility shift assay; IHC, immunohistochemistry; iNOS, induced nitric oxide synthase; nNOS, neuronal NOS; RPA, ribonuclease protection assay; RT-PCR, real-time polymerase chain reaction.

regulated within neurons, but have not yet been verified. In addition, several new targets of NFκB have been identified by studies within the CNS. These include the catalytic alpha subunit of cyclic AMP-dependent protein kinase A (PKA_{CA}). A promoter analysis indicates that PKA_{CA} could be a direct transcriptional target of NFκB, and the physiological relevance of regulation by NFκB is attested to by a decrease in forskolin-activated PKA_{CA}-dependent CREB phosphorylation observed in mice expressing the DNIκB inhibitor of NFκB (Kaltschmidt et al., 2006). Interestingly, the regulation of PKA_{CA} expression could also have important feedback implications for NFκB activity. PKA_{CA}-dependent phosphorylation of the p65 subunit strongly regulates the transactivation potential of NFκB by promoting its association with the CREB binding protein (CBP)/p300 (Zhong et al., 1997, 1998, 2002). Another potential target of NFκB is the enzyme glutamate decarboxylase (GAD65) that is involved in synthesis of the inhibitory neurotransmitter GABA. Both protein and mRNA for GAD65 were reported to be decreased in hippocampal lysates from mice expressing the DNIκB (O'Mahony et al., 2006). At present, it remains unknown whether transcriptional regulation of GAD65 expression is mediated directly by NFκB or is an indirect outcome of NFκB inhibition. The GAD65 promoter is TATA-less and contains multiple transcription initiation sites and transcription factor binding sites, but a putative NFκB consensus sequence has not yet been reported (Pinal et al., 1997)

Many additional genes which are reported to be regulated by NFκB appear likely to have significance in mechanisms of plasticity (see **Table 2**). Glutamate-responsive receptors and channels are known to contribute to multiple forms of plasticity, including LTP and LTD, and homeostatic and distance-dependent synaptic scaling. Several glutamate receptor subtypes are reported to be targets of NFκB. The regulatory regions of the gene encoding the GluR1 subunit of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-responsive glutamate receptors contains a consensus binding site for NFκB and GluR1 protein levels can be upregulated by stimuli that activate NFκB. In addition, protein levels of GluR1 are substantially reduced in the cortex and hippocampus of mice lacking the p50 subunit of NFκB (Borges and Dingledine, 2001; Yu et al., 2002). While comparisons of gene expression in wild-type mice versus mice lacking the p50 subunit of NFκB have been very useful in demonstrating NFκB regulation of genes, it should be kept in mind that these experiments do not define which NFκB dimers (e.g., p50:p50 vs. p50:p65) are most critical for the endogenous regulation of the gene. Brain-derived neurotrophic factor (BDNF) has been implicated in many processes involving plasticity both in the mature nervous system and during CNS development. The expression of this growth factor is regulated by several activity-dependent transcription factors, including CREB and NFκB. Interestingly, BDNF is not only transcriptionally upregulated by NFκB, but also serves as an activator of NFκB (**Tables 1 and 2**). This type of feedforward loop is a recurring theme in NFκB regulation, with a classic example being the transcriptional induction of TNFα and TNFβ by NFκB and activation of NFκB through the TNF receptor. An additional example with relevance for the CNS is the transcriptional regulation of nitric oxide synthetase (NOS) by NFκB and the activation of NFκB by nitric oxide (**Tables 1 and 2**).

4.28.8 Neurological Disorders with Cognitive Deficits

Dysregulation of the NFκB activation pathway has been implicated in a number of CNS disorders that exhibit cognitive impairment (for a recent review see Mattson and Meffert, 2006). These include Alzheimer's disease, Huntington's disease, and Parkinson's disease, as well as depression and age-related cognitive decline. Whether the dysregulation of NFκB is a causative agent in the cognitive deficits associated with these diseases, or simply a by-product of activating neuronal cell survival pathways, is not yet understood. In some cases, as with the mutant huntingtin protein (Khoshnan et al., 2004), activated NFκB is reported to contribute to the neurotoxicity, whereas in many other examples NFκB activation appears to preserve neuronal function.

Increases in basal NFκB activation associated with aging have been observed in multiple regions of the CNS (Korhonen et al., 1997). Age-associated increases in NFκB activation have also been observed outside the CNS, including in liver and skin, where they have been linked with accelerated inflammatory processes. An exacerbation of age-related neural degeneration has also been reported in the cortex, hippocampus, and caudate-putamen of mice deficient in the p50 subunit of NFκB (Lu et al., 2006). Mice deficient in p50 also exhibit an acceleration of both age-related and noise-induced hearing loss. The hearing loss occurs concomitantly with

enhanced degeneration of the auditory nerve spiral ganglion neurons (SGNs) in p50 knockout mice compared to wild-type controls (Lang et al., 2006).

It is plausible that a role for NFκB in cell growth and plasticity could imply a complementary function in cell survival and the maintenance of cognitive function. A potential role for NFκB in disorders of cognitive function in which neurodegeneration is not the most prominent component is also emerging. A serial analysis of frontal cortex gene expression in patients with bipolar disorder has revealed that the expression of NFκB pathway components are elevated compared to an unaffected control population (Sun et al., 2001). A link between NFκB signaling pathways and Down syndrome (trisomy 21) has also been established by a group studying the human Down syndrome critical region 1 gene (DSCR1) (Kim et al., 2006). Overexpression of the DSCR1.4 isoform was found to increase basal levels of the IκBα inhibitor of NFκB, likely by diminishing the steady-state degradation of the inhibitor. This resulted in an attenuation of NFκB activation and a downregulation of the pro-inflammatory NFκB target genes, cyclooxygenase-2 and interleukin1β in a glioblastoma cell line. Interestingly, increased mRNA levels of DSCR1 are reported not only in patients with trisomy 21, but also in postmortem brain samples from Alzheimer's patients (Fuentes et al., 2000; Ermak et al., 2001) and in the penumbra area of mouse brain after focal cerebral ischemia (Kim et al., 2006).

4.28.9 Conclusions

The regulation of gene expression by the NFκB family of transcription factors is emerging as a widespread mechanism involved in shaping cognitive processes such as learning, memory, and reconsolidation within the CNS of both vertebrates and invertebrates. A definitive role for NFκB in *in vitro* assays of plasticity, primarily within the hippocampus, has also been established. Whether NFκB might participate in additional aspects of cognition such as awareness, judgment, and problem solving remains to be defined. The molecular mechanisms by which NFκB modulates plasticity is an exciting area that remains to be explored. However, as the list of NFκB-regulated genes within the CNS continues to grow, some attractive potential targets have already been described (**Table 2**). Because NFκB is ubiquitously expressed within the CNS, experiments directed at cell type–specific functions of NFκB should also be illuminating. Future research toward understanding the transcriptional requirements essential to cognitive processes will undoubtedly necessitate investigating the ways in which activity-regulated transcription factors such as NFκB, CREB, immediate early genes, and other transcriptional regulators function independently and in cooperation.

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4.29 Dendritic Transport of mRNA, the IEG Arc, and Synaptic Modifications Involved in Memory Consolidation

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4.29.1 Introduction

It is widely believed that persistent modifications of synaptic properties induced by activity underlie long-term memory storage in the brain (Kandel, 2001; Dudai, 2002; Martin and Morris, 2002). Understanding the mechanisms underlying such synaptic modifications at a molecular level is a prominent goal of modern neuroscience. Two assumptions guide current thinking regarding memory storage

mechanisms. First, synaptic modifications that are triggered by activity and underlie memory storage are thought to occur in a synapse-specific fashion based on the history of activity in that particular synapse. Thus, to understand synaptic plasticity, one must understand how synaptic weights can be stably modified independent of the state of neighboring synapses. Although synapse specificity is generally postulated, there is an alternative view that synapses are not independently modified during the storage of information in the brain, but instead, clusters of co-active synapses are jointly regulated, independent of the history of activity of individual synapses (Govindarajan et al., 2006). In either case, modifications occur selectively at sets of synapses that are in close proximity. Second, enduring synaptic modifications, including the ones that underlie long-term memory storage, are thought to require gene transcription and mRNA translation (Kandel, 2001). These assumptions motivate searches for mechanisms that could allow activity-dependent alterations of individual synapses or small clusters of synapses in a way that involves gene transcription and mRNA translation. Here, we review one potential mechanism, which involves activity-induced expression of the immediate early gene (IEG) Arc, delivery of Arc mRNA to dendritic domains contacted by synapses that have experienced particular patterns of activation, and local synthesis of Arc protein. This mechanism is of particular interest because recent studies have shown that Arc protein is involved in an important aspect of late-phase synaptic plasticity that appears to be required for at least some forms of memory consolidation.

We begin with a brief overview of mRNA localization and local translation in dendrites. These topics have been considered in other recent reviews (Steward and Schuman, 2003; Pfeiffer and Huber, 2006; Hirokawa, 2006; Schuman et al., 2006) and so we will focus here on core concepts that set the stage for a detailed consideration of Arc. We will then discuss how and when Arc is expressed in response to synaptic activity and behavior, and how Arc mRNA is transported into dendrites and targeted to active synapses, and then consider the functions of Arc protein. Throughout, we will point out unresolved issues or assumptions for which definitive evidence is lacking. Key questions and future directions in Arc research will be discussed, with special emphasis on the consequences of an mRNA targeting mechanism for inducing, maintaining, and modulating synaptic plasticity. Finally, we will outline a hypothesis about how Arc expression may play a role in memory consolidation.

4.29.1.1 Activity-Dependent Synaptic Plasticity, Receptor Alterations, and the Process of Consolidation

Mechanisms of synaptic plasticity have been extensively studied and are discussed in other chapters of this volume. Here, we will mention only the core concepts that are of particular relevance for our consideration of dendritic protein synthesis and Arc.

There is an emerging consensus that changes in synaptic efficacy at excitatory glutamatergic synapses in the brain occur initially as a result of changes in the number and characteristics of glutamate receptor subtypes, especially changes in the number and subunit composition of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors (Malenka and Bear, 2004). For example, increasing evidence suggests that induction of long-term potentiation (LTP) is accompanied by, and presumably mediated by, insertion of additional AMPA receptors into the postsynaptic membrane, which increases postsynaptic currents and postsynaptic signaling responses within minutes after stimulation. Similar changes occur within neurons of the amygdala after fear conditioning (Rumpel et al., 2005).

Similarly, induction of certain forms of long-term depression (LTD) is accompanied by, and presumably mediated by, removal of AMPA receptors from the postsynaptic membrane by endocytosis, which leads to decrements in synaptic efficacy (for a review, see Malenka and Bear, 2004). Importantly, if synapses are modified on an individual basis, then experience-induced insertion and removal of AMPA receptors must occur selectively at synapses that are interspersed with others that are unmodified. Indeed, this can be shown for neurons in the lateral amygdala that receive projections from the auditory thalamus. Using viral delivery of glutamate receptor 1 (GluR1) subunits with altered rectification, it was shown that after auditory fear conditioning only some synapses on a given neuron showed GluR1 insertion (Rumpel et al., 2005).

Key questions remain, however, about the mechanisms that allow changes in synaptic weights to either persist or be converted into more stable forms. Several models have been proposed (Dudai, 2002; Malenka and Bear, 2004). One model incorporates the apparent constitutive recycling of heteromeric GluR2-GluR3 AMPA receptors in the postsynaptic membrane and

postulates that this receptor population incorporates and replaces the newly inserted heteromeric GluR1-GluR2 receptors that were inserted in association with a potentiation event (Shi et al., 2001). In this way, synaptic homeostasis is re-established at a new, enhanced level of AMPA receptors in the postsynaptic membrane. This mechanism would require some gauge that independently records or represents the number of AMPA receptors present in the postsynaptic membrane at a given time (that is, some way to determine the homeostatic set point). One possibility is that a component of the postsynaptic density identifies slots into which AMPA receptors can be reinserted after recycling (Schnell et al., 2002).

Another model is based on the fact that particular patterns of activity trigger changes in synapse shape; the idea is that changes in shape are propagated stably to perpetuate alterations in synaptic weighting. The postsynaptic membrane specializations of excitatory glutamatergic synapses are typically found on the tips of dendritic spines. Spines possess high levels of actin but not microtubules, and changes in spine shape require the action of the actin cytoskeleton (Matus, 2000). It has long been known that alterations in spine shape accompany alterations in synaptic efficacy (Desmond and Levy, 1983), and new aspects of the story continue to emerge (Engert and Bonhoeffer, 1999; Fischer et al., 2000; Matsuzaki et al., 2004; Nagerl et al., 2004). Increases in postsynaptic density size that accompany the enlargement of spines would provide a scaffold for the insertion of additional AMPA receptors. That is, the initial structural change would drive the functional modification and allow the modification to be maintained. But again, there must be some way to propagate this new homeostatic state, and so both models may be aspects of the same underlying mechanism.

There is one important disconnect, however, in the linkage between changes in receptor number and/or synapse size and shape and persistent changes in synaptic efficacy. The changes in molecular and structural properties as well as changes in synaptic efficacy occur essentially immediately (or at most within a few minutes) after the triggering event. Yet modifications in synaptic efficacy remain labile for hours, and then undergo some transition so that they become more stable. This conclusion is based primarily on the fact that blockade of protein synthesis (and sometimes RNA synthesis) causes both LTP and LTD to degrade over time. We will use the term consolidation to refer to this transition from a labile to a stable state, with the cautionary note that the term was applied originally to memory storage (see, for example, McGaugh and Alpern, 1966), which may involve different mechanisms than are involved in the consolidation of synaptic plasticity. The investigations that point to the requirement for protein synthesis form a separate line of inquiry into the basis for persistent changes in synaptic efficacy, and it remains to be established how protein synthesis-dependent processes relate to the early changes in receptor number and synapse size and shape that mediate the essentially immediate changes in synaptic efficacy after an inducing event.

It is noteworthy that the early events underlying LTP and LTD and also behavioral memory become stable (that is, undergo consolidation) in a way that is generally seamless in terms of the readout. Once induced, persistent LTP and LTD are reflected by a new level of synaptic strength. In a similar fashion, behavioral memory is generally manifested by stably enhanced behavioral performance. This fact has probably led us to favor models in which the initial changes (increases or decreases in receptor number for example) are somehow cemented during consolidation. It remains possible, however, that the later protein synthesis-dependent events cause different modifications than are responsible for early stage plasticity. As we will see in the next section, new information on the function of Arc protein has raised new possibilities in this regard.

4.29.1.2 Protein Synthesis at Synaptic Sites on Dendrites: A Possible Mechanism for the Selective Modification of Individual Synapses

4.29.1.2.1 Identification of polyribosomes at subsynaptic sites

The discovery that polyribosomes were selectively positioned beneath synaptic sites on dendrites (Steward and Levy, 1982) triggered a new line of thinking about how individual synapses could be modified in a protein synthesis-dependent fashion. This discovery suggested a mechanism whereby synaptic activation could induce the translation of mRNAs within dendrites, and the newly synthesized proteins could then act locally to alter properties of activated synapses. Many lines of evidence converge to support this model and help define its importance and scope in the stable modification of synaptic properties during synaptic plasticity and learning and memory. The discovery of Arc opened a new dimension in thinking because Arc mRNA was

induced by activity and then delivered into dendrites. Thus, synaptic activity could lead to the positioning of Arc mRNA near activated synapses and newly translated Arc protein could act locally to alter the properties of activated synapses. Much remains to be established about how this is coordinated. In considering what is known and what remains to be established, it is useful to begin by considering the nature of the protein synthetic machinery at synapses and the mRNAs that are potentially present.

4.29.1.2.2 Components for protein synthesis in dendrites, including mRNAs

Since the discovery of synapse-associated polyribosome complexes (SPRCs), significant effort has been directed toward demonstrating that components needed for local translation exist in dendrites and that dendrites are indeed capable of synthesizing proteins independently of the cell body (Steward and Schuman, 2001). Other components of the translation machinery (initiation and elongation factors) have also been shown to be present in dendrites, as have components of the endoplasmic reticulum and Golgi complex, which would allow posttranslational modifications of integral membrane proteins and proteins destined for release (for a review, see Steward and Schuman, 2001). It is interesting that recent studies have further confirmed the presence of Golgi outposts in dendrites, but also showed that the Golgi vesicles were mobile (Horton and Ehlers, 2003).

Some of the mRNAs that are present in dendrites of neurons in vivo have been identified (for a more comprehensive discussion of the localization and distribution of mRNAs other than Arc, see Steward, in press). Transcripts that are easily detectable by *in situ* hybridization in dendrites of neurons *in vivo* comprise a functionally diverse set of proteins, including protein components of the postsynaptic signaling complex such as Ca^{2+}/calmodulin-dependent protein kinase IIα (CaMKIIα) (first reported by Burgin et al., 1990), Shank (Bockers et al., 2004), and Arc. The mRNA for microtubule-associated protein-2 (MAP2) is present in the proximal third to half of dendrites of many neurons (first reported by Garner et al., 1988). The mRNA for the elongation factor EF1a is present in many dendrites (Huang et al. 2005), as is the mRNA for dendrin, a protein of unknown function (Herb et al., 1997). The mRNAs for some glutamate receptor subunits, including the *N*-methyl-D-aspartate (NMDA) receptor subunit NR1 (Benson et al., 1991) and the AMPA receptor subunit GluR2 (Grooms et al., 2006), appear to be present at lower levels. A noncoding pol-III transcript called BC1 is also present in many dendrites (Tiedge et al., 1991). A different subset of mRNAs are present constitutively in the dendrites of Purkinje cells of the cerebellum, including the mRNAs for L7 (Bian et al., 1996) and the inositol triphosphate (IP3) receptor (Furuichi et al., 1993). Finally, in addition to Arc, brain-derived neurotrophic factor (BDNF) mRNA is delivered into dendrites following seizures that lead to epileptic circuits (Tongiorgi et al., 2004). Amplification of mRNAs harvested from dendrites of young neurons growing in culture have identified other potential dendritic mRNAs (Miyashiro et al., 1994; Moccia et al., 2003), but definitive evidence for dendritic localization in the CNS by *in situ* hybridization is lacking for most of these. It is not known how the translation of the different mRNAs is regulated. This is likely to be crucial, because there are only a small number of ribosomes at individual synaptic sites in mature neurons and this is likely to be rate limiting for translation (for a consideration of this issue, see Schuman et al., 2006).

Importantly, no mRNAs other than Arc have been shown to rapidly redistribute in response to synaptic activation. Only a few have been tested, however (the mRNAs for CaMKII, MAP2, dendrin, and EF1α; Steward et al., 1998; Huang et al., 2005) and so it remains to be seen whether Arc is entirely unique in terms of its relocalization in response to synaptic activation.

4.29.1.3 mRNA Translation in Dendrites

Several studies have demonstrated that dendrites are capable of synthesizing proteins independently of the cell body, and in response to treatments that induce changes in synaptic efficacy (Kang and Schuman, 1996; Huber et al., 2000; Aakalu et al., 2001). In these experiments, dendrites of either single neurons in culture or neurons in brain slice preparations were mechanically separated from the cell body and new proteins were shown to arise from the isolated dendritic segments. In addition, very slowly diffusing fluorescent protein reporters have been used to demonstrate protein synthesis in the dendritic compartment of neurons in culture (Aakalu et al., 2001), as has a novel bi-arsenic-based fluorescent compound that allows the *in situ* labeling of proteins possessing a particular tetracysteine motif (Ju et al., 2004). Although important for demonstrating protein synthetic capacity within dendrites, studies of translation of exogenous mRNAs do not necessarily recapitulate mechanisms of translational regulation of native mRNAs *in vivo*.

There have also been a number of studies of protein synthesis in subcellular fractions containing pinched-off dendrites called either synaptodendrosomes or synaptoneurosomes depending on the method of isolation. Synaptodendrosomes are isolated by differential centrifugation in sucrose gradients (Rao and Steward, 1991), whereas synaptoneurosomes are prepared by filtration (Weiler and Greenough, 1991). Both types of fractions contain pinched-off nerve terminals that are connected to small pinched-off dendritic fragments and/or spines, which appear as snowman figures at the electron microscopic level. The fractions are similar, but can be contaminated by resealed fragments of neuronal and glial cell bodies (Rao and Steward, 1993). Nevertheless, when proper care is taken to ensure preparations are not contaminated (Bagni et al., 2000), the preparations can provide valuable information about the mRNAs that are present in dendrites (Rao and Steward, 1993) and the proteins that are locally synthesized (Rao and Steward, 1991).

Studies involving synaptodendrosomes and synaptoneurosomes have been especially useful for assessing how dendritic protein synthesis is influenced by depolarization (high potassium, for example) and bath application of neurotransmitters and growth factors (Weiler and Greenough, 1991, 1993). Measures include biochemical studies of metabolically labeled proteins (Villanueva and Steward, 2001a,b; Leski and Steward, 1996) and the degree of ribosome loading onto particular mRNAs (Zalfa et al., 2003).

An important caveat is that bath application of neurotransmitters may activate both synaptic and nonsynaptic receptors, whereas physiological release activates primarily the receptors present at synapses. This is important because recent studies have shown that activation of synaptic versus nonsynaptic receptors triggers quite different molecular cascades in the postsynaptic cell (Zhang et al., 2007).

4.29.1.3.1 Proteins associated with mRNA in dendrites

Both the delivery of mRNA into dendrites and the translation of mRNAs within dendrites are thought to be regulated by mRNA-binding proteins. The prevailing view is that mRNAs possess discrete sequences, usually in the 3′ untranslated region (3′UTR), that mediate dendritic transport (called dendritic transport sequences). Some RNA-binding proteins have been identified that bind to the dendritic transport sequences with some degree of selectivity (for a recent review, see Wells, 2006). This review focuses on the possible role of mRNA-binding proteins in translational repression, but some of these mRNA-binding proteins may play a role in dendritic transport. To our knowledge, however, there has been no compelling evidence that any particular mRNA-binding protein plays an obligatory role in the dendritic transport of any mRNA.

Clearly, a number of proteins associate with mRNAs during translation, including various initiation and elongation factors, and there is evidence for a role of these proteins in synaptic plasticity (Klann and Dever, 2004). Other mRNA-associated proteins may act as regulators of translation, for example cytoplasmic polyadenylation element binding factor (CPEB) (Wu et al., 1998) and fragile X mental retardation protein (FMRP) (Zalfa et al., 2003). A thorough consideration of how mRNA-binding proteins regulate dendritically localized mRNAs is beyond the scope of the present review, but it is noteworthy that several mRNA-binding proteins have been implicated in synaptic plasticity, especially FMRP (Valentine et al., 2000) and CPEB (Wells et al., 2001).

4.29.1.3.2 Evidence that local translation at synapses is important for synaptic function

One of the mRNAs that is abundant in dendrites is the mRNA for the alpha subunit of CaMKII, and several lines of evidence strongly suggest that CaMKIIα protein is actually synthesized from dendritically localized mRNA (Ouyang et al., 1999). But is local synthesis critical? In order to assess the function of dendritically synthesized CaMKIIα protein, Miller et al. (2002) created transgenic mice in which the dendritic targeting of CaMKIIα mRNA was abrogated by replacing the 3′UTR, which contains the dendritic targeting sequence, so that a fusion transcript would be produced that contains the 3′UTR of bovine growth hormone and the coding region and 5′UTR of CaMKIIα. As expected, the transcript encoded by the transgene remained in the cell body rather than being delivered into dendrites. While total levels of CaMKIIα protein were somewhat reduced in these mice, levels of CaMKIIα protein in the postsynaptic density (PSD) were reduced by about half. Tests of LTP revealed subtle but definite deficits in the late phase of LTP in hippocampal slices, and subtle deficits in spatial learning, associative fear conditioning, and object recognition learning. One caveat of this experiment, however, is that the decrease in steady-state levels of CaMKIIα at the synapse might

alter signal transduction competence (Steward, 2002). Thus, it remains unclear whether it is the absence of local synthesis of CaMKIIα or a change in CaMKIIα-mediated signal transduction at synapses that accounts for the deficits in LTP and memory.

4.29.1.3.3 Evidence that protein synthesis is required for late-phase long-term potentiation, long-term depression, and memory

One of the strengths of the local protein synthesis model has been the concordance of the many studies demonstrating requirements for dendritic protein synthesis in both long-term memory and synaptic plasticity (Steward and Schuman, 2003; Pfeiffer and Huber, 2006; Schuman et al., 2006). A thorough review of this extensive literature is beyond the scope of the present chapter, but there is general consensus that both memory and many forms of synaptic plasticity have a short-lived, protein synthesis-independent phase, and a longer phase that can be blocked by inhibiting protein synthesis (for a review, see Kandel, 2001).

One weak link in the logic chain, however, is that to block the late expression of synaptic plasticity or memory, protein synthesis inhibitors have to be present at the time of initial stimulation or very soon thereafter (Frey et al., 1988). Inhibition protein synthesis later on is without effect, even though it is during the later period that LTP and LTD decay in the presence of protein synthesis inhibitors. One possible interpretation of these findings is that the critical protein synthesis occurs at the time of stimulation, but that the newly synthesized proteins do not actually act until sometime after the triggering event. In this model, the essential products would act to maintain changes in synaptic efficacy and consolidate long-term memories, but would not play a role in the initial changes in synaptic efficacy, even though the proteins are synthesized near the time of stimulation.

Other recent experiments suggest that the persistence of LTP after treatment with protein synthesis inhibitors depends on the exact testing protocol (Fonseca et al., 2006). In this study, protein synthesis inhibitors were applied to acute brain slice preparations during tetanic stimulation of Schaffer collateral-cornu ammonis area 1 (CA1) synapses. The degree of blockade of LTP caused by these inhibitors was found to be dependent upon the frequency of test stimulation delivered subsequent to tetanization. Thus, the resulting level of stable potentiation depends upon events (test stimulation) that occur soon after tetanization (less than 1 h). These findings reveal that protein synthesis normally acts around the time of tetanization to prevent the decay of potentiation in the face of subsequent stimulation.

4.29.1.4 Are the Proteins Synthesized by Synapse-Associated Polyribosomes Targeted Selectively to an Individual Synapse or Are They Distributed More Widely?

The selective positioning of SPRCs beneath synaptic sites invites the speculation that locally synthesized proteins could be targeted selectively to an individual synapse (Steward and Levy, 1982). There is no compelling evidence one way or another about this hypothesis, however. Indeed, if all synapses are equally modifiable, then either protein synthetic machinery (polyribosomes) would have to move from synapse to synapse or the protein that is synthesized would have to be available to more than one synapse (see later discussion in the section 'Arc and synaptic homeostasis,' as well as Schuman et al., 2006).

There is evidence for a phenomenon called synaptic tagging, the properties of which suggest the possibility that patterns of activity at one synapse can create some sort of tag that enables the synapse to capture molecules that are produced as a result of activity of other, perhaps distant synapses (Frey and Morris, 1997; Sajikumar and Frey, 2004; reviewed in Martin and Kosik, 2002; Fonseca et al., 2004). In one approach, it was shown that weak patterns of synaptic stimulation that do not produce long-term enhancement of synaptic transmission can cause long-term enhancement when paired with strong stimulation of other synapses. The strong stimulation can either precede or follow the weak stimulation. The interpretation put forward for these results is that strong stimulation causes the production of plasticity factors, which are necessary and sufficient for converting transient changes in synaptic efficacy into stable ones. The idea is that these plasticity factors are distributed in the general vicinity of strongly stimulated synapses, and that synapses that have experienced a particular pattern of activity capture the plasticity factors through the use of a tag which is envisioned to act as a docking signal set up only at active synapses.

An interesting twist is that weak stimulation that would typically lead to transient depotentiation of

synaptic response can indeed cause stable depotentiation when paired with not only another strongly depressing stimulation pattern, but also a strongly enhancing stimulation pattern (Sajikumar and Frey, 2004). In other words, when they act, factors produced in response to strong stimulation preserve the direction of synaptic change at separate synaptic inputs at which a tag has been generated by weak activation. Thus, by implication, the sets of plasticity factors induced by LTP and LTD are the same, and this common pool contains factors that persistently enhance and persistently decrement synaptic efficacy.

In its purest form, the synaptic tagging hypothesis is agnostic as to where and how the putative plasticity factors are produced, or even what the factors are. The critical idea is that the factors are not tightly restricted to the synapses whose activity triggered the production of the factors; instead, the factors are available to other synapses and captured as a result of an activity-dependent tag. The conversion of short to long LTP at weakly activated inputs is blocked by protein synthesis inhibitors, however, suggesting that either the tag or the plasticity factors are newly synthesized proteins. If the factors are newly synthesized proteins, they could be synthesized either in the cell body or in dendrites. If the factors are the products of dendritic protein synthesis, then this would imply that proteins synthesized at one synapse would be available to other synapses. If so, then synapses within a particular dendritic domain may compete for locally synthesized proteins. At least some of the proponents of the synaptic tagging hypothesis consider it as an alternative hypothesis to the idea that critical molecules are synthesized locally at synapses. Also, it has been shown that antidromic stimulation is effective at rescuing LTP at weakly activated synapses on CA1 pyramidal cells in a manner similar to what occurs with strong synaptic activation (Dudek and Fields, 2002). This finding does not exclude the possibility that the critical factors are produced in dendrites (there could be back-propagating dendritic spikes, for example). In any case, there is not yet definitive evidence on the molecular nature of either the plasticity factors or the tag, and so the site of production of the factors remains open to debate.

There is in fact evidence for some form of competition for newly synthesized proteins during LTP induction that has come from experiments assessing synaptic plasticity after varying periods of protein synthesis inhibition (Fonseca et al., 2004). In these experiments, two independent pathways in the CA1 region of the hippocampus were tested. One was weakly tetanized and then reactivated, and the other was a strongly activated test pathway. As in the synaptic tagging experiments detailed previously, simultaneous delivery of the strong and weak stimulation resulted in LTP at both the reactivated pathway and test pathway. Subsequent weak stimulation of the reactivated pathway led to further potentiation of this pathway. However, when the preparation was treated with anisomycin while the reactivated pathway was stimulated again, the reactivated pathway was still potentiated but the test pathway was simultaneously decremented. The interpretation put forward for this result is that there is a limited supply of plasticity factor(s) that act, and redistribute, over the course of several hours, and that synapses compete for the factor(s).

4.29.2 Arc: A Rosetta Stone for Understanding Mechanisms of Synaptic Plasticity

With this general background, we can now consider how Arc fits into the story. Arc (also known as Arg 3.1) was initially discovered in two independent screens for IEGs, defined as genes whose transcription was induced by neural activity in a protein synthesis-independent manner (Link et al., 1995; Lyford et al., 1995). In both these experiments, electroconvulsive seizures were induced in animals that had been pretreated with protein synthesis inhibitors. Then mRNAs whose transcription was upregulated were identified through differential hybridization of mRNA from seizure-treated and control animals. A number of novel IEGs involved in synaptic structure and function were identified, including Arc, Homer, and NARP; these have been called effector IEGs because they have direct functions in contrast to IEG transcription factors such as c-fos and zif268 that induce transcription of other genes. Arc was unique, however, because its mRNA was rapidly delivered into dendrites, whereas the mRNAs for all other IEGs remained tightly localized in the neuronal cell body. Lyford et al. (1995) coined the name activity-regulated cytoskeleton-associated protein (Arc) based on biochemical studies that revealed that Arc protein remained in detergent-insoluble extracts in a manner similar to cytoskeletal proteins. Link et al. (1995) called the same gene activity-regulated gene 3.1 (Arg 3.1).

In addition to identifying Arc, these two studies provided the first evidence that Arc mRNA was delivered into dendrites, where it might be locally translated. A reasonable speculation is that translation occurred at synapse-associated polyribosome complexes, but this has not been shown definitively. Subsequent studies revealed that the Arc gene has an additional layer of regulation that at least so far has not been shown for other dendritically localized mRNAs in that Arc mRNA localizes selectively in dendritic domains contacted by recently activated synapses (Steward et al., 1998). The presence of at least two and possibly three independent mechanisms controlling the spatial domain of Arc protein expression (delivery into dendrites, localization at or near active synapses, and local translation) provides a compelling mechanism whereby Arc protein could be synthesized in local domains very near individual synapses that have recently experienced particular patterns of activation and ultimately targeted selectively to the active synapses. As will be discussed further, different parts of the mechanism have been demonstrated, but there is not yet direct evidence for selective targeting of either Arc mRNA or Arc protein to individual synapses or local regulation of Arc mRNA translation.

4.29.2.1 Activation of Arc Transcription by Synaptic Activity and Behavior

In the initial study by Lyford et al. (1995), it was shown that Arc transcription was strongly induced by strong synaptic activation (for example, the induction of LTP) and that levels of expression in the cerebral cortex are influenced by sensory experience (closure of one eye results in downregulation of Arc mRNA in the visual cortex). Subsequent studies have built upon these initial findings to show that Arc transcription is strongly induced in a number of behavioral settings, including ones that involve learning. Indeed, Arc mRNA and protein have been used to identify brain regions and neuron types that are activated during attentive brain states or experiences associated with learning. These experiments have informed us about where, when, and how Arc transcription in the brain links with experience of the external world.

A thorough review of these studies is beyond the scope of the present work, but selective consideration allows us to highlight general conclusions and unresolved issues. Studies of behavioral induction of Arc transcription also provide clues about what Arc does in the cells that express it and why and how Arc expression is induced. In the next two sections, we first consider what behavioral and neurophysiological induction tells us about the function of Arc and second, how patterns of induction of Arc expression inform us about neuronal subsets used to encode, and perhaps store, aspects of sensory–behavioral experience.

Initial experiments indicated that Arc transcriptional induction was rapid, in the manner of other IEGs. In these original studies, it was shown that a brief seizure or brief high-frequency stimulation of the perforant path *in vivo* led to Arc mRNA accumulation in the nucleus and cell body within 30 min (Link et al., 1995; Lyford et al., 1995). Subsequent studies using high-resolution fluorescent *in situ* hybridization (FISH) revealed that transcriptional induction began rapidly; 5 min after introducing a rat to a novel spatial environment, two discrete foci of Arc mRNA (the sites of Arc transcription) were evident in the nucleus of neurons in the CA1 region of the hippocampus, and newly synthesized Arc mRNA appeared in the cytoplasm within 20 min (Guzowski et al., 2005). The epochs of transcription induced by experience were brief, lasting as little as 16 min in CA1 neurons. This is in contrast to what is seen in the dentate gyrus following LTP or single seizures, where high levels of Arc mRNA persist for hours after a single episode of activity (Lyford et al., 1995; Wallace et al. 1998). It remains to be established whether the persistence of Arc mRNA in the dentate gyrus reflects continued transcription or delayed Arc mRNA degradation (the former is more likely).

4.29.2.2 Expression of Arc Following Stimulation Patterns that Lead to Long-Term Potentiation

In the studies that initially identified Arc, it was also shown that Arc transcription is strongly induced in dentate granule cells following induction of perforant path LTP *in vivo*. This has been confirmed in several subsequent reports. Other IEGs are also induced following induction of perforant path LTP (Abraham et al., 1993; Worley et al., 1993), although the threshold for induction for the different IEGs is somewhat different (Worley et al., 1993; see also Waltereit et al., 2001).

Although Arc transcription is typically induced in conjunction with perforant path LTP, the relationship between LTP induction and Arc expression is not completely established. For example, induction

of perforant path LTP in anesthetized rats by delivering thirty 8-pulse trains at 400 Hz reliably induces Arc transcription, whereas LTP can be induced by three 50-pulse trains at 250 Hz without accompanying induction of Arc expression in dentate granule cells (Steward et al., 2007). The LTP that is induced by 250-Hz stimulation persists for up to 5 h, but it is not known whether LTP that is induced in the absence of IEG expression persists for days and even months, as is true of the LTP induced by 400-Hz stimulation (for a review on LTP persistence, see Abraham, 2003).

It should be noted that Arc is induced if 250-Hz trains are delivered repeatedly for more prolonged periods than are necessary to induce perforant path LTP (Steward et al., 2007). It is noteworthy that a different study reported that induction of perforant path LTP with three trains of 50 pulses at 250 Hz led to increases in Arc protein in the stimulated dendritic lamina 2 and 4 h after inducing LTP, as assayed by electromagnetic immunocytochemistry (Rodriguez et al., 2005). There are concerns about these findings, however, because labeling was seen in both dendrites and glial processes, yet there is no evidence that glia express Arc mRNA. In any case, it seems that experimental details may determine whether or not Arc is induced in conjunction with perforant path LTP. Additional studies will be needed to resolve this question.

A relationship between LTP induction and Arc transcription is even less clear in other situations. For example, there have been no reports of induction of Arc transcription following induction of LTP in hippocampal slices. A technical complication may explain the lack of data, however. Arc and other IEGs can be strongly induced during preparation of the slice (Taubenfeld et al., 2002). In this case, high levels of Arc present as a result of preparing the slice might mask subsequent induction by synaptic stimulation. Indeed, one study (French et al., 2001) does report increases in Arc mRNA levels in hippocampal slices in the absence of LTP and no increase in Arc mRNA following LTP induction. Thus, a relationship between hippocampal LTP and Arc transcription remains to be established.

4.29.2.3 Behavioral Activation of Arc Expression

Although evidence linking Arc transcription and different forms of LTP is not compelling or extensive, there is ample evidence that Arc transcription is strongly induced by behavioral experience. Arc is strongly induced in cells throughout the forebrain by simple exposure to a novel environment (Temple and Steward, 2003), and in particular cell populations following training in any of a number of tasks (see following). In specific training paradigms, the neurons that express Arc differ to some extent. In general, however, one is impressed more by the similarities in expression patterns in different settings than the differences.

Can Arc induction serve as a marker of neurons that are engaged during particular behavioral experiences? This general question has been considered previously in terms of other IEGs. A comprehensive review of this area is beyond the scope of the present chapter (but for an example of the approach, see Gall et al., 1998). The benefits of using IEG transcription to monitor neural activation in the brain include: (1) the ability to assay the entire brain in a single experiment, (2) the ability to interrogate essentially every cell in a given section, (3) the presence of a defined and uniform readout of neural activation of an essentially digital nature, and (4) the possibility that IEG expression reflects not merely neural activity but neuronal activation that accompanies long-term changes in neural transmission. Indeed, many studies were probably motivated originally by the hope that Arc induction might mark neurons that were engaged in learning.

The first question is whether Arc expression, or the expression of any other IEG for that matter, reflects overall levels of neuronal activity (number of action potentials per time unit) or some other aspect of neuronal activation, for example, a sufficient level of synaptic activation to trigger a postsynaptic signal transduction cascade. The latter idea is embodied in the concept of a genomic action potential in which particular patterns of activity trigger signal transduction cascades that broadly activate gene expression (Clayton, 2000). The answer to this simple question is not entirely clear. Detailed studies comparing the number of neurons in which Arc transcription is activated versus the number of neurons activated by spatial environments have revealed that similar proportions of neurons in the CA1 region of the hippocampus respond transcriptionally and physiologically to exploration of a novel spatial environment (Guzowski et al., 1999). Indeed, this correspondence extends to cases in which two different environments are presented sequentially. In these experiments, similar fractions of neurons respond to the first environment, the second, or both using

either Arc transcription or place cell firing as a readout. At first pass, this might be interpreted as indicating that transcriptional activation of Arc is related to simple neuronal activity. But there is evidence against this interpretation. For example, repeated exposure to an environment over a single day reduces the percentage of hippocampal neurons in which Arc is induced, whereas the number of cells that respond physiologically is similar (Guzowski et al., 2006). Thus, Arc expression is not simply a readout of neural activity of place cells, implying that the coupling between neural activity and Arc expression is not direct.

So, what types of behavioral experience activate Arc transcription? The simplest experiment is one in which animals are taken out of their home cage and allowed to explore a novel environment (Temple and Steward, 2003). This experience strongly activates Arc transcription in neurons throughout the forebrain. Thus, one interpretation is that Arc is induced as a result of any novel experience.

Although Arc expression is strongly induced in neurons throughout the forebrain by exposure to a novel environment, the relationship between novelty and Arc induction may be complex. Indeed, the threshold for activating Arc appears to vary by task and the number and spacing of repetitions. Repeated exposure to the same environment, when separated by 1 day between exposures, activates the same portion of cells in the hippocampus as a single exposure (Guzowski et al., 2006). Thus, in this case novelty *per se* is not a prerequisite for Arc expression. At the same time, as noted above, repeated exposure to an environment over a single day reduces the percentage of hippocampal neurons in which Arc is induced (Guzowski et al., 2006). There are several interpretations of this finding, including that the animal recognizes that the environment is not novel when repeated exposures occur over the course of a single day, or that there is some refractory period for repeated genomic activation that prevents full transcriptional activation when repeated exposures are closely spaced in time.

Arc transcription is also activated following explicit training in a variety of different learning tasks. Arc is preferentially induced in newly trained verses overtrained animals in both an operant lever-pressing task (Kelly and Deadwyler, 2002) and the Morris water maze (Guzowski et al., 2001). Similarly, Arc is induced after two training sessions in an *in vitro* eyeblink conditioning preparation but not after five sessions (Mokin et al., 2006). These differences in induction may reflect the novelty of the task or the stage of learning (early learning vs. reinforcing a previously learned association).

Although Arc induction is useful for mapping cells activated by various patterns of neural activity, Arc is not unique in this regard. Many studies of this type were undertaken with other IEGs (for reviews, see Herrera and Robertson, 1996; Tischmeyer and Grimm, 1999). Nevertheless, the unique pattern of Arc mRNA localization within dendrites suggests that Arc may be particularly useful for monitoring brain states associated with long-term changes in synaptic properties. Moreover, Arc might be a better choice for monitoring learning-induced changes in the brain than other commonly used IEGs. Comparison of the expression levels between Arc, c-fos, and zif268 after spatial, cued, and reversal variants of the Morris water maze suggests that induction of Arc is more closely associated with learning tasks than induction of the other two IEGs (Guzowski et al., 2001). Also, comparisons of the expression of Arc versus that of other IEGs is useful because comparisons between IEG expression patterns can help define the nature of the genomic response to neural activity. For example, in most, perhaps all situations, there are multiple genomic responses to neural activity, and it remains to be established whether these are independent or form a related series (Clayton, 2000; Guzowski et al., 2001).

The approach of assessing behavioral induction of a single IEG suffers from some drawbacks, including a lack of within-subject comparisons (leading to a lack of precision in comparing control and experimental populations) and an inability to discern the degree of overlap in expression profiles induced by experiences that differ only in a few key details (Guzowski et al., 2005). These problems have been circumvented in part through the use of an *in situ* hybridization protocol that captures expression patterns from two distinct, though recent, time periods in the past. Because this protocol typically uses Arc expression as a readout, precise localization patterns and subtle aspects of neural encoding have been associated with the expression of Arc, but not other IEGs. This technique, termed catFISH (for cellular compartment analysis of temporal activity by FISH), relies upon the brief pulse of Arc transcription induced by discrete behavioral–sensory experiences together with high-resolution *in situ* hybridization to follow the temporal progression of Arc transcript maturation and nucleocytoplasmic transport at the subcellular level in tissue sections (Guzowski et al.,

1999). Newly synthesized Arc mRNA can be detected in the nucleus as paired transcriptional foci. Twenty minutes after induction, the newly synthesized mRNA begins to exit the nucleus to enter the cytoplasm. Labeling of nuclear foci and cytoplasmic structures by Arc mRNA can be easily distinguished by confocal microscopy. Thus, expression within individual cells can be associated with either recent activation (nuclear labeling only), activation at least 20–30 min previously (cytoplasmic labeling), or both.

This technique has not only identified brain regions differentially responsive to closely related behavioral–sensory experiences, but has also been used to extract general principles of neural processing in the active brain regions. Using Arc CatFISH, it was shown that different environments are encoded by overlapping, but not identical, subsets of hippocampal pyramidal neurons in CA1 (Guzowski et al., 1999). Similar studies demonstrate differences between CA3 and CA1 in encoding the spatial environment, with CA1 exhibiting a more graded transition in response to changes in the spatial environment and CA3 exhibiting a more discontinuous response (Vazdarjanova and Guzowski, 2004). CatFISH was also used in the experiments demonstrating that Arc expression can be decoupled in part from neural activity during spatial exploration if the environment is repeatedly presented to the rat on the same day (Guzowski et al., 2006). Arc CatFISH has also been used to reveal the extent to which cortical layers in the prefrontal and gustatory cortex are responsive to spatial context (Burke et al., 2005). In this case, the more superficial layers were preferentially activated during context discrimination.

Arc expression can be used not only to determine where aspects of behavioral tasks might be processed in the brain, but also when. After exploration of a novel environment, Arc protein is reinduced between 8 and 24 h after initial behavioral induction (Ramirez-Amaya et al., 2005). Reinduction was accompanied by close correlation of Arc expression between brain regions, suggesting that network reactivation underlies this late phase of Arc induction and enabling Arc to participate in network-driven memory consolidation processes. Others have identified two phases of Arc induction in mice in response to a one-trial novel gustatory stimulus (Montag-Sallaz et al., 1999) and a one-trial inhibitory avoidance task (Montag-Sallaz and Montag, 2003).

Other IEGs are induced together with Arc, so what do the patterns of co-expression tells us about the existence and nature of the genomic program(s) induced by activity? Early studies demonstrated that all IEGs are not induced to the same extent by neurons activated by LTP-inducing stimulation delivered *in vivo*, with zif268 requiring fewer stimulus trains than junB, c-jun, or c-fos (Worley et al., 1993; Abraham et al., 1993). Similarly, during induction of perforant path LTP, Arc is induced by fewer stimulus trains than c-fos (Waltereit et al., 2001). As mentioned above, Arc expression is dissociable from and may be more closely associated with learning-associated tasks than zif268 or c-fos expression (Guzowski et al., 2001). While expression of zif268, c-fos, and Arc after water maze training are correlated in the entorhinal and visual cortices, and expression of Arc and zif268 are correlated in the hippocampus as well, the degree to which each of these pairs of genes is correlated varies widely (Guzowski et al., 2001). In chick, Arc and zif268 are both induced by auditory imprinting, but in different regions (Bock et al., 2005). Together, these studies indicate that there is not a unitary program induced by activity and suggest a degree of independence and cell type specificity to programs that induce IEGs in the brain. In contrast, Arc is coexpressed with Homer 1a to a very high degree, strongly suggesting that a single genomic program induces both of these genes (Vazdarjanova et al., 2002).

4.29.2.4 Signal Transduction Pathways Responsible for Arc Induction by Synaptic Activation and Behavior

Having summarized the situations in which Arc transcription is induced, it is useful to briefly summarize what is known about the signal transduction cascade(s) that actually trigger the transcriptional activation. We focus here on experiments in intact animals where there is a greater probability that the signal transduction cascades that are identified are actually the ones that are engaged physiologically.

The foundation for the first and most definitive conclusion comes again from the initial study that identified Arc (Lyford et al., 1995), which showed that Arc induction following seizures is blocked by NMDA receptor antagonists (MK801). There is a confounding factor with the approach of delivering antagonists systemically, however, because the antagonist is likely to affect neural activity generally (for example, reducing the duration of a seizure). Thus it is important to explore the question in situations where the pattern of activity used to induce Arc transcription

is closely controlled. A critical role for NMDA receptor activation has been confirmed in subsequent studies involving activation of glutamatergic pathways. For example, activation of Arc transcription in perforant path LTP is completely blocked by NMDA receptor antagonists (Steward and Worley, 2001a). Signal transduction pathways downstream from the NMDA receptor remain to be identified.

It is unclear whether nonglutamatergic neurotransmitter systems activate Arc independently of the NMDA receptor and if so, what signal transduction pathways are involved. A comprehensive review of this question is beyond the scope of the present chapter, but it is clear that the issue may be hard to parse out in the intact brain. To give a single example, local injections of agonists for beta-adrenoreceptors into the amygdala activate Arc expression in the hippocampus (McIntyre et al., 2005). However, this is likely due to muscarinic activation of glutamatergic neurons in the amygdala, which then synaptically activate hippocampal neurons. In this case, Arc transcription could ultimately be activated via NMDA receptors on hippocampal neurons. Elucidating the role of different neurotransmitter and receptor subtypes will likely require studies *in vitro*, but the problem in this case will be translating the conclusions to physiological settings *in vivo*.

4.29.2.5 Dendritic Transport of Arc mRNA

Once induced, Arc mRNA is rapidly delivered into dendrites. Because Arc is expressed as an IEG, it is possible to roughly define the time course of transport of Arc mRNA *in vivo* by killing animals at different times after a stimulus and determining the distribution of newly synthesized mRNA over time. These studies have provided important information about the time course of changes in overall mRNA distribution *in vivo*. Recent studies using live cell imaging *in vitro* have provided more detailed information about the movement of individual packets of mRNA. As described in the next section, delivery is mediated by a dendritic transport mechanism that moves Arc mRNA at a variety of rates, some of which strongly suggest a microtubule-based transport mechanism. Whether the process of dendritic transport is regulated remains to be determined.

4.29.2.5.1 Delivery of Arc mRNA to dendrites in vivo

The original estimates of the rate of movement of Arc mRNA into dendrites were derived by inducing Arc expression with an electroconvulsive seizure (ECS) and assessing the distribution of Arc mRNA over times using *in situ* hybridization (Wallace et al., 1998). These experiments indicated that Arc mRNA moved from the nucleus to the distal tips of dendrites of dentate granule cells (approximately 300 μm) within 60 min, indicating a minimal rate of 300 μm/h. It was recognized that this was an underestimation, however, because of the time required for Arc mRNA to be transcribed and undergo nucleocytoplasmic transport (approximately 20–30 min; see earlier). This paper also revealed that Arc mRNA was delivered into dendrites in the presence of protein synthesis inhibitors, indicating that dendritic delivery did not require sequences within the nascent protein and that dendritic transport was determined by sequences within the Arc mRNA itself. As will be discussed further, signals that mark Arc mRNA for dendritic delivery are localized in the 3′UTR, as is also true for most other mRNAs that are localized in different cell types (for a review, see Steward and Singer, 1997).

4.29.2.5.2 Multiple rates of transport of Arc mRNA-containing granules

We have recently used an exogenous expression approach to directly visualize the transport of transcripts containing the Arc 3′UTR (Dynes and Steward, 2007). In these experiments, the localization of messages from transiently transfected constructs was visualized in living rat cortical neurons by way of coexpressed green fluorescent protein (GFP)-MS2 phage coat protein fusions, which bind to an MS2 binding site inserted into the transcribed sequences containing the Arc 3′UTR. These exogenously expressed transcripts assemble into particles, which we refer to as Arc/MS2 mRNA particles. By following the movement of Arc/MS2 mRNA particle movement in unstimulated cortical neurons *in vitro* using live cell imaging, it was shown that Arc/MS2 mRNA particles are transported bi-directionally at a variety of rates. If Arc mRNA particles were to move continuously at the most rapid rate seen in these experiments (almost 70 μm/min), they could move from the nucleus to a synapse hundreds of micrometers distant within minutes, which is much faster than previous estimates would suggest. This rapid movement could allow transcription and translation-dependent synaptic modifications to occur much faster than previously imagined.

Because of differences in experimental parameters (such as neuron type and maturity, image acquisition rate, incubation temperature), direct comparisons of

data from our study with data from other studies of dendritic mRNA transport are difficult. Moreover, because a continuous range of velocities was seen, measures of central tendency (either mean or median) may not be very meaningful. Nevertheless, some general qualitative comparisons are reasonable. The mean and maximum instantaneous velocities calculated for Arc/MS2 mRNA particles differ from those reported for CaMKIIα mRNA (four- to sixfold slower than Arc; Rook et al., 2000) and the actin mRNA-binding protein ZBP1 (four- to sixfold faster than Arc; Tiruchinapalli et al., 2003). However, transport rates for Arc are not markedly different from those for complexes containing the mRNA binding proteins Staufen (Kohrmann et al., 1999), FMRP (De Diego Otero et al., 2002), CPEB (Huang et al., 2003), and RNA particles of unknown type labeled with syto 14 (Knowles et al., 1996). It is noteworthy that complexes that contain KIF5A and Pur α also move more slowly (maximum of 7.4 μm/min) and for shorter distances (maximum of 8 μm) (Kanai et al., 2004) than the Arc mRNA containing particles. Possibly, the KIF5A motor may be used by Arc mRNA particles for the slow, local phase of movement (see following).

Analysis of Arc/MS2 mRNA particle movement revealed the existence of several phases of movement. Although transport velocities for orthograde and retrograde Arc/MS2 mRNA were similar, orthograde translocations were typically longer than retrograde translocations. This suggests the use of multiple types of motor proteins or multiple modes of motor regulation. Arc/MS2 mRNA particles also moved slowly and progressively on the submicron scale. The fast and slow movements occurred at rates that are consistent with microtubule and actin- or myosin-based transport, respectively, but there was also a continuous distribution of velocities that were intermediate between what is considered typical for microtubule-based and actin- or myosin-based mechanisms.

Our experiments using the MS2 system were done in the absence of stimulation, and so there was no explicit signal to cause Arc mRNA targeting and docking at active synapses (see following). Indeed, at the ages examined (9–16 days of age), synapses are just forming on the dendrites. Nevertheless, some Arc/MS2 particles stopped during the recording period, and the behavior of Arc/MS2 mRNA particles as they stopped was suggestive of some form of local targeting. Nearly half of the particles that stopped during the recording period exhibited stereotyped reversals in direction just before stopping. These movements were nonrandom in direction, slow, and progressive, and thus are difficult to explain as being due to random dissociations from the transport machinery. In addition, none of the particles exhibiting stereotyped reversals before stopping resumed rapid transport during the rest of the imaging session. While this behavior is consistent with some final targeting based on local cues, it may also result from asymmetries in the cytoskeleton in the vicinity of stopping mRNA particles.

It remains to be established whether the principles derived from studies of Arc/MS2 mRNA apply to native Arc mRNA. Using an induction protocol that involves the washout of previously added tetrodotoxin from rat cortical neurons in culture, Rao et al. (2006) reported that newly induced Arc mRNA is localized to granules in dendrites, but it remains to be established whether the granules formed by native Arc mRNA are the same as the ones in which Arc/MS2 localizes. It is important to recall that fusion transcripts expressed by transfected DNAs have not undergone the same nuclear processing as native mRNAs (for example, splicing). If proteins involved in splicing remain with the mRNA and are important for proper localization, then the movement and localization of fusion transcripts may fail to reflect what actually occurs with native mRNAs.

4.29.2.6 Targeting Arc mRNA to Active Synaptic Sites

One of the most striking features about Arc mRNA is that it localizes selectively in dendritic segments contacted by synapses that have been recently activated. So far, no other mRNA has demonstrated a similar response. Localization near recently activated synapses was demonstrated in studies in which high-frequency stimulation was delivered to the perforant path in anesthetized rats (Steward et al., 1998). The perforant path terminates in a topographically organized fashion along the dendrites of dentate granule cells; projections from the medial entorhinal cortex terminate in the middle molecular layer on mid proximal-distal dendrites, whereas projections from the lateral entorhinal cortex terminate in the outer molecular layer on distal dendrites (Steward, 1976). Hence, by stimulating different parts of the entorhinal cortex, it is possible to selectively activate sets of synapses that terminate selectivity on different dendritic segments. This selectivity allows one to define how mRNA becomes localized in response to synaptic activation.

Our studies revealed that delivery of eight pulse trains at 400 Hz at 0.1-s intervals for 1–2 h strongly induced Arc expression and caused the newly synthesized mRNA to localize in the activated dendritic lamina (Steward et al., 1998). Stimulation of the medial perforant path caused Arc mRNA to localize in the middle molecular layer; stimulation of the lateral perforant path caused Arc mRNA to localize in the outer molecular layer.

The minimal stimulation required for inducing localization has not been precisely defined. Simple induction of LTP, which can be achieved by delivering thirty 400-Hz trains at 0.1-s intervals, strongly induces Arc transcription, but when Arc mRNA reaches the dendrites 45 min to 1 h later, there was little evidence for selective localization in the activated lamina (Steward and Worley, 1998). Instead, the mRNA is distributed uniformly throughout dendrites. In contrast, when high-frequency stimulation was delivered at a rate of 0.1 s for 1–2 h, when Arc mRNA is being transported into dendrites, the mRNA localizes selectively in the activated lamina (Steward et al., 1998).

There are two possible explanations for the failure to localize after simple LTP induction: (1) localization may require repeated stimulation over long periods of time and (2) the signals generated by activity may be relatively short-lived. Evidence against the first possibility came from experiments in which *Arc* expression is induced by an ECS; time was allowed for the mRNA to migrate into dendrites, and then the newly synthesized mRNA was targeted to particular dendritic domains by subsequent synaptic activation. Rats received an ECS, and then high-frequency trains were delivered to the perforant path beginning 1.75 h after the ECS. Remarkably, 15 min of synaptic activation was sufficient to cause Arc mRNA to redistribute to the activated dendritic lamina (Steward and Worley, 2001a). Indeed, in this situation, delivering thirty 400-Hz trains was sufficient to generate a detectable band of Arc mRNA in the activated lamina. Hence, a signal sufficient for localization can be generated with short periods of stimulation, although stimulation for longer periods (30 min to 1 h) is required to cause a full redistribution of Arc mRNA to the activated lamina. Because 30 trains is sufficient to cause localization of Arc mRNA that is already present in dendrites, the likely explanation of the failure of Arc mRNA to localize in a simple LTP paradigm is that by the time the Arc mRNA that is induced by the stimulation reaches the dendrite, the signal for localization has waned.

What are the signals that mediate localization of Arc mRNA in the region of activated synapses? Again, the NMDA receptor plays an obligatory role. Using the localization assay described above, in which Arc is first induced by a generalized seizure and then targeted by synaptic stimulation, Steward and Worley (2001a) provided definitive evidence that activation of NMDA receptors was critical. NMDA receptor antagonists completely prevented localization despite continued strong synaptic activation via AMPA receptors. Instead of localizing at active synaptic sites, Arc mRNA remained diffusely distributed throughout the dendrite. The signal transduction pathways that are downstream of the NMDA receptor remain to be defined.

Studies of Arc mRNA localization using light microscopic *in situ* hybridization document that Arc mRNA is targeted to regions of dendrites contacted by active synapses, but it remains to be established whether the mRNA is actually selectively targeted to active synapses or spines. There is evidence that synaptic activation can cause mRNAs and ribosomes to move from the dendritic shaft into spines, which could represent the final stage of the localization process. For example, electron microscopic studies following tetanic stimulation of the Schaffer collateral system in hippocampal slices from 15-day-old rats revealed that an increased percentage of spines contained polyribosomes, and there was a corresponding decrease in polyribosomes in dendritic shafts (Ostroff et al., 2002). The likely explanation is a translocation of polyribosomes from the shaft into the spine proper. In another study, perforant path LTP was induced in unanesthetized adult rats, and subcellular fractionation techniques were then used to isolate synaptoneurosomes which contain pinched-off spines (Havik et al., 2003). Real-time polymerase chain reaction was then used to determine the levels of the mRNA for CaMKIIα in the synaptoneurosomes and also in total homogenates. It was found that levels of CaMKIIα mRNA increased approximately 2.5-fold in the synaptoneurosomes after inducing LTP, whereas there was no change in CaMKIIα mRNA levels in total homogenates. These findings suggest that CaMKII mRNA translocates into spines as a result of stimulation that induces perforant path LTP.

There were also increases in the levels of Arc mRNA in synaptoneurosomes, but these were accompanied by increases in Arc mRNA levels in the total homogenates. Thus, the increases in synaptoneurosomes could reflect the overall increase in Arc mRNA

levels and not necessarily translocation of Arc mRNA into spines. Thus, although these results are provocative, it remains to be established whether Arc mRNA localizes selectively at activated synapses or is instead targeted to the general region of the dendrite contacted by active synapses.

4.29.2.6.1 Arc mRNA-binding proteins and ribonucleoprotein transport complexes

In many cell types, mRNA transport and localization in particular subcellular domains are mediated by mRNA-binding proteins that recognize particular sequences on the mRNA (see, for example, Ainger et al., 1997). Nevertheless, no protein has yet been identified that selectively binds to particular domains in Arc mRNA, although there are few published reports of proteins that associate with Arc mRNA message (Zalfa et al., 2003; Kanai et al., 2004). Perhaps newly synthesized Arc mRNA is bound by precisely placed but sequence nonspecific factors, which are required for subsequent binding of additional proteins that bind selectively. These factors may not be assembled properly *in vitro*. Alternatively, association of Arc mRNA with the critical RNA-binding proteins may require Arc to fold into a tertiary structure that does not form *in vitro*.

Interestingly, Arc mRNA contains introns that are spliced out in the nucleus prior to export. Comparison of the cDNA with the genomic sequence indicates that while the Arc coding region and 5′UTR do not contain introns, the 3′UTR possesses two introns whose size and relative position are conserved between humans and rodents (NCBI Unigene; Rao et al., 2006). This is important because recent evidence suggests that components of the exon junction complex (EJC) might remain with spliced mRNAs and play a role in localization. For example, components of the EJC are essential for anterior localization of oscar mRNA during *Drosophila* oogenesis (Hachet and Ephrussi, 2004). Little is known of the early events of Arc mRNA maturation, except that they are completed within 20 min after being induced, by which time the mRNA has begun to leave the nucleus (Guzowski et al., 1999). High-resolution *in situ* hybridization reveals that intronic sequences are only found in the nucleus of hippocampal neurons after behavioral induction and thus must be spliced out of the mature mRNA (Guzowski et al., 2006).

It remains to be established whether components of the EJC remain associated with Arc mRNA. Because components of the EJC participate in nonsense-mediated decay of mRNA when present downstream of the stop codon, these proteins may contribute to the short half-life of Arc mRNA.

FMRP has also been reported to associate with Arc mRNA with the noncoding pol-III transcript BC1 RNA serving as a linker (Zalfa et al., 2003). FMRP also associates with a large number of other mRNAs that are not transported into dendrites, and so the significance of this association in the case of Arc is not clear.

Arc mRNA has also been shown to coprecipitate with huge, 1000S ribonucleoprotein complexes that contain the kinesin KIF5 (Kenai et al., 2004; Hirokawa, 2006). Forty-one other proteins that cosediment in these large complexes were identified, including Pur alpha and beta, Staufen, FMRPs, EF1 alpha, and other RNA-associated proteins. As noted earlier, Kanai et al. (2005) further showed that granules labeled with GFP fused to Pur alpha, which is tightly associated with KIF5, exhibited slow (maximum of 7.4 μm/min), short-distance (maximum of 8 μm) movements in dendrites of hippocampal neurons in culture. This behavior is unlike the movement of Arc/MS2 particles that move at rates approaching 70 μm/min over distances of at least tens of micrometers (Dynes and Steward, 2007). Many technical factors could account for these differences, and additional work will be required to identify and characterize the primary transport structures for Arc mRNA, especially *in vivo*.

It is important to recall that mRNA may be present in different types of structures at different times. For example, there are large numbers of Arc/MS2 granules that show no movement during recording sessions, which could represent a storage depot for Arc mRNA. Also, it remains to be established how Arc mRNA (or any other dendritic mRNA, for that matter) is degraded; some of the granules that contain Arc mRNA may represent sites of mRNA degradation rather than being structures involved in transport. It is also possible that Arc mRNA destined for degradation must be transported back to structures in the cell body (more on this in the following section). Clearly, there are a host of unresolved questions about the trafficking of Arc mRNA that will motivate many studies over the coming years.

4.29.2.6.2 Arc mRNA degradation

While docking is an attractive model for the mechanism underlying the localization of Arc mRNA to the vicinity of recently active synapses, local RNA

degradation may contribute to the selectivity of the localization. After a seizure, Arc mRNA is localized throughout the dendrites of dentate granule cells. If the medial entorhinal cortex is stimulated in rats that previously experienced a seizure, Arc mRNA is rapidly redistributed to the activated dendritic region (the middle molecular layer). However, the total amount of Arc mRNA summed throughout granule cell dendrites is reduced on the side of the synaptic activation in comparison to the contralateral side that received the ECS only. This result is consistent with the degradation of Arc mRNA present in adjacent lamina after local activation.

Although degradation in inactive dendritic laminae is possible, we favor an alternative interpretation that is based on processes that are plausible but not yet shown to occur in the case of Arc. First, it is likely that the Arc mRNA induced by a seizure is being transported back and forth in the absence of a localizing signal. Clearly, synaptic activation causes the mRNA to localize in the activated lamina, and if the mRNA is being transported back and forth in the dendrite, the capture of the moving mRNA in the active region would slowly deplete mRNA from other parts of the dendrite. How then can the progressive decreases in total mRNA levels be explained as stimulation continues? Our working hypothesis is based on two other processes that remain to be documented. The first is the idea that docking of the mRNA at active synapses activates translation (a speculation at this point). The second is that Arc mRNA is subject to translation-dependent mRNA degradation. If all of this actually does occur, then capture of the mRNA in the active lamina would deplete the mRNA from other parts of the dendrite, and the enhanced translation would then cause the mRNA to undergo degradation. Together, these processes would cause an activity-dependent depletion of mRNA from the dendrite. Although speculative, the model invokes processes that are biologically plausible and that have been shown to operate in other settings. Nevertheless, two key pieces of the model remain to be documented, specifically, that localization enhances translation and that translation enhances degradation.

4.29.2.7 Functions of Arc Protein

The biological functions of Arc protein remained a mystery for 11 years after Arc was first discovered. Nevertheless, very recent elegant studies have provided a compelling story about the role of Arc protein in GluR recycling. We begin by reviewing the basic biochemical characteristics of Arc protein and then the recent evidence regarding its physiological role at glutamatergic synapses.

4.29.2.7.1 Biochemistry of Arc protein

The original studies that identified Arc revealed that the Arc gene encodes a hydrophilic protein of 45 kDa with limited homology to other proteins in the genome (Link et al., 1995; Lyford et al., 1995). Apparent homologs of Arc are found in mammals, birds (chicken – by sequence homology; Bock et al., 2005), and reptiles (turtle – by antibody cross reactivity; Mokin et al., 2006) but not in fish or invertebrates. Arc has limited amino acid sequence similarity with the actin-binding protein alpha-spectrin. Arc also possesses a region of amino acid similarity with the retroviral gag protein, although the functional significance of this finding is unclear (NCBI Unigene).

As its name indicates, Arc protein associates with components of the cytoskeleton. Arc has been shown to associate with F-actin by cosedimentation, potentially through a separate actin-binding protein (Lyford et al., 1995). Arc protein is surprisingly insoluble, and its inclusion in the pellets of salt- and triton-extracted hippocampal lysates after seizure indicates that newly synthesized Arc protein also associates with the cytoskeleton. Arc protein has been shown to associate with microtubules by taxol precipitation from nerve growth factor-stimulated PC12 cells; attenuation of MAP2 antibody binding after Arc induction suggests that Arc associates with MAP2 as well (Fujimoto et al., 2004).

There is evidence of other protein–protein interactions involving Arc. Arc has been shown to associate with CaMKIIα *in vitro* and when jointly expressed in Nb2a neuroblastoma cells (Donai et al., 2003). Arc contains one consensus site for CaMKIIα phosphorylation, and CaMKIIα can phosphorylate Arc *in vitro*. Another protein, Amida, has been identified using a two-hybrid screen for proteins associated with Arc; in this case Arc appears to modulate the pro-apoptotic activity of Amida (Irie et al., 2000).

4.29.2.7.2 Localization of Arc protein in the postsynaptic density

Several lines of evidence indicate that Arc protein associates with the proteinaceous material comprising the postsynaptic membrane specialization. Arc protein is enriched in subcellular fractions of PSDs (Steward and Worley, 2001), and proteomic analyses have revealed its presence in the collection of proteins that coprecipitate with the NMDA receptor, which

are thought to be part of a large multimolecular signaling complex in the postsynaptic membrane (Husi et al., 2000). In addition, electron microscopic immunocytochemical studies have demonstrated that Arc protein is localized to the PSD of dendritic spines in the rat dentate gyrus (Moga et al., 2004) and caudate putamen (Wang and Pickel, 2004). Arc-GFP fusion proteins also localize to spines when expressed in neurons in culture, suggesting that Arc protein targets the fusion protein to the postsynaptic region (Fujimoto et al., 2004). Importantly, Arc protein that is synthesized in response to strong patterns of synaptic activation also localizes in or near the PSD (Moga et al., 2004). It remains to be seen whether the same is true of the Arc protein that is synthesized in response to behavior.

Arc protein synthesized in response to strong synaptic activation localizes in a pattern similar to that of the mRNA. When induced by a seizure, both mRNA and protein are localized in the cell body and throughout dendrites (Lyford et al., 1995). When induced by activating particular sets of synapses, both mRNA and protein are present in cell bodies, and within dendrites, the protein localizes predominantly in the region of the active synapses (Steward and Worley, 2001b). The similarities in the subcellular distribution of Arc mRNA and protein are consistent with the hypothesis that the selective localization of Arc protein is determined by the distribution of the mRNA, but this has not been shown definitively. It remains possible that Arc protein is targeted independently of the mRNA, although separate and redundant mechanisms seem unlikely. As noted, it remains to be shown whether or not the newly synthesized protein in the activated dendritic lamina associates with the PSD.

It is interesting that after strong synaptic activation, there is strong immunostaining for Arc protein in the nucleus in addition to the staining in the activated dendritic lamina (Steward and Worley, 2001b). What role, if any, Arc protein plays in the nucleus remains to be defined.

4.29.2.8 Evidence That Arc Is Critical for Long-Term Synaptic Changes and Long-Term Memory

Arc has been implicated in long-term synaptic changes and long-term memory by studies using antisense oligonucleotides to inhibit expression of Arc protein and, more recently, from studies of Arc knockout mice. Infusion of Arc antisense oligonucleotides into the hippocampus 1.5 h before delivery of LTP-inducing stimuli inhibited the maintenance but not the initial potentiation of the perforant path. Infusion of antisense oligonucleotides either 3 h before or immediately after training blocked spatial memory retention assayed 2 days later using the Morris water maze (Guzowski et al., 2000). These studies implicate Arc in late-phase LTP and memory.

Initial attempts to generate Arc-knockout mice were not successful because of embryonic lethality (Liu et al., 2000). In this experiment, a neomycin cassette remained and only part of the Arc coding region was deleted, so that lethality may have been caused by dominant negative effects or fusion peptides. More recent studies have been successful in generating Arc knockout mice that survive to maturity and appear healthy. One paper reported the generation of an insertion mutation in which a destabilized version of the GFP gene was inserted into the Arc gene (Wang et al., 2006). Homozygous mice with insertions of GFP into the Arc locus lived to adulthood. The presence of the GFP tag allowed imaging of Arc expression patterns in living mice. These experiments also revealed a potential role for Arc in visual cortical plasticity. The mice were repeatedly exposed to a patterned visual environment, and Arc/GFP expression patterns were monitored repeatedly in the primary visual cortex using two-photon microscopy. The pattern of expression indicated the expected orientation selectivity of visual cortical neurons in that a subset of neurons was activated by stimuli of a particular orientation. Daily presentation of the same stimulus led to reactivation of a progressively smaller population of cells, and this adaptation process was not disrupted in homozygous mice lacking Arc. At the same time, the number of cells with low orientation selectivity was greater and tuning curves to visual stimulation were broader in mice that were homozygous for the transgene. These findings suggest a role for Arc in promoting the development of orientation selectivity, which is an important form of activity-dependent plasticity.

A more recent study has extensively evaluated different forms of synaptic plasticity and learning ability in Arc/Arg 3.1-knockout mice (Plath et al., 2006). In the Morris water maze task, the knockout mice showed the same initial learning, as measured by decreases in the time required to navigate to a hidden platform, but failed to continue to improve their performance during the late acquisition phase (training blocks 6–9). The homozygous knockout

mice also exhibited substantial deficits in learning a new platform location. The knockout mice showed normal acquisition in a fear conditioning task (measured by the time spent freezing after a CS-US trial in which tone and context signaled delivery of shock) but exhibited retention deficits (less freezing in response to both tone and context) when tested 24 h after initial training. Knockout mice also were deficient in a conditioned taste aversion task and in long-term object recognition as measured by the time spent exploring a novel object versus objects seen 24 h previously. Taken together, the experiments provide a consistent picture of normal initial acquisition of different forms of memory but a deficit in the formation of long-term memories of various types.

Arc/Arg 3.1-knockout mice also displayed deficits in the late phase of perforant path LTP *in vivo* induced by 400-Hz trains and in Schaffer collateral LTP in CA1 neurons in hippocampal slices induced by pairing synaptic stimulation with depolarization. At the same time, there were no detectable differences in basal synaptic properties. Interestingly, early-phase LTP was actually greater in the knockout mice in comparison to controls, but response amplitude decayed back to baseline within about 2 h in the case of perforant path LTP and 70 min in the case of Schaffer collateral LTP. Taken together, the physiological results suggest a deficit in the conversion of early LTP to the form of LTP that is able to persist for hours or longer.

4.29.2.9 A Role for Arc Protein in AMPA Receptor Endocytosis

An extraordinary set of studies has recently established a physiological role for Arc protein in AMPA receptor endocytosis. First, biochemical assays revealed that Arc protein binds to two proteins (endophilin 3 and dynamin 2), which are involved in the endocytosis of membrane vesicles (Chowdhury et al., 2006). This binding occurs via separate nonoverlapping domains in Arc. When expressed alone in HeLa cells, Arc and endophilin 3 were distributed diffusely, but when coexpressed in HeLa cells, Arc and endophilin localized in vesicular structures near the plasma membrane that also contained transferrin receptors, which are considered to be a marker for early endosomes. When coexpressed in primary neurons in culture, Arc and endophilin 3 colocalized in puncta that were distributed along the length of dendrites. Interestingly, internalized GluR1 receptors were also present in some of the Arc/endophilin-positive structures, as was endocytosed transferrin. These results suggested that the Arc/endophilin-positive structures were receptor-trafficking endosomes.

Probably the most important finding was that transgenic expression of Arc caused a marked reduction in surface GluR1 receptors, suggesting that Arc protein actually regulates glutamate receptor endocytosis. GluR1 endocytosis was selectively affected because surface NMDA receptors were not significantly altered (and were perhaps even slightly increased). To further test this hypothesis, Chowdhury et al. took advantage of the Arc/Arg 3.1-knockout mice generated by Plath et al., and showed that hippocampal neurons from knockout mice exhibited twice as many surface GluR1 receptors in comparison to neurons from control mice. Also, the rate of endocytosis of GluR1 receptors was lower in neurons from the knockout mice. Thus, overexpression of Arc protein is associated with increases in GluR1 endocytosis and decreases in the number of surface receptors, whereas deletion of Arc protein decreases GluR1 endocytosis, causing increases in the number of surface GluR receptors.

Another paper in the series filled in other aspects of the story, showing that overexpression of recombinant Arc in neurons in hippocampal slices reduced the amplitude of AMPA-mediated synaptic currents (Rial Verde et al., 2006). This effect was blocked by knocking down Arc expression with RNAi and was not seen when the portion of recombinant Arc protein that interacts with endophilin 3 was deleted. This paper also confirmed that overexpression of Arc promoted GluR endocytosis. Another interesting finding was that overexpression of recombinant Arc occluded LTD induced by 1-Hz stimulation, suggesting that the mechanism for LTD involves the same type of GluR endocytosis that occurred in response to overexpression of Arc.

Still another paper in the series focused on the role of Arc in a phenomenon called homeostatic scaling (Shepherd et al., 2006). Previous studies have shown that chronic treatment of neurons in culture with tetrodotoxin (TTX), which blocks action potentials, leads to a compensatory increase in surface AMPA receptors, whereas increasing activity by chronically blocking gamma-aminobutyric acid-mediated inhibition with bicuculline leads to decreases in surface AMPA receptors (O'Brien et al., 1998; Turrigiano and Nelson, 2004). These responses are thought to be homeostatic adjustments that allow neurons to maintain a constant level of synaptic

drive. Using the same types of manipulations, Shepherd et al. demonstrated that Arc expression by primary neurons in culture is regulated by neuronal activity; Arc expression was reduced following long-term TTX blockade and increased following treatment with bicuculline, consistent with it playing a role in homeostatic scaling. Overexpression of Arc led to the expected cell-wide decreases in GluR1 surface receptor levels in the same way as chronic treatment with bicuculline. Critically, overexpression of Arc also blocked the homeostatic increases in Glu1Rs that were otherwise seen following chronic TTX treatment. Finally, using the Arc-knockout mice prepared by Plath et al., Shepherd et al. showed that neurons from knockout animals expressed high levels of surface GluRs and larger AMPA receptor-mediated excitatory postsynaptic currents. Most critically, neurons from Arc-knockout mice did not exhibit the homeostatic rescaling in response to chronic activity blockade with TTX or chronic blockade of inhibition with bicuculline. Taken together, these results suggest that Arc protein plays a critical role in the mechanisms underlying homeostatic rescaling of AMPA receptor number.

4.29.2.10 Arc and Synaptic Homeostasis

Taking all the information into consideration, a novel function for Arc protein was proposed that is consistent with the timing of its expression following LTP induction and its physiological role in regulating GluRs (Rial Verde et al., 2006). The basic idea is that induction of LTP increases efficacy at some synapses, causing an increase the total synaptic weight. If this was not adjusted, progressive increases in total synaptic strength could overactivate the neuron. Accordingly, the idea is that induction of LTP at some synapses triggers a later homeostatic adjustment to adjust overall synaptic weights to return total input strength to a preset level that the neuron can accommodate.

The model proposed by Rial-Verde et al. is illustrated in **Figure 1(a)** using a drawing that shows a realistic representation of the distribution of spine synapses along a dendrite and the distribution of protein synthetic machinery beneath the spines. As in the model proposed by Rial-Verde et al., synaptic activation triggers strong LTP at some synapses and weaker LTP at others, and leaves other synapses unaffected. Their model proposes that homeostatic adjustments would occur equivalently across synapses irrespective of the history of activity, which would preserve the differential in strength between the synapses while importing a late reduction in synaptic strength at synapses that initially did not experience LTP. An impartant feature of the model is that there would be heterosynaptic LTD at synapses that did not initially undergo LTP, which would develop slowly over time after a potentiating event. No such slowly developing LTD has been reported, but that may be because the appropriate studies have not been carried out.

Another important implication of the model is that if Arc is involved in the homeostatic reductions in synaptic strength, and if these occur at synapses that did not initially experience LTP, then Arc protein must be delivered to synapses that were 'not' activated by the initial stimulation. Putting this together with the fact that Arc protein is selectively localized to dendritic domains contacted by active synapses, the implication is that homeostatic adjustments in synaptic strength might occur locally in parts of the dendrite, not generally over the entire postsynaptic receptive surface. It will be interesting to see if the key phenomena predicted by this model can be demonstrated (slowly developing LTD and targeting of Arc protein to synapses that were not initially activated).

In considering the model, it is useful to consider limitations imposed by the nature and distribution of the machinery for protein synthesis. Several points are noteworthy. (1) Quantitative electron microscopic studies indicate that not all spines in adult neurons *in vivo* have underlying polyribosomes. For example, in the middle dendritic regions of dentate granule cells (the site of termination of the perforant path) only approximately 25% of the spines have polyribosomes (Steward and Levy, 1982). (2) There is no reason to think that inputs would selectively activate synapses with polyribosomes, and so activity-induced modifications (such as LTP) would presumably occur at synapses with and without polyribosomes. For example, **Figure 1** illustrates a biologically realistic dendrite with spines in which the distribution of polyribosomes is indicated. If Arc protein is targeted to synapses that have experienced a particular pattern of activity, either the protein that is made at one synapse must be available to other nearby synapses or the machinery for protein synthesis must move from one synapse to another. This

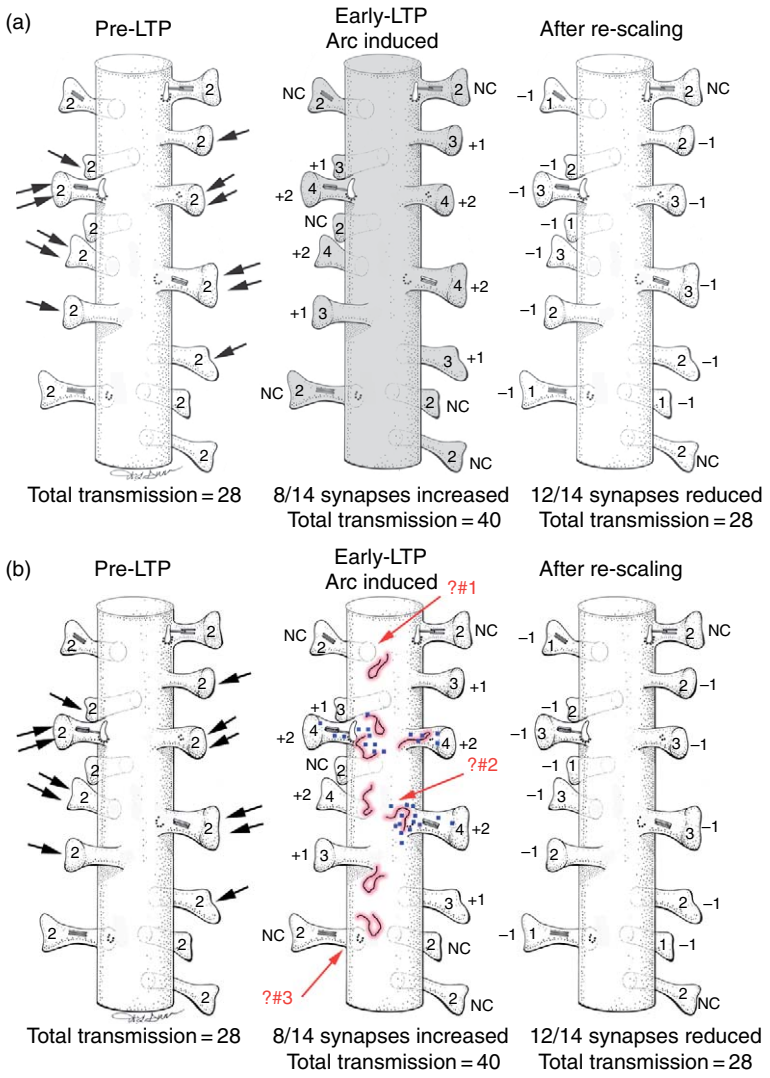


Figure 1 (a) illustrates the model proposed by Rial-Verde et al (2007) using a drawing that shows a realistic representation of the distribution of spine synapses along a dendrite and the distribution of protein synthetic machinery beneath the spines. The model assumes that synaptic activation triggers strong LTP at some synapses (double arrows), weak LTP at others (single arrows), and leaves other synapses unchanged (NC). The shaded dendrite (middle) illustrates the situation after induction of LTP and before homeostatic adjustments have occurred, and the dendrite on the right illustrates the situation after homeostatic rescaling. One difference between the model shown and the one proposed by Rial-Verde is that the proposed synaptic modifications occur selectively in a localized region of the dendrite. In the Rial-Verde model, rescaling occurs equivalently across synapses irrespective of the history of activity, which would preserve the differential in strength between the synapses while returning the total synaptic strength to its initial level. Thus, there would be heterosynaptic LTD at synapses that did not initially undergo LTP, which would develop slowly over time. (b) illustrates questions regarding the delivery of Arc mRNA and protein to the synapses undergoing rescaling. Arc mRNA is shown as a line with red halo. Arc protein is shown as blue squares. If Arc is involved in the homeostatic reductions in synaptic strength, and if these occur at synapses that did not initially experience LTP, then Arc protein must be delivered to synapses that were NOT activated by the initial stimulation, which raises several questions. Question #1 (?#1): Is Arc mRNA targeted selectively to synapses that were NOT activated, but that undergo rescaling? If the synapse does not already have ribosomes, where do the ribosomes come from to translate the mRNA? Question #2 (?#2): If Arc mRNA is targeted exclusively to synapses that were activated in a way as to induce LTP, can the Arc protein that is synthesized at one synapse be captured by nearby synapses that were NOT initially activated? Also, in the case of synapses that capture Arc mRNA but do not already have ribosomes (the synapse on the left side of the dendrite at the arrow), do ribosomes relocalize in order to translate captured Arc mRNA or do ribosomes come along with the mRNA when it is transported from the cell body? Question #3 (?#3). If a synapse that was not initially activated has polyribosomes, does the process of rescaling enable it to capture and translate Arc mRNA?

could involve movements of polyribosome assemblies or individual ribosomes.

The combined results on Arc protein function can also be accommodated by other models. For example, the proposed homeostatic adjustments in synaptic strength could occur at the same synapses that had undergone LTP in the first place. This model would predict a slow decrease in synaptic strength from the initial peak after induction of LTP during the period of Arc expression. An important feature of this model is that the homeostatic changes would involve the same synapses that had initially undergone LTP.

4.29.2.11 Speculations on the Role of Arc in Cellular and Molecular Mechanisms Underlying Memory Consolidation

The models for Arc function at synapses take on special interest in the context of the studies that implicate Arc in memory consolidation. If Arc's role is to promote receptor endocytosis and reduce synaptic strength, then the implication would be that this process plays a role in memory consolidation. One can easily imagine, for example, that the initial alterations in synaptic strength triggered by LTP create an inherently unstable synaptic circuit that becomes stabilized by the homeostatic adjustments mediated by Arc. In the absence of such homeostatic adjustments, the initial alterations in synaptic strength would thus decrement in a nonspecific fashion, erasing the synaptic representation of the memory trace. This novel concept opens up a new dimension in thinking about the cellular and molecular mechanisms of memory consolidation. It is likely that the new findings regarding the functions of Arc protein will trigger a host of follow-up studies testing predictions and seeking to demonstrate the predicted synaptic modifications.

In thinking about mechanisms of synaptic plasticity that require transcription and translation, it is relevant that mature Arc transcripts can be synthesized within minutes after a period of stimulation (Guzowski et al., 2005) and Arc mRNA can be transported within dendrites at rates in excess of 1 μm per second (Dynes and Steward, 2006). Thus it is possible to transcribe an mRNA and deliver it to any point of a dendrite within minutes of the inducing stimulus. Studies of Arc thus reveal a mechanism that was not previously anticipated.

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4.30 Glutamate Receptor Trafficking in LTP

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4.30.1 Introduction

For several decades now, long-term potentiation (LTP) has been studied as a putative mechanism for memory formation. Because of this potential link to memory (see, for example, Whitlock et al., 2006, but see also Shors and Matzel, 1997), there has been a tremendous amount of interest in understanding the molecular mechanisms of LTP. Two complimentary molecular mechanisms have been widely recognized as potentially underlying LTP. The first of these, protein phosphorylation, was shown to be correlated with LTP in the late 1970s and early 1980s (Williams and Rodnight, 1977; Browning et al., 1979a,b; Lovinger et al., 1985; Hu et al., 1987; Sweatt and Kandel, 1989). Subsequent studies using inhibitors of protein kinase C (PKC) (Hu et al., 1987; Malinow et al., 1988; Klann et al., 1992; Osten and Sacktor, 1992; Hvalby et al., 1994), Ca^{2+}/calmodulin kinase II (CaMKII) (Finn et al., 1980; Malenka et al., 1989; Malinow et al., 1989; Sweatt and Kandel, 1989), and Src-family tyrosine kinases (Lu et al., 1998; Huang and Hsu, 1999) have provided compelling evidence for a role of phosphorylation in the induction of LTP. Interestingly, in adult animals, cAMP-dependent protein kinase (PKA) appears to have little role in the induction of LTP, but this kinase is thought to be critical for the long-lasting maintenance of LTP (Frey et al., 1993; Matthes and Reymann, 1994; Nayak et al., 1998). In contrast, PKA appears to be required for the induction of LTP early in development (Esteban et al., 2003; Yasuda et al., 2003). The second mechanism, receptor trafficking,

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has more recently emerged as a leading candidate for a mechanism underlying LTP (Boehm and Malinow, 2005; Malinow and Malenka, 2002). The focus of this chapter is to examine the evidence for such receptor trafficking in LTP and to describe how protein phosphorylation may play a role in this trafficking. Any review of LTP is plagued by the huge number of publications in this field. Because of space limitations, one must be selective of one's coverage of the topic, and this necessarily requires that many outstanding studies go unrecognized. For this chapter, we focus primarily on studies using *in situ* hippocampal slices, *in vitro* organotypic hippocampal slice cultures, or dissociated neuronal cultures. The rationale for this focus is described next.

4.30.2 Basic LTP Experimentation Practices

4.30.2.1 Neuronal Preparations for Studies of LTP

LTP was initially discovered in the dentate region of the hippocampus in an *in vivo* (i.e., in a live animal) rabbit preparation (Bliss and Lomo, 1973). Subsequent work in the 1970s extended these studies using *in situ* hippocampal slices, with the CA1 region being the focus of most studies. The advent of the hippocampal slice offered many advantages for studies of LTP, including easy access to a stable preparation for intracellular and extracellular as well as whole-cell recordings. The *in situ* slice preparation also greatly facilitated pharmacological studies of LTP because of the ease with which one could control the extracellular milieu by simply changing the constituents of the buffer bathing the slice. However, there are also numerous disadvantages with the *in situ* preparation, including the long-lasting effects of sacrifice of the animal and the widespread loss of cells and synaptic proteins, as well as the fact that the slices do not survive well when maintained at 37 °C. A number of other preparations, including *in vitro* cultured neurons or organotypic slice cultures and nonneuronal preparations such as human embryonic kidney (HEK) cells, have been used to study LTP and trafficking. As with the *in situ* hippocampal slice, such reduced preparations have both advantages and limitations. The *in vitro* systems permit a variety of genetic manipulation, and it is also possible to visually monitor receptor trafficking. However, it is not possible to elicit LTP in neuronal cultures using the classical LTP induction protocol. Moreover, cultured neurons and organotypic slices are virtually always prepared from embryonic or neonatal tissue in which massive activity-dependent trafficking is ongoing because this postnatal period is when most synaptogenesis occurs. Nonneuronal systems (cell lines) using expression of recombinant receptors have provided some of the greatest insight into the role played by individual receptor subunits in trafficking. However, major limitations of such preparations are that they cannot exhibit LTP and they lack the plethora of synaptic proteins that are thought to participate in glutamate receptor trafficking in neurons. For these reasons, and because of the focus of this chapter, we emphasize studies that use neuronal systems, particularly those using *in situ* hippocampal slices prepared from neonatal or adult animals as well as *in vitro* cultured neurons and organotypic slice cultures. We attempt to identify exactly which type of preparation is used in each of the studies we discuss.

4.30.2.2 LTP Induction Paradigms

In addition to the myriad systems used to study LTP, there are several different electrical stimulation patterns used to induce LTP. Among these are high-frequency stimulation (HFS), theta burst stimulation (TBS), primed burst stimulation (PBS), and pairing (**Table 1**). The most common paradigms used when studying glutamate receptor trafficking are HFS and pairing. Stimuli such as theta burst and primed burst are sometimes used, as these appear to more closely mimic endogenous firing patterns of the hippocampus (Diamond et al., 1988; Larson and Lynch, 1989). HFS is typically used with extracellular recordings and entails from one to four trains of 100 stimuli at 100 Hz given at 10- to 30-s intervals. Although the hippocampus does fire *in vivo* at rates of 100 Hz, such firing rarely lasts more than 50 ms. Pairing stimulation involves delivering 100 stimuli at 2 Hz concomitant with depolarization of the neuron. It is important to note that the pairing protocol is typically used in whole-cell patch clamp experiments where the membrane potential of the postsynaptic neuron can be controlled. An important, and often overlooked, caveat with whole-cell experiments is that such recording results in dialysis of the cell with the electrode buffers and thus leads to dilution of the intracellular content of the neuron. Indeed, the ability to induce LTP using the whole-cell configuration declines rapidly after the first 30–45 min of recording. This issue may be particularly germane to controversies that develop concerning the relative contributions of intracellular signaling molecules to LTP.

Table 1 The range of stimuli used in the various LTP induction paradigms[a]

Paradigm	*Stimulus*	*Reference*
HFS	1 burst of 100 pulses at 100 Hz, 1–4 trains 10–30 s apart	Bliss and Lomo (1973) Shi et al. (1999) Grosshans et al. (2002)
TBS	3–10 bursts of 4–6 pulses at 100 Hz at 5 Hz (1 burst = 4–6 pulses at 100 Hz), 1–4 trains 10 s–5 min apart	Larson et al. (1986) Larson and Lynch (1989)
PBS	1 pulse followed 140 or 170 ms later by 2–10 pulses at 100 Hz	Diamond et al. (1988)
Pairing	10–200 pulses at 0.1–2 Hz, while depolarizing the postsynaptic neuron	Gustafsson et al. (1987) Liao et al. (1995) Durand et al. (1996)

[a]HFS, high-frequency stimulation; TBS, theta burst stimulation; PBS, primed burst stimulation.

4.30.2.3 Brain Regions and LTP

LTP or LTP-like potentiation has been described in a great many different brain regions. For example, in the CA3 region of the hippocampus, two apparently distinct forms of LTP have been described. One of these is classical *N*-methyl-D-aspartate (NMDA) receptor (NMDAR)–dependent LTP (Zalutsky and Nicoll, 1990). The other, exhibited by the mossy fiber input to this region, is a form of LTP not dependent on NMDAR activation but, rather, requires activation of PKA and is possibly presynaptic in nature (Weisskopf et al., 1994). However, in this chapter we focus primarily on NMDAR-dependent LTP in the CA1 region of the hippocampus.

Having established the ground rules for our discussion, we are now ready to discuss the role of glutamate receptor trafficking in LTP. We also discuss the molecular mechanisms that may underlie this trafficking. Given the different roles played by the alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor (AMPAR) and NMDAR in LTP, and because their trafficking mechanisms appear to differ, we discuss trafficking of the AMPAR and the NMDAR separately.

4.30.3 AMPA Receptor Trafficking in LTP

Excitatory postsynaptic potentials (EPSPs) in the CA1 are composed largely of AMPAR currents, with smaller contributions made from NMDAR currents. LTP is principally expressed as an enhancement of the slope and amplitude of the EPSP and as such is largely a product of an increase of AMPAR current, although LTP can also be measured as an increase in pharmacologically isolated NMDAR responses (Bashir et al., 1991; Grosshans et al., 2002). The mechanism of enhanced AMPAR function following LTP has been a topic of intense debate for decades. One possibility is that LTP increases channel currents without an increase in the number of receptors at the synapse (Banke et al., 2000; Benke et al., 1998). However, an increasingly more popular idea is that LTP increases the number of glutamate receptors at synapses. In the case of AMPARs, the argument for LTP-induced trafficking of receptors to the synapse was fueled by the discovery of silent synapses, which lack functional AMPARs but contain a detectable number of NMDARs (Isaac et al., 1995; Kullmann, 1994; Liao et al., 1995). The idea of an increased number of synaptic glutamate receptors as a mechanism of LTP was first proposed in the 1980s (Baudry et al., 1986). For decades, this provocative hypothesis was obscured by the heated debate over pre- versus postsynaptic expression of LTP. More recently, studies of acute and cultured hippocampal slices have provided conclusive evidence that both spontaneous activity and LTP induction lead to trafficking of AMPARs to the synapse in these preparations from neonatal animals (Hayashi et al., 2000; Zhu et al., 2000; Oh et al., 2006).

The two potential mechanisms of LTP, conductance change and trafficking of AMPARs, are not mutually exclusive, as both seem likely to play a role in LTP. Moreover, both mechanisms may require protein phosphorylation (Benke et al., 1998; Shi et al., 1999; Grosshans et al., 2002). For example, CaMKII activation leads to increases in AMPAR conductance and may also contribute to AMPAR trafficking via phosphorylation of the AMPAR auxiliary protein stargazin (Derkach et al., 1999; Tomita et al., 2005). However, for the purposes of this chapter, we focus on AMPAR trafficking and LTP.

4.30.3.1 Molecular Biology of AMPARs

AMPARs gate Na^+ entry into neurons and mediate fast excitatory neurotransmission in response to glutamate release. Various combinations of four homologous subunits (GluR1 to GluR4) form tetrameric ion channels with unique characteristics (Lambolez et al., 1992; Hollmann and Heinemann, 1994). Each subunit is encoded by a separate gene with developmentally and regionally regulated expression patterns (review: Palmer et al., 2005). All four subunits exist as two alternatively spliced forms, flip and flop (Borges and Dingledine, 1998). The flop form predominates in adult CA1 and desensitizes less slowly than the flip form (Dingledine et al., 1999). Coimmunoprecipitation studies suggest that AMPARs are primarily GluR1/GluR2 or GluR2/GluR3 heterotetramers in the adult hippocampus (Wenthold et al., 1996). The presence of GluR2 renders the receptor impermeable to calcium (Hollmann et al., 1991; Verdoorn et al., 1991). Interestingly, a recent study suggests that GluR2-lacking receptors may be present at the synapse for a brief time period following plasticity events such as LTP (Plant et al., 2006). Expression of GluR4 is restricted to the immature hippocampus (Zhu et al., 2000). AMPAR subunit composition may also regulate receptor trafficking, as GluR2/3 receptors are thought to cycle continuously, whereas trafficking of GluR1/2 and GluR4/2 receptors can be regulated by synaptic activity (Hayashi et al., 2000; Zhu et al., 2000; Shi et al., 2001). These differences in trafficking correlate with C-terminal tail length, as GluR1 and GluR4 have long tails, whereas GluR2 and GluR3 have short tails. These intracellular C termini mediate AMPAR interactions with scaffolding proteins as well as components of the actin cytoskeleton and trafficking machinery (Dong et al., 1997; Leonard et al., 1998; Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998; Dev et al., 1999; Xia et al., 1999; Chen et al., 2000; Shen et al., 2000).

4.30.3.1.1 Phosphorylation of AMPARs

The C-terminal tails also contain several sites of phosphorylation that may mediate protein–protein interactions and/or alter channel kinetics. Electrophysiological studies have demonstrated that CaMKII and PKA regulate AMPAR channel gating by increasing open channel conductance and probability, respectively (Derkach et al., 1999; Banke et al., 2000). Site-directed mutagenesis and phosphopeptide mapping studies suggest that these effects are mediated by direct phosphorylation of GluR1 subunits at serine 831 and serine 845 (Roche et al., 1996; Barria et al., 1997). Production of site-specific phosphoantibodies confirmed that serine 831 can be phosphorylated by CaMKII and PKC, whereas serines 845 is phosphorylated by PKA (Mammen et al., 1997). Knock-in mutations of both serines 831 and 845 suggest that at least one of these sites is important for AMPAR trafficking, LTP, and learning in the adult hippocampus (Lee et al., 2003). Interestingly, phosphorylation of serine 845, but not serine 831, is necessary for synaptic incorporation of GluR1 subunits in slices from neonates (Hayashi et al., 2000; Esteban et al., 2003). Furthermore, a recent report suggests that phosphorylation of serine 845 delivers GluR1 to the extrasynaptic membrane, and coincident synaptic activation induces lateral translocation to the synapse (Oh et al., 2006). PKA has also been shown to regulate surface expression of GluR4 subunits via direct phosphorylation of serine 842 (Carvalho et al., 1999; Esteban et al., 2003; Gomes et al., 2004).

In contrast to the consistently demonstrated potentiating effects of PKA and CaMKII, other kinases seem to have differential effects of AMPAR function and localization. The result of phosphorylation correlates with the location of the phosphorylated residue within the C terminus of AMPAR subunits. For example, PKC and Src-family tyrosine kinase phosphorylation of the distal C terminus of GluR2 can downregulate AMPAR transmission and surface expression (Seidenman et al., 2003; Ahmadian et al., 2004; Hayashi and Huganir, 2004). Conversely, recent evidence demonstrates that PKC phosphorylation of serine 818 in the membrane proximal region of the GluR1 C terminus correlates with increased AMPAR transmission and surface expression observed following LTP in juvenile hippocampus (Boehm et al., 2006).

Activated PKC is translocated to dendritic spines by PICK1, a protein that also interacts with GluR2 subunits. This translocation correlates with phosphorylation of serine 880 on GluR2 and reduced surface expression of exogenous GluR2 subunits (Perez et al., 2001). Loss of surface expression is consistent with phosphoserine 880– and phosphotyrosine 876–mediated disruption of GluR2 binding to the synaptic scaffolding proteins glutamate receptor-interacting protein (GRIP) and AMPA-binding protein (ABP) (Matsuda et al., 1999; Chung et al., 2000; Hayashi and Huganir, 2004). However, a recent

study suggests that tyrosine dephosphorylation, possibly at a different site in the C-terminal tail of GluR2, regulates internalization of GluR1 subunits during metabotropic glutamate receptor–mediated long-term depression (LTD) (Moult et al., 2006). Thus, both phosphorylation and dephosphorylation events are likely to mediate specific mechanisms of AMPAR trafficking in response to various stimuli.

Additional PKC phosphorylation sites include serine 863 on GluR2 and potentially threonine 830 on GluR4 (Carvalho et al., 1999; McDonald et al., 2001). The functional consequence of PKC phosphorylation at these sites has not been investigated in neurons.

4.30.3.1.2 Developmental profile of AMPAR subunit expression

Because *in situ* hippocampal slices and organotypic hippocampal slice cultures are commonly prepared from rodents at various stages of postnatal development, it is appropriate that we review the developmental profiles of AMPAR subunit expression. Immunoblot analysis of hippocampal homogenates reveals that expression of GluR1, GluR2, and GluR3 (as determined with anti-GluR2/3 antibody) subunits gradually increases from P0 to steady state around P15 (Zhu et al., 2000). An *in situ* hybridization study demonstrated that total brain expression of the long-tail splice variant of GluR2, GluR2L, peaks during the second postnatal week (P7–P14) of development (Kolleker et al., 2003). High levels of expression were observed in the hippocampus at these developmental stages. Conversely, expression of the GluR4 subunit peaks from P0 to P2 before rapidly declining to 20% of maximum expression level at P9. Expression of GluR4 is near undetectable by postnatal day 15 (Zhu et al., 2000). Interestingly, when GluR4 expression is highest (P2), the relative abundance of GluR1 and GluR2 subunits is similar to that of GluR4. The distinct developmental profiles of AMPAR subunit expression suggest that each protein may mediate unique mechanisms of synaptic plasticity at different stages of postnatal development.

4.30.3.2 Evidence for AMPAR Trafficking in LTP

4.30.3.2.1 Early postnatal development

The studies discussed in this section were conducted primarily in acute hippocampal slices prepared from neonatal (<P10) rodents or in organotypic hippocampal slice cultures prepared from postnatal day 5–8 rats. The cultured slices were generally infected with recombinant AMPAR proteins 0–2 days following preparation and maintained in culture for 2–3 days prior to experimental observation. These studies are therefore likely to reflect mechanisms that contribute to synaptic plasticity during the first ~7–11 days of life, hence 'early' postnatal development. Given that GluR4 subunits are highly expressed during the first few days of postnatal development, receptors containing these subunits may play a key role in mechanisms of plasticity during this time period.

4.30.3.2.1.(i) GluR4 Pyramidal neurons infected with recombinant GFP-tagged GluR4 subunits show enhanced AMPAR transmission when compared to uninfected control neurons (Zhu et al., 2000). To determine whether the enhanced transmission was due to synaptic incorporation of the recombinant subunits, this group used an electrophysiological assay developed in their laboratory (Hayashi et al., 2000). Coimmunoprecipitation and electrophysiological studies indicate that GFP-GluR4 subunits form homomeric channels that display inward rectification (Zhu et al., 2000). Because most endogenous hippocampal AMPARs exhibit a linear current–voltage relationship (Verdoorn et al., 1991; Wenthold et al., 1996), this characteristic of GFP-GluR4 homomers permitted electrophysiological monitoring of the functional incorporation of the recombinant channels (Hayashi et al., 2000). Indeed, AMPAR EPSCs recorded from GFP-GluR4-infected cells exhibit greater rectification than uninfected control cells (Zhu et al., 2000). Furthermore, the observed enhancement of AMPAR transmission and rectification was occluded by synaptic activity blockade or treatment with APV, suggesting that spontaneous activity delivers GluR4 to synapses in an NMDAR-dependent manner. Subsequent experiments revealed that PKA phosphorylation of GluR4 on serine 842 was necessary for activity-dependent delivery of GluR4 to synapses (Esteban et al., 2003). Additional work utilizing chimeric GluR1/GluR4 subunits indicates that the membrane proximal region of the GluR4 intracellular C-terminal tail is required for delivery of GluR4 by spontaneous activity (Boehm et al., 2006).

4.30.3.2.1.(ii) GluR1 Although expression of GluR4 peaks during the first few days of postnatal development, the relative abundance of GluR1 is

near equivalent to that of GluR4 at postnatal day 2 (Zhu et al., 2000). It is therefore possible that GluR1 subunits contribute to mechanisms of synaptic plasticity during this time period. Electron microscopy studies of recombinant GFP-tagged GluR1 expressed in organotypic hippocampal slice cultures suggest that these subunits are largely excluded from synapses (<1% localized to postsynaptic densities; Shi et al., 1999). Similar results (~3% localized to postsynaptic densities) were obtained for endogenous GluR1 subunit expression at postnatal day 10. Tetanic stimulation (two trains 100 Hz for 1 s separated by 20 s) induced NMDAR-dependent delivery of GluR1-GFP to spines and clustering of GluR1-GFP in dendrite shafts as assayed by two-photon laser scanning microscopy (Shi et al., 1999). Similar results have been obtained using acute hippocampal slices prepared from postnatal day 9 rats and biochemical measures of AMPAR trafficking (Grosshans et al., 2002). Studies of dissociated hippocampal neurons in culture have also provided evidence for NMDAR- and activity-dependent recruitment of AMPARs to previously silent synapses (Liao et al., 2001a; Lu et al., 2001; Pickard et al., 2001).

The immunofluorescence method utilized by Shi et al. (1999) does not discriminate between synaptic and extrasynaptic receptors. Subsequent coimmunoprecipitation and electrophysiology experiments demonstrated that the recombinant GluR1-GFP subunits form homomeric, rectifying channels (Hayashi et al., 2000). Thus, as described for GFP-GluR4 homomers, functional incorporation of these receptors into synapses can be assessed electrophysiologically. Expression of the GluR1-GFP had no effect on AMPAR transmission or rectification under basal conditions (Hayashi et al., 2000). This result supports the conclusion of the previous report that these subunits are largely excluded from synapses at this stage of development (Shi et al., 1999). Following induction of LTP via a pairing protocol, synaptic delivery of GluR1-GFP was observed as increased rectification of AMPAR responses relative to uninfected control cells (Hayashi et al., 2000). Mutation of a threonine residue within the PDZ ligand near the C terminus of GluR1 (T887A) led to short-lasting potentiation, which eventually decayed to a depression at 45 min post–pairing stimulation. Furthermore, at least two reports have indicated that the canonical PDZ protein PSD-95 is necessary and sufficient for LTP during various developmental stages (Stein et al., 2003; Ehrlich and Malinow, 2004). Taken together, these data suggest that GluR1 delivery following LTP stimulation likely involves an interaction between GluR1 and a PDZ-domain-containing protein. However, extensive studies of knock-in mice with the last seven amino acids of GluR1 deleted provide *in vivo* evidence that the GluR1 PDZ ligand is not required for NMDAR-dependent LTP in mature animals (aged 3 weeks to 7 months; Kim et al., 2005). Clearly, further study is necessary to determine the contribution of AMPAR-PDZ interactions to mechanisms of AMPAR trafficking and LTP. One possibility is the sequential involvement of multiple-PDZ-domain-containing proteins such as PSD-95 and stargazin-like TARPs (review: Malenka, 2003).

Although constitutively active CaMKII is sufficient for synaptic delivery of recombinant GluR1, CaMKII phosphorylation of GluR1 at serine 831 seems not to be required for this process, as mutation of this residue (S831A) had no effect on CaMKII-induced amplification of AMPAR transmission and rectification (Hayashi et al., 2000). Unfortunately, the effect of this mutation on LTP-induced delivery of recombinant GluR1 was not tested in this study. However, work from another group suggests that hippocampal LTP does not require CaMKII activity during early postnatal development (Yasuda et al., 2003). Taken together, these studies suggest that CaMKII may be sufficient – yet not necessary – for LTP during early postnatal development.

4.30.3.2.2 *Late postnatal development*

The studies discussed in this section were conducted primarily in acute hippocampal slices prepared from 2- to 3-week-old rodents or in organotypic hippocampal slices prepared from postnatal day 5–7 rats. The slices were then cultured *in vitro* for 5–14 days before introduction of recombinant AMPAR proteins and were maintained in culture for an additional 1–3 days prior to experimental observation. Thus, these slices correspond to an approximate developmental age of 11–24 days and are likely to suggest mechanisms that contribute to synaptic plasticity during 'late' postnatal development. This period is also characterized by peak levels of synaptogenesis.

4.30.3.2.2.(i) GluR1 The LTP-dependent delivery of recombinant GluR1-GFP to synapses observed by Hayashi et al. (2000) has been revisited more recently (Boehm et al., 2006; Esteban et al., 2003). In the more current studies, organotypic slice cultures were maintained *in vitro* for 7–10 days prior to

infection with GluR1 to more closely mimic the endogenous expression profile of GluR1 subunits. These experiments confirmed previous findings that constitutively active CaMKII drives delivery of recombinant GluR1 subunits to synapses. However, mutation of GluR1 (S818A) blocked CaMKII-induced potentiation (Boehm et al., 2006). Additional experiments demonstrated that serine 818 is phosphorylated by PKC both *in vitro* and in brain homogenates and that increased phosphorylation of this site correlates with synaptic incorporation of GluR1 following induction of LTP. These data suggest that both CaMKII and PKC, either independently or in concert, contribute to mechanisms of AMPAR trafficking following induction of LTP.

These and other lines of evidence suggest that various protein kinases may be linked to AMPAR trafficking and LTP via multiple signaling pathways. For example, CaMKII appears to activate Ras signaling as constitutively active Ras mimics CaMKII-induced delivery of GluR1 to synapses (Zhu et al., 2002). In addition, PKA phosphorylation of GluR1 on serine 845 is necessary, but not sufficient, for LTP and CaMKII-dependent delivery of GluR1 (Esteban et al., 2003). Furthermore, activation of PKA correlates with increased localization of GluR1 within extrasynaptic membrane fractions purified from acute hippocampal slices prepared from slightly older rats (4–6 weeks; Oh et al., 2006). Subsequent TBS resulted in stronger LTP, suggesting that PKA, perhaps via serine 845 phosphorylation, primes extrasynaptic membrane with AMPARs readily available for synaptic incorporation on synaptic activation. However, another group reported that PKA activity is not required for induction of LTP in hippocampal slices prepared from animals older than postnatal day 27 (Yasuda et al., 2003). Clearly there are numerous mechanisms of AMPAR trafficking mediated by an intricate mix of signaling pathways. It will be exciting to see how future work is able to tease out the contributions of various signaling molecules to mechanisms of synaptic plasticity during different stages of postnatal development.

Our discussion thus far has been restricted to studies carried out in the hippocampus. We diverge here briefly to discuss a study conducted in the amygdala (Rumpel et al., 2005). The authors used a GFP-tagged construct containing the last 81 amino acids of the GluR1 C terminus to evaluate the contribution of AMPAR trafficking to associative learning. The phrase 'plasticity block vector' was used to describe the construct, as it was previously shown to block LTP in organotypic hippocampal slices and neurons cultured from visual cortex (Shi et al., 2001; Watt et al., 2004). The more recent paper replicated these results in the amygdala and extended the findings by correlating associative learning with synaptic incorporation of recombinant GFP-GluR1. Importantly, the source of the newly incorporated subunits is unknown. It is therefore possible that these subunits are trafficked from the extrasynaptic membrane rather than an intracellular compartment. Additional *in vivo* experiments demonstrated that the plasticity block vector impairs fear acquisition (Rumpel et al., 2005). It will be exciting to see how similar studies in the hippocampus and other brain regions strengthen the ever-elusive correlation between molecular mechanisms of LTP and learning.

4.30.3.2.2.(ii) GluR2 The alternative splice variant of GluR2, GluR2L, is highly expressed in the hippocampus during the second and third weeks of postnatal development (Kolleker et al., 2003). Interestingly, GluR2L seems to function independently from GluR2, as neither endogenous nor recombinant GluR2L coassembles with GluR2. Conversely, endogenous GluR2L was found in complex with GluR1 and GluR3 in homogenates of neonatal hippocampus (Kolleker et al., 2003). Like GluR1 and GluR4, GluR2L subunits have long C-terminal tails and therefore may be subject to activity-dependent regulation of trafficking during this developmental period (Shi et al., 2001). To test this hypothesis, Kolleker et al. (2003) transfected organotypic hippocampal slice cultures with recombinant GFP-tagged GluR2L subunits containing the R586Q mutation (flop form). Much like the GluR1 and GluR4 constructs described earlier, these receptors form homomeric channels that display inward rectification. Indeed, infected cells exhibit increased amplitude and rectification of AMPAR excitatory postsynaptic currents (EPSCs) compared to uninfected cells, and these effects were occluded by synaptic activity blockade (Kolleker et al., 2003). These results suggest that spontaneous activity delivers GluR2L subunits to synapses during late postnatal development. A Ras signaling pathway, possibly linked to PI3-kinase, is likely involved in this process, as a previous study demonstrated that neurons coexpressing dominant negative Ras and GFP-GluR2L exhibit depressed AMPAR transmission and normal rectification (Zhu et al., 2002; Man et al., 2003).

In the absence of continued spontaneous activity, the newly incorporated GluR2L-containing

AMPARs are replaced by GluR2-containing receptors (Kolleker et al., 2003). This result is consistent with a recent study that reported transient synaptic incorporation of calcium-permeable, GluR2-lacking AMPARs during the first 10 min following LTP induced by a pairing protocol (Plant et al., 2006). Furthermore, activation of these calcium-permeable AMPARs may be required for subsequent stabilization of LTP via replacement of the GluR2-lacking AMPARs with GluR2-containing channels. Taken together, these findings suggest that activity-dependent delivery of GluR2L may mediate mechanisms of LTP. Supporting this idea, when synaptic activity is reduced to block delivery of GluR2L, subsequent induction of LTP delivers GluR2L to synapses, as determined by increased amplitude and rectification of AMPAR EPSCs (Kolleker et al., 2003). Furthermore, expression of the C-terminal tail of GluR2L completely abolishes LTP in GluR1 knock-out mice (Jensen et al., 2003; Kolleker et al., 2003). Therefore, activity-dependent delivery of GluR2L subunits may mediate GluR1-independent mechanisms of LTP during late postnatal development.

4.30.3.2.3 Adult

Very few studies of molecular mechanisms of receptor trafficking during LTP are conducted in preparations of adult hippocampus. One exception is work from our lab using acute hippocampal CA1 minislices prepared from 6- to 8-week-old rats (Grosshans et al., 2002). LTP of field EPSPs (fEPSPs) was induced by application of HFS. More specifically, four 1-s trains of 100-Hz stimulation were separated by 30 s. Importantly, a multirake electrode was used to stimulate 16 points per minislice. This method ensures saturation of LTP within each minislice and increases the ratio of stimulated to unstimulated tissue used for biochemical analyses (Clayton et al., 2002a; Nayak et al., 1996). Both stimulated and control minislices were harvested 30 min after LTP induction and subjected to three independent biochemical assays to evaluate the subcellular distribution of glutamate receptors. All three measures indicated that neither the intracellular pool nor the total surface expression of the AMPAR subunits GluR1 and GluR2 was altered by LTP in adult rat CA1 (Grosshans et al., 2002). Importantly, these data do not address the possibility that AMPAR may be trafficked from extracellular sites to the synapse. Rather, these studies indicate that LTP-related trafficking from an intracellular pool to the synapse, as seen during the first few weeks of postnatal development (Hayashi et al., 2000; Esteban et al., 2003; Boehm et al., 2006), does not appear to occur in the adult. This result is likely due to the significantly smaller intracellular pool of AMPAR subunits present in adult CA1 (12–18%) as compared to 30–35% intracellular AMPAR subunits in CA1 hippocampus at postnatal day 9 (Grosshans et al., 2002). These results are consistent with electron microscopy studies demonstrating that synaptic AMPAR expression increases during development (Petralia et al., 1999). In the absence of altered AMPAR surface expression during early-phase LTP in adult hippocampus, the enhancement of AMPAR transmission may be largely due to increased channel conductance (Benke et al., 1998; Luthi et al., 2004). Alternatively, as mentioned earlier, AMPARs may be trafficked within the surface membrane (i.e., from extrasynaptic to synaptic sites).

In our forgoing discussions, we have only examined evidence that implicates AMPAR trafficking in early-phase LTP. There is also evidence to suggest a role for AMPARs in late-phase LTP. In one study, adult rats (250–500 g) were anesthetized, and hippocampal CA1 fEPSPs were recorded *in vivo* (Heynen et al., 2000). Robust LTP was achieved by application of 10 trains of HFS. More specifically, two 1-s trains of 100 Hz separated by 30 s were applied every 15 min for 1 h. Approximately 5.5 h after stimulation, GluR1 and GluR2 levels increased ~20% in synaptosomal fractions purified from LTP stimulated hippocampus versus nonstimulated yoked controls. This result is consistent with another study from our laboratory that suggests that maintenance of late-phase LTP correlates with a 45–55% increase in *de novo* synthesis of GluR1 and GluR2/3 subunits (Nayak et al., 1998). This increase in AMPAR synthesis was dependent on both transcription and translation. Additional experiments performed by Heynen et al. (2000) included application of low-frequency stimulation (LFS) to induce LTD; LFS was applied alone and in combination with LTP stimulation. The results of these experiments revealed a strong correlation between changes in GluR2 and GluR1 protein levels and changes in synaptic strength during late-phase LTP (Heynen et al., 2000). A key issue that was not addressed in these studies was how the newly synthesized AMPARs could be trafficked specifically to potentiated synapses. Because LTP is confined to stimulated synapses, some sort of targeted trafficking would be required for specific synaptic delivery of

new AMPARs resulting from transcriptional and translational events. We discuss briefly how NMDARs could play a role in such targeted trafficking in a later section of this chapter.

4.30.3.3 Summary

It has become more widely accepted that multiple mechanisms of hippocampal LTP evolve throughout the course of postnatal development (Grosshans et al., 2002; Malenka, 2003; Malinow, 2003; Yoshimura et al., 2003; Palmer et al., 2004). It is primarily from this perspective that we have examined studies of AMPAR phosphorylation and trafficking as mechanisms of hippocampal LTP (**Table 2**). It is also quite interesting that recent work indicates that AMPAR trafficking can occur during learning (Rumpel et al., 2005). Thus, the various mechanisms that contribute to LTP and learning may be complimentary and even additive, such that more complex mechanisms become required as animals mature.

Early in postnatal development, homomeric GluR4 receptors are delivered to silent synapses by spontaneous activity (Zhu et al., 2000). This process requires the membrane proximal region of the GluR4 C terminus, NMDARs, and PKA phosphorylation but not CaMKII (Zhu et al., 2000; Esteban et al., 2003; Boehm et al., 2006). GluR1 subunits may also contribute to mechanisms of synaptic plasticity during early postnatal development. Studies conducted in preparations corresponding to postnatal day ~9 have reported surface or spine delivery of GluR1 following induction of LTP (Shi et al., 1999; Hayashi et al., 2000; Grosshans et al., 2002). The mechanism of GluR1 trafficking remains unclear, but it likely involves CaMKII activity and/or interaction with one or more PDZ domain–containing proteins (Hayashi et al., 2000; Malenka, 2003; Yasuda et al., 2003; Kim et al., 2005).

Trafficking of GluR1 has been studied more intensely in the juvenile hippocampus, where NMDARs, CaMKII, Ras, PKC, and PKA signaling pathways all seem to make significant contributions to synaptic delivery of AMPARs following induction of LTP (Zhu et al., 2002; Esteban et al., 2003; Boehm et al., 2006; Oh et al., 2006). Interestingly, robust LTP occurs in the juvenile hippocampus of GluR1 knockout mice (Jensen et al., 2003), and considerable evidence suggests that GluR2L subunits may mediate GluR1-independent mechanisms of LTP during this stage of postnatal development (Zhu et al., 2002; Kolleker et al., 2003). Although the source of these newly incorporated AMPARs has not been widely studied, it is likely that the large intracellular pool of receptors constitutes a stock of AMPARs available for synaptic incorporation following LTP stimulation in these preparations (**Figure 1**; Grosshans et al., 2002; Park et al., 2004).

Although studies of knock-out mice demonstrate that GluR1 subunits are required for LTP at mature synapses (Zamanillo et al., 1999; Mack et al., 2001), the contribution of AMPAR trafficking to mechanisms of LTP is less well characterized in adult animals. It is likely that the intracellular pool does not constitute an adequate source for synaptic incorporation of AMPARs during early-phase LTP

Table 2 Glutamate receptor subunits trafficked during hippocampal LTP at various developmental stages, and the phosphorylation sites involved[a]

Subunit trafficked	*Equivalent developmental stage*	*Phosphosite implicated*	*Experimental preparation*	*Reference*
GluR1	P8–11	pS831 (CaMKII) not required	Organotypic hippocampal slices	Shi et al. (1999) Hayashi et al. (2000)
GluR1 and GluR2	P9	None evaluated	Hippocampal slices	Grosshans et al. (2002)
GluR1	P13–15	pS845 (PKA)	Organotypic hippocampal slices	Esteban et al. (2003)
GluR1	P17–18	pS818 (PKC)	Organotypic hippocampal slices	Boehm et al. (2006)
GluR2-lacking AMPARs	P14–21	None evaluated	Hippocampal slices	Plant et al. (2006)
NR1, NR2A, and NR2B	6–8 weeks	Unspecified Tyr(s) pY1472 (Fyn) on NR2B	Hippocampal slices	Grosshans et al. (2002) Nakazawa et al. (2001)

[a]Studies of other brain regions, dissociated neurons in culture, LTP-independent AMPAR trafficking, and chemically induced LTP are not included.

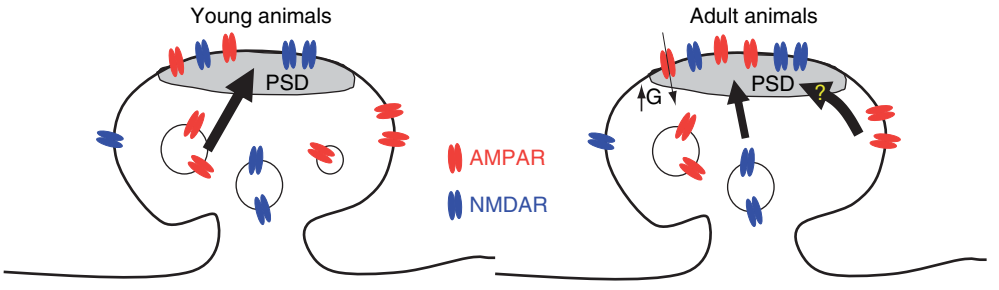


Figure 1 Proposed model for developmentally divergent mechanisms of AMPAR enhancement during LTP in the hippocampus. During postnatal development, hippocampal LTP leads to trafficking of AMPARs (red) from intracellular compartments to the postsynaptic density (PSD) of potentiated synapses (left). This mechanism does not occur in the hippocampus of mature animals, probably because the intracellular pool of AMPARs is much smaller than that in juvenile animals. Two alternative mechanisms, increased conductance (G) of existing AMPARs and lateral movement of AMPARs from extrasynaptic to synaptic sites, likely contribute to LTP of AMPARs in the adult hippocampus (right). Trafficking of NMDARs (blue) may contribute to one or both of these possibilities, as discussed in **Figure 2**.

(Grosshans et al., 2002). However, it remains possible that AMPARs are trafficked within the membrane (**Figure 1**). Alternatively, modulation of AMPAR channel conductance may be primarily responsible for enhanced transmission during the early phase of LTP in the adult hippocampus (Luthi et al., 2004).

4.30.3.4 Gaps in Our Knowledge

The precise mechanism of AMPAR potentiation in adult hippocampus remains one of the most intangible results in studies of LTP. As mentioned earlier, two possible mechanisms are (1) increased conductance of AMPARs already localized to the synapse and (2) lateral movement of extrasynaptic AMPARs to the postsynaptic density (**Figure 1**). There is clear evidence to suggest that the first mechanism occurs, at least in juvenile rats (Benke et al., 1998; Luthi et al., 2004). In support of the lateral trafficking mechanism, electrophysiological and single-molecule tracking studies indicate that AMPARs are highly mobile and move laterally within the plasma membrane (Choquet and Triller, 2003; Triller and Choquet, 2005). Additional studies suggest that synaptic and extrasynaptic glutamate receptors may be subject to differential regulation by synaptic activity and phosphorylation (Clark and Cull-Candy, 2002; Oh et al., 2006). Furthermore, detergent extraction studies indicate that distinct pools of glutamate receptors exist within the plasma membrane. Importantly, these pools exhibit differential association with the PSD (Alvestad et al., 2005). It is exciting to speculate that receptors that are loosely associated with the PSD represent a pool, possibly localized to extrasynaptic membrane, that may be subject to dynamic regulation in response to stimuli such as induction of LTP.

4.30.4 NMDA Receptor Trafficking in LTP

As discussed in the previous section, potentiation of AMPARs constitutes a majority of the enhanced postsynaptic responses seen after high-frequency stimulation of the hippocampal Schaffer collateral-CA1 synapse (Muller and Lynch, 1988). However, pharmacologically isolated NMDAR responses also undergo robust, synapse-specific LTP that can last for hours in many different LTP paradigms (Bashir et al., 1991; Xie et al., 1992; Grosshans et al., 2002; but also see Kauer et al., 1988). As mentioned previously, LTP induction requires NMDAR activation (Collingridge et al., 1983; Coan et al., 1987; Bashir et al., 1990) and tyrosine phosphorylation (O'Dell et al., 1991; Lu et al., 1998). In addition, NMDAR function is highly regulated by tyrosine phosphorylation (Wang and Salter, 1994; Chen and Leonard, 1996; Wang et al., 1996; Yu et al., 1997; Lu et al., 1999; Pelkey et al., 2002), and LTP of isolated NMDAR responses is blocked by tyrosine kinase inhibitors (Grosshans et al., 2002). Importantly, trafficking of NMDARs is also regulated by tyrosine phosphorylation (Dunah and Standaert, 2001; Roche et al., 2001; Vissel et al., 2001; Lavezzari et al., 2003, 2004; Goebel et al., 2005; Prybylowski et al., 2005; Snyder et al., 2005; Hallett et al., 2006; Nakazawa et al., 2006), and recent studies suggest that NMDAR surface expression is enhanced following LTP in an Src-family tyrosine kinase-dependent manner in area CA1 of the adult hippocampus (Grosshans et al., 2002). Therefore, it seems likely that potentiation and trafficking of NMDARs via tyrosine phosphorylation play key roles in LTP. We will now review evidence

concerning the mechanisms that underlie NMDAR trafficking and discuss the potential role of such trafficking in LTP in area CA1 of the hippocampus.

4.30.4.1 Molecular Biology of NMDARs

At depolarized membrane potentials, such as those achieved during high-frequency stimulation, NMDARs are permeable to Na^{2+}, K^{2+}, and Ca^{2+} (Ascher and Nowak, 1988). This influx of Ca^{2+} through the NMDAR is critical for LTP induction (Lynch et al., 1983) and is thought to lead to LTP via activation of downstream signaling cascades. To date, seven genes encoding the NMDAR subunits have been identified: NR1, NR2A-D, and NR3A-B (Moriyoshi et al., 1991; Meguro et al., 1992; Ishii et al., 1993; Ciabarra et al., 1995; Sucher et al., 1995; Chatterton et al., 2002). Functional NMDARs are thought to be tetrameric receptor complexes assembled as a pair of dimers formed by two NR1 subunits and two NR2 subunits (Ishii et al., 1993; Sheng et al., 1994; Laube et al., 1998; Schorge and Colquhoun, 2003), although the exact composition of synaptic NMDAR complexes remains unclear. (For a more comprehensive review of NMDAR molecular biology and structure, *See* Chapter 4.20.)

4.30.4.1.1 Developmental profile of NMDAR subunit expression

NR1, NR2A, and NR2B subunits are the most highly expressed NMDAR subunits in the adult hippocampus (Monyer et al., 1994; Nishi et al., 2001; Wong et al., 2002). NR1 mRNA (Monyer et al., 1994) and protein (Sheng et al., 1994; Sans et al., 2000) are abundant at birth and remain relatively constant throughout adulthood. Each of the three NR1 splice cassettes can be found in the hippocampus (Laurie and Seeburg, 1994; Coultrap et al., 2005); however, the C1 and C2 C-terminal cassettes are the predominant ones expressed in area CA1 of the adult hippocampus (Coultrap et al., 2005). Importantly, NR2 subunits have distinct patterns of developmental expression in the hippocampus. *In situ* hybridization studies show that NR2B is the primary NR2 mRNA expressed at birth in the hippocampus; however, in adults, NR2A and NR2B mRNA are both highly expressed, especially in areas CA1 and CA3 (Monyer et al., 1994). Interestingly, Sans et al. (2000) report that NR2A and NR2B proteins display opposing patterns of expression in the hippocampus. Specifically, this study reported that NR2A protein expression is undetectable at postnatal day 2 and substantially increases from postnatal day 10 to 6 months (Sans et al., 2000). Conversely, NR2B protein expression peaks early in development (postnatal days 2–10) and gradually decreases to 30% of maximum at 6 months (Sans et al., 2000). Importantly, NR2A and NR2B are still the most prevalent NR2 subunits expressed in the adult hippocampus (Monyer et al., 1994). NR2A and NR2B subunits also have distinct protein expression profiles within the different subregions of the hippocampus (Coultrap et al., 2005). For example, NR2A protein expression does not significantly differ among the three subregions; however, NR2B protein expression is significantly greater in area CA1 than the CA3 and dendate gyrus (Coultrap et al., 2005).

4.30.4.1.2 Tyrosine phosphorylation of NMDARs

It is well understood that NMDAR function can be modulated by tyrosine kinases and phosphatases. Src-family tyrosine kinases enhance NMDAR function (Wang and Salter, 1994; Lu et al., 1998), whereas tyrosine phosphatases decrease NMDAR function (Wang and Salter, 1994; Pelkey et al., 2002). Given that tyrosine kinases are required for induction of LTP (O'Dell et al., 1991; Lu et al., 1998), it is possible that tyrosine phosphorylation modulates NMDAR function during LTP induction. In fact, tyrosine phosphorylation of the NR2A (Grosshans et al., 2002) and NR2B (Rosenblum et al., 1996; Rostas et al., 1996; Nakazawa et al., 2001) subunits is enhanced following LTP. Moreover, Salter (1998) has proposed a model in which LTP induction leads to phosphorylation and enhancement of NMDARs via Src-family tyrosine kinase activation.

Although one group reported that NR1 surface expression is modulated by phosphorylation of a tyrosine residue (tyrosine 837) on NR1 in HEK293 cells (Vissel et al., 2001), NR1 does not appear to be tyrosine phosphorylated in brain (Lau and Huganir, 1995). Conversely, both NR2A and NR2B are tyrosine phosphorylated in brain homogenates (Lau and Huganir, 1995). Indeed, the intracellular C-terminal tails of NR2A and NR2B each contain 25 tyrosine residues that can be phosphorylated by nonreceptor tyrosine kinases, including Fyn and Src (Tezuka et al., 1999; Cheung and Gurd, 2001; Nakazawa et al., 2001; Yang and Leonard, 2001).

Studies in HEK293 cells report four potential sites of Src-mediated phosphorylation on the NR2A subunit: tyrosine 1105, tyrosine 1292, tyrosine 1325, and tyrosine 1387 (Zheng et al., 1998; Yang and Leonard,

2001). Two of these sites, tyrosine 1105 and tyrosine 1387, appear to modulate NR2A channel current by Src (Zheng et al., 1998); however, the role of tyrosine 1292 and tyrosine 1325 remains uncertain. Phosphorylation of another tyrosine residue on NR2A, tyrosine 1267, seems to play an important role in Src-induced potentiation of NR2A-containing receptors by reducing tonic inhibition of NMDARs by zinc (Zheng et al., 1998). Furthermore, phosphorylation of NR2A tyrosine 842 appears to inhibit internalization of NR1/NR2A recombinant receptors in HEK293 cells (Vissel et al., 2001). Although these studies demonstrate that the NR2A subunit can be tyrosine phosphorylated in heterologous cell systems, the exact sites of NR2A phosphorylation and their function in neurons remain incompletely understood. To the best of our knowledge, phosphorylation of only one of the aforementioned NR2A sites, tyrosine 1387, has been reported in brain (Besshoh et al., 2005). Thus, further work is required to determine the NR2A phosphorylation sites in neurons.

The NR2B subunit is reportedly the most tyrosine phosphorylated protein found within the PSD (Moon et al., 1994). *In vitro* kinase assays and work in HEK293 cells demonstrate that the NR2B subunit can be phosphorylated by Fyn at tyrosine 1252, tyrosine 1336, and tyrosine 1472 (Nakazawa et al., 2001). Importantly, tyrosine 1472 appears to be phosphorylated by both exogenous and endogenous PSD-associated Src-family tyrosine kinases (Cheung and Gurd, 2001). Phosphorylation of NR2B tyrosine 1472 has been proposed to regulate synaptic localization of NR2B-containing NMDARs (Roche et al., 2001; Lavezzari et al., 2003, 2004; Goebel et al., 2005; Prybylowski et al., 2005). It is important to note that phosphorylation of NR2B tyrosine 1472 increases after LTP induction in the hippocampus (Nakazawa et al., 2001). Phosphorylation of tyrosine 1252 and tyrosine 1336 has also been reported in neurons (Yaka et al., 2003); however, their role in LTP and NMDAR trafficking is incompletely understood. Because very little is known about the remaining 22 tyrosine residues located on the NR2B subunit, it is important for future studies to determine which additional NR2B residues are phosphorylated in neurons and to examine their role in regulating NMDARs.

4.30.4.1.3 Serine/threonine phosphorylation of NMDARs

Inhibitors of PKC block induction of LTP (Malinow et al., 1989). In addition, PKC activity and autophosphorylation are persistently elevated during LTP induction and maintenance (Klann et al., 1991, 1993; Sweatt et al., 1998). As such, PKC-mediated phosphorylation of its substrates appears to be an important part of LTP. We have shown that intracellular application of the constitutively active fragment of PKC potentiates NMDA-evoked currents by increasing the probability of NMDAR channel opening in cultured neurons and isolated CA1 hippocampal neurons (Xiong et al., 1998). This PKC-induced potentiation of NMDA-evoked currents in neurons is also dependent on the activation of Src-family tyrosine kinases (Lu et al., 1999). Notably, one study in oocytes suggests that PKC-induced potentiation of NMDARs is not due to direct phosphorylation of NR1 (Zheng et al., 1999) but is a result of phosphorylation of adaptor proteins, such as SNAP-25 (Lan et al., 2001).

Perhaps the most well characterized NMDAR PKC sites are found on the NR1 subunit. Tingley et al. (1997) reported that PKC activation increased phosphorylation of serine 890 and serine 896 in hippocampal slice homogenates. Phosphorylation of serine 890, but not serine 896, has been shown to increase NR1 surface expression in heterologous cell lines (Tingley et al., 1997). Two additional NR1 PKC sites have been identified *in vitro* (serine 889 and threonine 879); however, these sites have not been evaluated in neurons (Tingley et al., 1997). NR2A and NR2B can also be phosphorylated by PKC *in vitro* (Leonard and Hell, 1997). One study reported that NR2A can be phosphorylated by PKC at serine 1416 and suggested that this phosphorylation event inhibited CaMKII binding *in vitro* (Gardoni et al., 2001). Another group showed that PKC phosphorylation of NR2B at serine 1303 and serine 1323 enhances NR1/NR2B currents in oocytes (Liao et al., 2001b). However, the consequence of phosphorylating these sites remains incompletely understood in neurons.

PKA potentiation of NMDAR function has also been reported. In particular, PKA activation appears to overcome the constitutive activity of protein phosphatase 1, enabling PKA to enhance NMDAR currents in cultured neurons (Raman et al., 1996). A more recent report suggests that PKA facilitates LTP induction early in development by increasing Ca^{2+} entry through NMDARs (Skeberdis et al., 2006). Tingley et al. (1997) showed that the NR1 subunit is phosphorylated by PKA at serine 897 in brain homogenates. Notably, although both NR2A and NR2B can be phosphorylated by PKA *in vitro* (Leonard and Hell, 1997), the exact sites are unknown.

Other serine/threonine kinases are also capable of phosphorylating the NMDAR. NR2A appears to be phosphorylated by CaMKII at serine 1289 *in vitro* (Gardoni et al., 1999); however, it is unknown whether this residue is phosphorylated in neurons. NR2B serine 1303 is a major site of CaMKII phosphorylation in neurons (Omkumar et al., 1996), and this phosphorylation event appears to inhibit binding of CaMKII to NR2B in heterologous cells (Strack et al., 2000). Moreover, phosphorylation of NR2A by cyclin-dependent kinase 5 at serine 1232 has been proposed to be involved in LTP induction (Li et al., 2001). Furthermore, casein kinase II has been shown to phosphorylate serine 1480 on the NR2B subunit (Chung et al., 2004).

4.30.4.2 General Trafficking Mechanisms of NMDARs

Although NMDARs were previously thought of as a stable component of the PSD, mounting evidence in recent years has shown that NMDARs are actually a dynamic component of the PSD that can be trafficked between intracellular and plasma membrane compartments (reviews: Wenthold et al., 2003; Prybylowski and Wenthold, 2004), as well as between extrasynaptic and synaptic membrane compartments (Tovar and Westbrook, 2002). In addition, tyrosine phosphorylation has been proposed to modulate NMDAR function by regulating NMDAR trafficking. Roche et al. (2001) showed that endocytosis of NR2B-containing NMDARs in heterologous cells and dissociated neuronal cultures is dependent on the presence of a consensus tyrosine-specific internalization motif (YXXØ; Y = tyrosine, X = any amino acid, Ø = hydrophobic amino acid) located within the distal NR2B C-terminal tail. Binding of the clathrin-associated adaptor protein 2 (AP-2) $\mu 2$ chain to the NR2B subunit seems to require unphosphorylated tyrosine 1472, which is located within the YXXØ motif on the NR2B C-terminal tail (Lavezzari et al., 2003). Consistent with this hypothesis, recent evidence suggests that NR2B tyrosine 1472 phosphorylation and association with AP-2 are inversely correlated (Nakazawa et al., 2006). As a result, phosphorylated NR2B subunits remain bound to the cytoskeletal synapse-anchoring protein, α-actinin 2 (Nakazawa et al., 2006). Indeed, expression of a NR2B Y1472A mutant increases the number of synaptic NMDARs in dissociated neuronal cultures (Prybylowski et al., 2005). A similar YXXØ motif has been identified in the proximal C terminus of the NR2A subunit (Vissel et al., 2001). Mutation of the tyrosine residue within this motif (tyrosine 842) to phenylalanine enhances NR1/NR2A peak current in HEK293 cells, an effect that appears to be due to decreased clathrin-mediated endocytosis of NR1/NR2A receptors (Vissel et al., 2001).

Together, these studies provide evidence that phosphorylation of specific tyrosine residues regulates NMDAR trafficking in cultured systems. These mechanisms of NMDAR trafficking are also mirrored *in situ*. In particular, treatment of hippocampal slices with a tyrosine phosphatase inhibitor or Src-family tyrosine kinase inhibitor increases or decreases, respectively, NR2A and NR2B tyrosine phosphorylation, as well as the concentration of NMDARs in synaptosomal membrane fractions (Goebel et al., 2005). Notably, phosphorylation of tyrosine 1472 on the NR2B subunit is enhanced when hippocampal slices are treated with a tyrosine phosphatase inhibitor, suggesting a correlation between increased NR2B tyrosine 1472 phosphorylation and increased surface expression of the NMDAR (Goebel et al., 2005). Consistent with this hypothesis, Dunah and Standaert (2001) have established that dopamine D1 receptor activation leads to NMDAR movement to the membrane in striatal slices; this effect appears to be dependent on tyrosine phosphorylation. Moreover, in rat forebrain, ischemic challenge enhances NR2A and NR2B tyrosine phosphorylation and increases association of these subunits in the PSD (Besshoh et al., 2005).

We cannot exclude the possibility that phosphorylation of residues other than tyrosine 1472 regulates NMDAR trafficking and protein–protein binding interactions in the PSD. For example, phosphorylation of serine 1480 on the NR2B subunit negatively regulates binding of NR2B to the synaptic scaffolding proteins PSD-95/SAP90 and decreases NMDAR surface expression in cultured cortical neurons (Chung et al., 2004). Moreover, coordinated phosphorylation of specific PKC and PKA sites on the NR1 subunit (serine 896 and serine 897, respectively) has been shown to overcome ER retention signals and enhance surface delivery of NR1-containing receptors in heterologous cells and cultured neurons (Scott et al., 2003). It is also important to note that phosphorylation-mediated events are not likely the only means of modulating NMDAR trafficking. Indeed, recent evidence suggests that trafficking of the NR2A subunit is regulated by a dileucine motif in heterologous cells (Lavezzari et al., 2004).

4.30.4.3 Evidence for NMDAR Trafficking in LTP

The data just discussed have naturally led to the question of what is/are the physiological stimulations that trigger NMDAR trafficking to the synapse. Of particular importance to this review, Grosshans et al. (2002) reported that LTP stimulation leads to rapid redistribution of NMDARs from intracellular compartments to the synaptosomal membrane in area CA1 of the adult rat hippocampus (Grosshans et al., 2002). As described in the previous section, these authors saw no change in AMPAR surface expression in CA1 mini-slices obtained from adult animals following LTP. These data are in marked contrast to the selective increase in AMPAR surface expression that seems to occur early in postnatal development after LTP induction (Shi et al., 1999; Hayashi et al., 2000; Grosshans et al., 2002; Esteban et al., 2003; Boehm et al., 2006). In adults, up to 40% of total NR1, NR2A, and NR2B reside intracellularly in area CA1, providing a substantial pool of intracellular NMDARs that are available for recruitment to the synaptic membrane following LTP (Grosshans et al., 2002). Moreover, it is important to note that the increase in NMDAR surface expression seen following LTP in adults occurs in a PKC- and Src-family tyrosine kinase-dependent manner that persists for at least 3 h (Grosshans et al., 2002). As such, these findings highlight differential involvement of AMPAR and NMDAR trafficking in LTP induction between the neonatal and adult hippocampus. Because NMDARs do not significantly contribute to the enhancement of postsynaptic responses after LTP (Muller and Lynch, 1988), perhaps trafficking of NMDARs to the synaptic membrane in adults plays an indirect role in enhancing AMPAR function.

Consistent with the hypothesis that NMDARs can be trafficked as a result of synaptic activity, Quinlan et al. (1999) reported that NR2A-containing receptors are selectively trafficked to synaptoneurosomes in the adult primary visual cortex following light exposure, which is a form of sensory experience–dependent synaptic plasticity. Furthermore, recent work suggests that NMDAR-dependent LTD causes internalization of NMDARs via dynamin-dependent endocytosis and thus leads to a depression in isolated NMDAR currents (Montgomery et al., 2005; but see Morishita et al., 2005). Although the precise molecular mechanisms underlying NMDAR trafficking during these various types of synaptic plasticity are not well characterized, tyrosine phosphorylation likely plays an important role.

To date, relatively few studies have examined the correlation between NMDAR tyrosine phosphorylation, NMDAR surface expression, and LTP. As previously discussed, one exception is previous work from Nakazawa et al. (2001), which showed increased phosphorylation of NR2B tyrosine 1472 following LTP induction in the hippocampus. This study, along with work showing that nonphosphorylated tyrosine 1472 is important for endocytosis of NR2B-containing receptors (Roche et al., 2001; Lavezzari et al., 2004; Prybylowski et al., 2005), suggests the possibility that LTP increases tyrosine 1472 phosphorylation and blocks endocytosis of NR2B-containing receptors, thereby enhancing NMDAR function during LTP (Bashir et al., 1991; Xie et al., 1992; Grosshans et al., 2002). In agreement, our lab has reported that NMDAR surface expression is enhanced following LTP in the adult hippocampus (Grosshans et al., 2002). This study also provided evidence for the sequential activation of PKC and Src-family tyrosine kinases after LTP as a means to increase NMDAR tyrosine phosphorylation and suppress internalization of synaptic NMDARs (Grosshans and Browning, 2001; Grosshans et al., 2002). Moreover, a recent study by Nakazawa et al. (2006) showed that amygdaloid LTP is impaired in mice with a knock-in mutation of NR2B tyrosine 1472 to phenylalanine (Y1472F). Interestingly, localization of NR2B was found in more peripheral and perisynaptic regions of the PSD in the Y1472F mice (Nakazawa et al., 2006). These results suggest that phosphorylation of tyrosine 1472 and synaptically localized NR2B are essential for expression of LTP in the amygdala (Nakazawa et al., 2006). Although it has been shown that LTP increases NR2B tyrosine 1472 phosphorylation in the hippocampus (Nakazawa et al., 2001), hippocampal LTP was not affected in the Y1472F mice (Nakazawa et al., 2006). The authors suggest that some unknown compensatory mechanism may have developed in the Y1472F mice to suppress the hippocampal phenotype observed in the Y1472F mice (Nakazawa et al., 2006). Another possibility is that residues or subunits other than or in addition to tyrosine 1472 and NR2B, respectively, are responsible for hippocampal synaptic plasticity (Nakazawa et al., 2006).

4.30.4.4 NMDAR Subunit Requirements for Synaptic Plasticity

The contribution of the different NMDAR NR2 subunits to the expression of synaptic plasticity has been a

topic of intense debate in recent years. Clayton et al. (2002b) previously demonstrated that selective knock-down of the NR2B subunit using antisense oligonucleotides decreased isolated NMDAR fEPSPs and completely inhibited NMDAR-dependent LTP in the hippocampus. Furthermore, this study showed that NR2B expression and hippocampal-dependent spatial learning were significantly correlated, suggesting that the NR2B subunit has an important role in the expression of hippocampal LTP and behavioral learning (Clayton et al., 2002b). Notably, Tang et al. (1999) reported that overexpression of the NR2B subunit results in 'smart' mice, where LTP and spatial learning are enhanced.

More recent studies have utilized pharmacological antagonists to determine the role of NR2A and NR2B in LTP and LTD (Williams, 1993; Auberson et al., 2002). Using antagonists at doses purported to exhibit NR2A and NR2B selectivity, some studies concluded that NR2A-containing receptors are responsible for LTP induction, whereas NR2B-containing receptors are responsible for LTD induction (Liu et al., 2004; Massey et al., 2004). However, others provide compelling evidence that both the NR2A and NR2B subunits are capable of generating LTP and/or LTD (Berberich et al., 2005; Toyoda et al., 2005; Weitlauf et al., 2005; Zhao et al., 2005). Importantly, Neyton and Paoletti (2006) recently described the necessity of careful interpretation when analyzing data obtained using such NR2A and NR2B pharmacological antagonists. In particular, it seems as though the NR2A antagonist, NVP-AAM077, does not discriminate well between NR2A- and NR2B-containing receptors at the doses used by the studies cited above (Liu et al., 2004; Massey et al., 2004). In fact, one group suggested that this compound is not NR2A-selective at any concentration (Neyton and Paoletti, 2006). Furthermore, until recently, there has been no evidence to describe the selectivity of these pharmacological antagonists on triheteromeric NR1/NR2A/NR2B-containing receptors (Hatton and Paoletti, 2005), even though good evidence indicates that triheteromeric NMDARs exist in brain (Sheng et al., 1994; Cull-Candy and Leszkiewicz, 2004). One of the future challenges of the NMDAR field will be to reconcile these conflicting results with pharmacological tools that are able to take the complex, heteromeric nature of NMDARs into account.

4.30.4.5 Summary

Mounting evidence demonstrates that NMDARs can be trafficked between intracellular compartments and the plasma membrane, as well as between extrasynaptic and synaptic membrane compartments. In addition, phosphorylation of key tyrosine residues enhances the surface expression of NMDARs (Roche et al., 2001; Vissel et al., 2001; Lavezzari et al., 2004; Goebel et al., 2005; Prybylowski et al., 2005). Given that tyrosine phosphorylation of NR2A and NR2B subunits is increased following LTP induction (Rosenblum et al., 1996; Rostas et al., 1996; Nakazawa et al., 2001; Grosshans et al., 2002), it is reasonable to hypothesize that LTP also increases NMDAR surface expression. Indeed, work from our laboratory showed that the surface expression of NR1, NR2A, and NR2B is enhanced following LTP in area CA1 of the adult hippocampus (Grosshans et al., 2002). This process requires the sequential activation of PKC and Src-family tyrosine kinases, which is consistent with the hypothesis that tyrosine phosphorylation regulates NMDAR surface expression (Grosshans et al., 2002).

The notion that NMDARs are trafficked to the synapse following synaptic plasticity is not new. NR2A-containing receptors are selectively enriched in synaptoneurosomes prepared from the adult primary visual cortex following light exposure (Quinlan et al., 1999). NMDARs also appear to be internalized during LTD (Heynen et al., 2000; Montgomery et al., 2005). The mechanisms of NMDAR trafficking in these two forms of synaptic plasticity remain unclear, but they may involve tyrosine phosphorylation of the NMDAR.

The contribution of the different NR2 subunits in LTP is clearly still up for debate. Using pharmacological antagonists, some groups suggest that the NR2A subunit, but not the NR2B subunit, is required for LTP (Liu et al., 2004; Massey et al., 2004). However, other studies have shown that NR2B also plays an important role in LTP (Tang et al., 1999; Clayton et al., 2002b). Because recent reports cast doubt on the use of the NR2A pharmacological antagonist to determine the role of NR2A in LTP (Berberich et al., 2005; Weitlauf et al., 2005; Zhao et al., 2005), more sophisticated tools that are able to take the heteromeric nature of NMDARs into account are required to settle this problem.

4.30.4.6 Gaps in Our Knowledge

Key questions still remain regarding the role of NMDAR trafficking in LTP. Because the majority of LTP is expressed as an enhancement of AMPAR-mediated responses (Muller and Lynch, 1988), it remains unclear how increasing NMDAR surface expression contributes to LTP in the adult

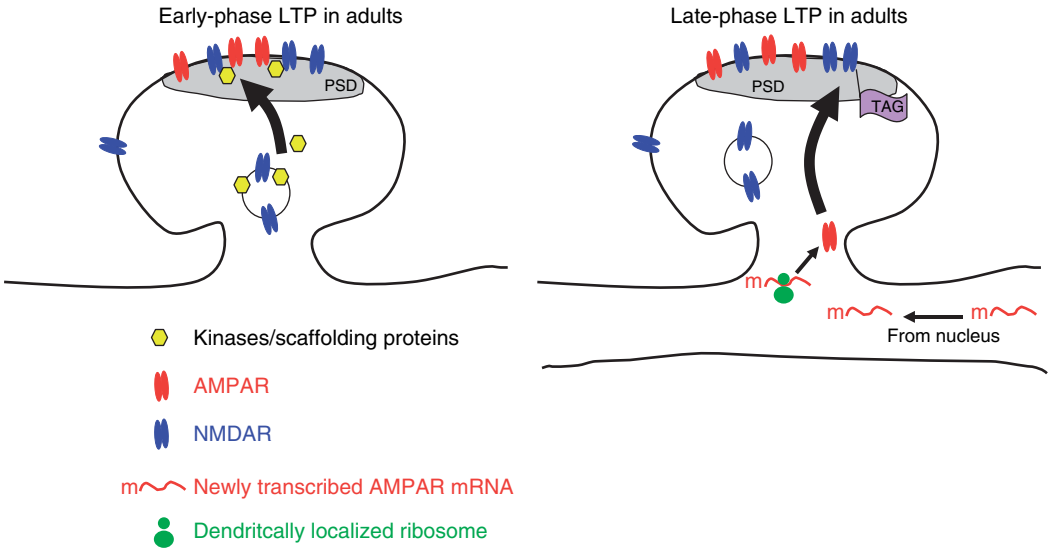


Figure 2 Proposed model for how increased *N*-methyl-D-aspartate receptor (NMDAR) surface expression during long-term potentiation (LTP) may enhance alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor (AMPAR) function in the adult hippocampus. During early phase LTP in adults, NMDAR (blue) surface expression is increased. Because AMPARs (red) still constitute a majority of the enhanced excitatory postsynaptic potential after LTP, a mechanism other than increased AMPAR surface expression is required to potentiate the response. Certainly one possibility is lateral movement of AMPARs from extrasynaptic to synaptic sites, as proposed in **Figure 1**. It is also possible that NMDARs may act as chaperone molecules to transport kinases and/or scaffolding proteins (yellow) from intracellular compartments to the membrane during early-phase LTP (top). Translocation of these molecules to the postsynaptic density (PSD) may lead to increased conductance of AMPARs already present in the synapse. A third possibility is that increased NMDAR surface expression serves as a synaptic tag for targeted insertion of AMPARs during late-phase LTP (bottom). These receptors may be newly synthesized AMPARs shuttled from intracellular compartments or extrasynaptic membrane.

hippocampus. The data we reviewed here suggest that it may play an important role. In particular, inhibitors that decrease NMDAR surface expression also inhibit LTP (O'Dell et al., 1991; Lu et al., 1998; Dunah and Standaert, 2001; Goebel et al., 2005). Perhaps NMDARs are acting as chaperones and transporting kinases or scaffolding proteins to the synaptic membrane, where they can then enhance AMPAR responses (**Figure 2**). It is exciting to speculate that activated CaMKII may move with NMDARs to the synapse during LTP, where it can then phosphorylate serine 831 on GluR1-containing receptors (Barria et al., 1997) already localized in the synapse. CaMKII has been shown to bind tightly to NMDARs (Strack and Colbran, 1998; Leonard et al., 1999) and translocates to the PSD following synaptic stimulation (Shen and Meyer, 1999; Otmakhov et al., 2004). Given that phosphorylation of serine-831 by CaMKII potentiates GluR1 channel current (Barria et al., 1997), and AMPAR channel conductance is enhanced following LTP (Benke et al., 1998), it is conceivable that NMDARs chaperone CaMKII to the PSD and are responsible, at least in part, for mediating LTP in adults. Another intriguing possibility is that synapses with enhanced NMDAR surface expression serve as a tag for targeted insertion of newly synthesized AMPARs at potentiated synapses during the late phase of LTP (**Figure 2**; Nayak et al., 1998). Further investigation is required to rigorously address these questions and determine the role(s) of NMDAR trafficking following LTP in adults.

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4.31 AMPA Receptor Regulation and the Reversal of Synaptic Plasticity – LTP, LTD, Depotentiation, and Dedepression

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4.31.1 Introduction

Activity-dependent bidirectional changes in synaptic strength are recognized to be critical in mediating various functions of the brain, including learning and memory. Long-term potentiation (LTP) and long-term depression (LTD) are the two most studied forms of activity-dependent synaptic plasticity mechanisms, and both are implicated to play a role in memory formation. While LTP and LTD have been described in various synapses in the nervous system (reviewed in Malenka and Bear, 2004), molecular mechanisms of LTP and LTD are perhaps best understood at one particular set of synapses in the central nervous system: the Schaffer collateral inputs synapsing onto CA1 pyramidal neurons. The more commonly studied forms of LTP and LTD at this set of synapses are dependent on the activation of *N*-methyl-D-aspartate (NMDA) receptors, which act as coincident detectors of pre- and postsynaptic activity (reviewed in Malenka and Bear, 2004). A consequence of NMDA receptor activation is a rise in intracellular calcium, which acts on various signaling molecules, including several types of protein kinases and protein phosphatases, which can alter the biochemical makeup of the synapse. There is mounting evidence that one of the downstream molecules affected by NMDA receptor activation is alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor. AMPA receptors are glutamate-gated ion channels, which mediate the majority of fast excitatory synaptic transmission in the central nervous system. They comprise four types of subunits, glutamate receptors (GluRs) 1–4 (or GluR A–D), which assemble in a combinatorial fashion (Nakanishi et al., 1990; Wenthold et al., 1992) into a tetrameric channel (Rosenmund et al., 1998; Safferling et al., 2001). Different subunits confer distinct properties to the receptor complex and show distinct developmental and spatial expression patterns (reviewed in Hollmann and Heinemann, 1994). For example, incorporation of GluR2 into the AMPA receptor complex makes the channel impermeable to calcium and contributes to the linear current-voltage relationship of the current flux (Hollmann et al., 1991; Verdoorn et al., 1991; Washburn et al., 1997). At least in the adult hippocampus, the majority of AMPA receptors comprise GluR1 and GluR2 subunits (Wenthold et al., 1996). As will be discussed further, regulation of AMPA receptor function has emerged as a critical endpoint for bidirectional synaptic plasticity (Malinow and Malenka, 2002; Lee, 2006a).

4.31.2 Reversibility of LTP and LTD

A key property of LTP and LTD is that they are readily reversible, which suggests that they are probably acting on common mechanisms for expression. Earlier evidence demonstrating reversibility came from electrophysiological studies using the fact that LTP and LTD can be saturated by multiple bouts of

high-frequency stimulation (HFS) and low-frequency stimulation (LFS), respectively. It was shown that after saturating LTP, LFS that elicits LTD 'unsaturates' LTP, so that a subsequent HFS can result in further potentiation (Dudek and Bear, 1993). Conversely, following LTD saturation, HFS can reverse LTD and increase the responses near the original baseline, and a further LFS can once again depress synaptic responses (Mulkey and Malenka, 1992). These results indicate that, when LTP increases synaptic weight through a cellular process, LTD can reverse this to decrease the synaptic strength, and that both synaptic changes are mediated by changing the same parameters but in opposite directions.

The observations that LTD and LTP mutually reverse each other led to the question as to whether LTP or LTD in a 'naive' pathway is the same as LTP or LTD in previously depressed or potentiation pathways. To distinguish these processes, the reversal of LTP is referred to as depotentiation (DeP) and reversal of LTD is called dedepression (DeD). Questions as to whether LTP or LTD *per se* is different from their reversal counterparts arose especially from earlier studies where several groups could not induce LTD in naive pathways, while still successfully inducing DeP (Barrionuevo et al., 1980; Staubli and Lynch, 1990; Fujii et al., 1991; Larson et al., 1993; O'Dell and Kandel, 1994). Although this may have come from differences in experimental preparations and/or stimulation paradigms, there is now accumulating evidence that the molecular underpinnings of LTD and DeP may indeed be different. Similarly, molecular mechanisms of LTP and dedepression seem to differ as well.

This review will focus on the regulation of AMPA receptors in LTP, LTD, DeD, and DeP. As will be discussed later, there is a form of DeP that can only occur within a critical time window after LTP induction, which is termed 'deconsolidation' (Bear and Abraham, 1996). We will compare the mechanisms of deconsolidation to DeP proper. In addition, mechanisms that lead to 'decay' of LTP (i.e., inability to maintain LTP) in terms of AMPA receptor regulation will also be discussed. A schematic comparison of all these processes is provided in **Figure 1**.

4.31.3 AMPA Receptor Regulation and LTP

Since the initial proposal of Lynch and Baudry (1984), regulation of AMPA receptors has emerged as one of the core mechanisms controlling synaptic strength. There is now a wealth of evidence that LTP is associated with an increase in synaptic AMPA receptor function. Activity-dependent changes in synaptic AMPA receptor function can be mediated by both alterations in the number of functional AMPA receptors or by changes in existing AMPA receptor function by mechanisms such as phosphorylation. There are data supporting both methods of AMPA receptor regulation during LTP, and these two mechanisms may be linked to each other.

An initial indication that LTP is associated with an increase in the number of functional AMPA receptors came from studies demonstrating the existence of 'silent synapses,' which lack functional AMPA receptors (Isaac et al., 1995; Liao et al., 1995). Importantly, these silent synapses could become functional after LTP induction (Isaac et al., 1995; Liao et al., 1995), which suggests that an LTP-inducing stimulus somehow allows functional AMPA receptors to be expressed at synapses. One of the first convincing demonstrations that LTP is indeed associated with synaptic incorporation of AMPA receptors came from Malinow's group, which visualized green fluorescent protein-tagged GluR1 subunits moving into spines following LTP using two-photon microscopy (Shi et al., 1999). Subsequent studies showed that these newly inserted AMPA receptors are functional and participate in synaptic transmission by measuring synaptic responses from electrophysiologically tagged GluR1 homomeric receptors (Hayashi et al., 2000; Shi et al., 2001). Synaptic insertion of AMPA receptors by LTP is not a universal feature of all AMPA receptor subunits, but only of GluR1 (Shi et al., 1999; Hayashi et al., 2000; Shi et al., 2001). On the other hand, exogenously transfected homomeric GluR2s are constitutively recycled into synapses independent of synaptic activity (Passafaro et al., 2001; Shi et al., 2001). The signal for the subunit-specific regulation was shown to be contained in the intracellular carboxy terminal regions of each subunit (Passafaro et al., 2001; Shi et al., 2001). The activity-dependent synaptic insertion of GluR1-containing AMPA receptors requires Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) activity (Hayashi et al., 2000; Shi et al., 2001) and depends on phosphorylation of GluR1-S818 (Boehm et al., 2006) and GluR1-S845 (Esteban et al., 2003) residues, but not GluR1-S831 (Hayashi et al., 2000). Proposed roles of these phosphorylation sites in LTP will be discussed in detail. An emerging view is that activity-dependent insertion of GluR1-containing AMPA receptors occurs at extrasynaptic sites followed by a lateral movement into synapses (Passafaro et al.,

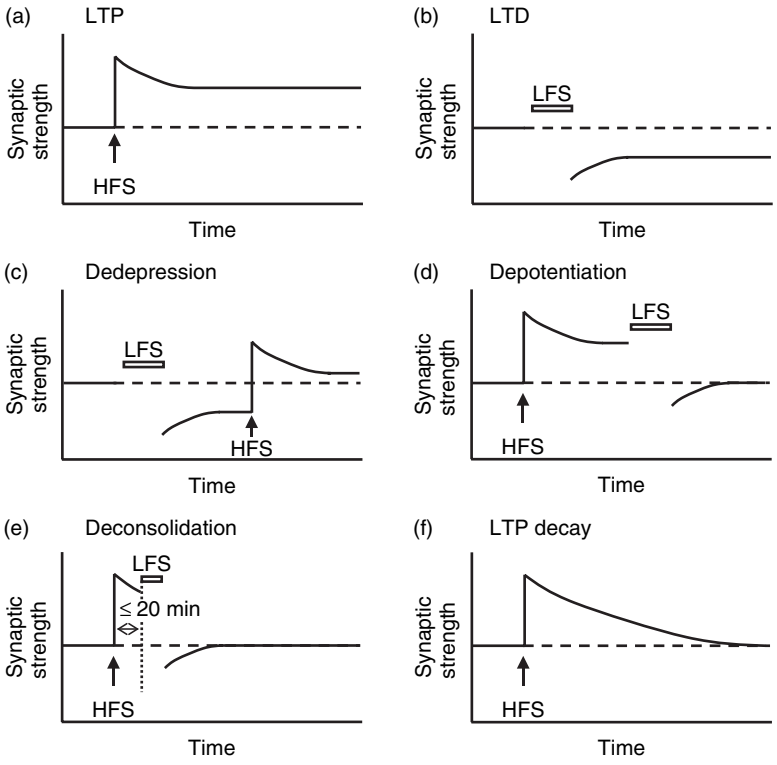


Figure 1 Different forms of synaptic plasticity. (a) Long-term potentiation (LTP). Brief trains of high-frequency stimulation (HFS) at a range of 100 Hz or theta burst pattern leads to a long-lasting increase in synaptic strength. (b) Long-term depression (LTD). A prolonged train of low-frequency stimulation (LFS) in the range of 1–3 Hz leads to a long-lasting decrease in synaptic strength. (c) Dedepression. LTD can be reversed by the same types of HFS used for inducing LTP. (d) Depotentiation. LTP can be reversed by a long train of LFS in the range of 1–5 Hz, often the same stimulation used for LTD induction. (e) Depotentiation that can only occur within a critical time window after LTP induction is sometimes referred to as deconsolidation. Stimulations that normally would not produce LTD (e.g., a shorter train of LFS) can result in deconsolidation. (f) LTP decay. Sometimes LTP fails to maintain stable expression and decays back to the original baseline.

2001; Andrasfalvy et al., 2003; Park et al., 2004). Recent data suggest that these newly inserted receptors containing GluR1, at least the ones lacking GluR2, are then replaced over time by GluR2-containing receptors (McCormack et al., 2006; Plant et al., 2006). Heteromeric GluR1s/GluR2s may also insert into synapses following LTP, since GluR1 acts dominantly over GluR2 (Shi et al., 2001).

The initial excitement over the role of AMPA receptor phosphorylation in LTP came from observations that phosphorylation of GluR1-S831 (Barria et al., 1997b; Lee et al., 2000), which may occur by CaMKII (Barria et al., 1997a; Mammen et al., 1997) and/or protein kinase C (PKC) (Roche et al., 1996), is increased following LTP. However, phosphorylation of this site increases single-channel conductance of only the homomeric GluR1s (Derkach et al., 1999; Oh and Derkach, 2005), while having no effect on heteromeric GluR1s/GluR2s (Oh and Derkach, 2005). This is interesting in light of recent data suggesting that there may be a transient insertion of homomeric GluR1s following LTP (McCormack et al., 2006; Plant et al., 2006). It is likely that GluR1-S831 phosphorylation may mediate the observed increase in AMPA receptor single-channel conductance following LTP induction (Benke et al., 1998; Poncer et al., 2002; Luthi et al., 2004). However, since the newly inserted homomeric GluR1s are replaced by GluR2-containing receptors over time (McCormack et al., 2006; Plant et al., 2006), this predicts that the increase in single-channel conductance would be transient. Whether this happens is unknown at present, but the time-dependent switch in AMPA receptor subunit composition may explain the cell-to-cell variability in detecting changes in AMPA receptor unitary conductance following LTP induction (Poncer et al., 2002; Luthi et al., 2004).

Phosphorylation of GluR1-S845 residue is also thought to participate in LTP by regulating CaMKII-dependent synaptic insertion of AMPA receptors (Esteban et al., 2003). This site is phosphorylated by protein kinase A (PKA) (Roche et al., 1996), which increases mean open probability of AMPA receptor channels (Banke et al., 2000). However, S845 phosphorylation is not significantly increased following LTP induction (Lee et al., 2000). A recent study suggested that phosphorylation of GluR1-S845 may 'prime' receptors for synaptic insertion by trafficking them to extrasynaptic sites (Oh et al., 2006). Collectively, these data support a model where basal phosphorylation of GluR1-S845 is permissive to LTP, but is not sufficient (Lee et al., 2000) (**Figure 2**).

In addition to their role in controlling AMPA receptor single-channel properties and synaptic trafficking, GluR1-S831 and S845 phosphorylation sites may participate in stabilizing LTP (Lee et al., 2003; Lee, 2006a). Recent characterization of a transgenic mouse line expressing GluR1 with both S831 and S845 sites mutated to alanine ('double phosphomutants') still expressed LTP, albeit in a lesser magnitude when compared to that of wild-type littermates (Lee et al., 2003). Specifically, LTP induced in double phosphomutants decayed faster than in wild type, which suggests that GluR1 phosphorylation may be important for stabilizing newly inserted AMPA receptors (Lee et al., 2003; Lee, 2006a). Based on the data from the double phosphomutants, we surmise that phosphorylation of GluR1 at S831 or

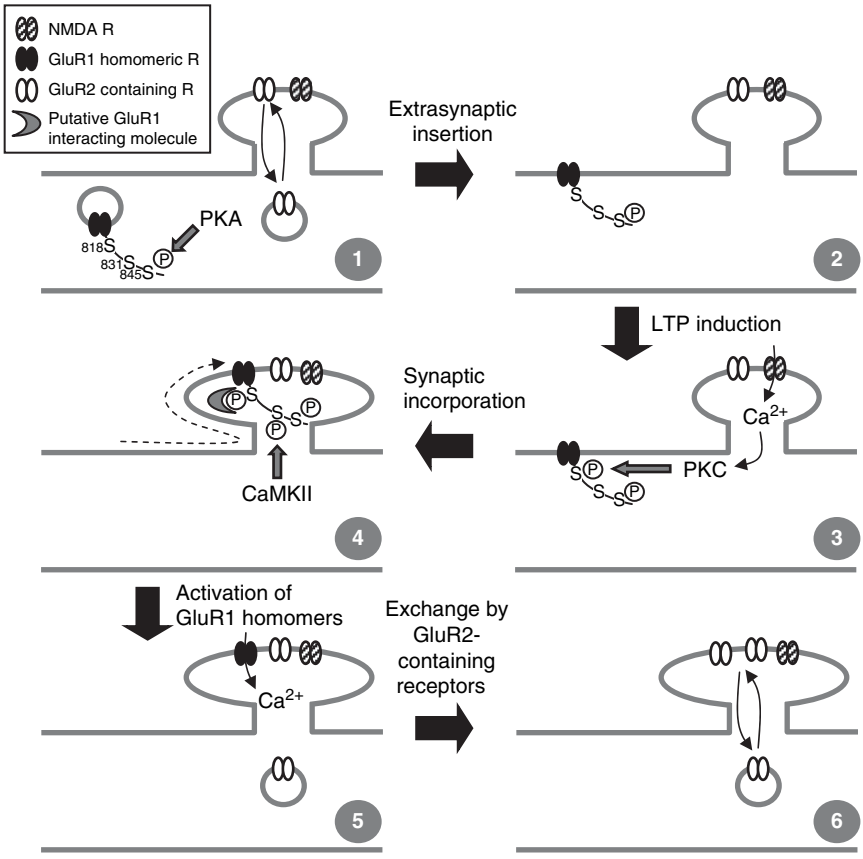


Figure 2 A model of AMPA receptor regulation during LTP. PKA phosphorylation of GluR1-S845 primes AMPA receptors for LTP by trafficking the receptors to extrasynaptic sites. Activation of NMDA receptors by LTP-inducing stimuli causes calcium influx and results in phosphorylation of GluR1-S818, which is likely mediated by PKC. Phosphorylation of GluR1-S818 is thought to bind a putative GluR1 binding protein, which ultimately incorporates AMPA receptors to synapses. GluR1 homomeric receptors inserted into synapses then may be activated to allow calcium influx that exchanges them with GluR2-containing receptors. This process may resemble calcium-permeable AMPA receptor plasticity (CARP) observed in cerebellar granule cells. Phosphorylation of synaptic GluR1 homomeric receptors at S831 by constitutively active CaMKII at synapses may facilitate the exchange process by perhaps allowing a larger influx of calcium.

S845 is not critical for synaptic insertion by LTP-inducing stimuli. However, this is does not exclude the proposed role of S831 in mediating the increase in AMPA receptor conductance following LTP or the priming role of S845. A possible role of GluR1-S831 and S845 phosphorylation in regulating LTP stability will be discussed in a later section.

A recent study highlighted another key phosphorylation site (S818) on the GluR1 subunit as being critical for LTP expression (Boehm et al., 2006). GluR1-S818 is phosphorylated by PKC and lies on the membrane-proximal region of the intracellular carboxy terminus (Boehm et al., 2006). While GluR1-S818 phosphorylation is increased following LTP, its function seems to depend also on phosphorylation of both S831 and S845 (Boehm et al., 2006). Mutating S818 to aspartate to mimic phosphorylation was itself insufficient for driving GluR1 into synapses, but adding aspartate mutations to S831 and S845 greatly increased synaptic incorporation of these receptors (Boehm et al., 2006). This suggests that the several phosphorylation sites on GluR1 may act synergistically in activity-dependent trafficking of the receptors. Boehm et al. (2006) further presented evidence that phosphorylation of S818 allows GluR1 to interact with a putative trafficking protein that allows synaptic incorporation of the receptors.

Collectively, the evidence so far suggests a model of LTP depicted in **Figure 2**. According to this model, the first step that happens before LTP induction is PKA phosphorylation of GluR1-S845, which primes the receptors for synaptic incorporation by inserting them at extrasynaptic sites (Oh et al., 2006). This is consistent with our previous model where GluR1-S845 phosphorylation is a prerequisite for LTP (Lee et al., 2000; Lee, 2006a). The next step is PKC phosphorylation of GluR1-S818, which allows the receptor to interact with a putative protein involved in delivering GluR1 to synapses (Boehm et al., 2006). This putative protein may be regulated by CaMKII, since activity of this enzyme is required for synaptic incorporation of GluR1 (Hayashi et al., 2000). In order for this model to work, both PKC and CaMKII activity should be coupled to NMDA receptor activation that results from LTP-inducing stimuli, while PKA activity may, in principle, precede NMDA receptor activation. There are reports that LTP-inducing stimuli do indeed activate both PKC and CaMKII (Akers et al., 1986; Klann et al., 1991; Fukunaga et al., 1993; Sacktor et al., 1993; Barria et al., 1997b; Ouyang et al., 1997) and both these enzymes are critical for LTP expression (Lovinger et al., 1987; Reymann et al., 1988; Malenka et al., 1989; Malinow et al., 1989; Silva et al., 1992; Abeliovich et al., 1993; Otmakhov et al., 1997; Giese et al., 1998). Activity of CaMKII and/or PKC may also phosphorylate GluR1-S831 (Barria et al., 1997b; Lee et al., 2000), which may increase the conductance of homomeric GluR1s (Oh and Derkach, 2005) that are newly anchored to synapses. Activation of these homomeric GluR1s may then drive their replacement by GluR2-containing receptors which are impermeable to calcium (Plant et al., 2006). This process may be similar to the calcium-dependent AMPA receptor plasticity (CARP) that has been described in cerebellar stellate neurons (Liu and Cull-Candy, 2000; Gardner et al., 2005). These GluR2-containing AMPA receptors then undergo constitutive recycling (Shi et al., 2001), which may allow for stable expression of LTP. While there is evidence that homomeric GluR1s are inserted following LTP induction (Plant et al., 2006), we cannot rule out synaptic insertion of heteromeric GluR1/GluR2 complexes after LTP. Indeed, exogenously expressed GluR1/GluR2 complexes are incorporated into synapses following LTP induction (Shi et al., 2001). Therefore, it is very likely that native heteromeric GluR1s/GluR2s may also insert into synapses following LTP. If this is the case, newly inserted heteromeric GluR1s/GluR2s will behave differently from homomeric GluR1s, since they are not calcium permeable (Hollmann et al., 1991; Washburn et al., 1997), and phosphorylation of GluR1-S831 does not affect their conductance (Oh and Derkach, 2005).

4.31.4 AMPA Receptor Regulation and LTD

Regulation of AMPA receptors is also critical for the expression of synaptic depression. LTD is known to involve endocytosis of synaptic AMPA receptors (reviewed in Carroll et al., 2001) and has been correlated with changes in phosphorylation of both GluR1 and GluR2 subunits (reviewed in Lee, 2006a). Specifically, LTD of 'naive' synapses in the CA1 region of hippocampus is associated with dephosphorylation of GluR1-S845 (Lee et al., 2000) and phosphorylation of GluR2-S880 (Kim et al., 2001). Furthermore, these changes in GluR1 and GluR2 phosphorylation sites accompany endocytosis of AMPA receptors (Chung et al., 2000; Ehlers, 2000; Perez et al., 2001; Lee et al., 2003). Indeed, LTD induction leads to synaptic loss of both GluR1 and GluR2 subunits (Heynen et al., 2000), and it is thought

that alterations in AMPA receptors phosphorylation may be responsible (reviewed in Lee, 2006a).

GluR1-S845 dephosphorylation by LTD was first demonstrated using a chemical method of LTD induction (chemLTD) that employs a brief bath application of NMDA (20 μmol l^{-1} for 3 min) (Lee et al., 1998). The dephosphorylation of GluR1-S845 was also observed following the induction of NMDA receptor–dependent LTD using a conventional LFS (1 Hz, 900 pulses) protocol (Lee et al., 2000). These findings suggest that the dephosphorylation of GluR1-S845 may be central to LTD expression. However, LTD induced by bath application of a metabotropic GluR (mGluR) agonist, (S)-3,5-dihydroxyphenylglycine (DHPG), resulted in an increase, not a decrease, in GluR1-S845 phosphorylation (Delgado and O'Dell, 2005). This is interesting in light of the observation that both NMDA- and DHPG-induced LTD cause a decrease in surface AMPA receptors (Nosyreva and Huber, 2005; Snyder et al., 2005; Holman et al., 2006). Together these results suggest that expression mechanisms of NMDA receptor–dependent and mGluR-dependent LTD may differ, one of the differences being the regulation of GluR1-S845 phosphorylation. The significance of the differential changes in GluR1-S845 during NMDA- and DHPG-induced LTD is unclear at this point. Nonetheless, at least for NMDA receptor–dependent LTD, dephosphorylation of GluR1-S845 (Lee et al., 1998, 2003; Ehlers, 2000; Vanhoose and Winder, 2003) and endocytosis of AMPA receptors (Ehlers, 2000; Lee et al., 2003; Nosyreva and Huber, 2005; Snyder et al., 2005; Holman et al., 2006) have been consistently observed. Whether these two processes are causal in nature or two independent parallel processes is currently undetermined. Nevertheless, the uncoupling of these two events during mGluR-dependent LTD clearly indicates that AMPA receptor endocytosis can occur independently from GluR1-S845 dephosphorylation.

One of the key properties of GluR1-S845 dephosphorylation following LTD is that it is long-lasting (Lee et al., 1998, 2000). This dephosphorylation is likely mediated by activity of a protein phosphatase, calcineurin (PP2B) (Lee et al., 1998). Since the phosphorylation level of GluR1-S845 is likely maintained by a balance of activity of PKA and PP2B (Lee and Huganir, 1999; Lee et al., 2000), the persistent dephosphorylation of GluR1-S845 may be achieved by either a persistent increase in calcineurin activity or a persistent decrease in PKA activity by LTD-inducing stimuli. It is recognized that both PP2B and PKA are placed in close proximity to GluR1 subunit by their association with A-kinase anchoring protein (AKAP), which in turn interacts with GluR1 through its binding protein SAP97 (Colledge et al., 2000). A ternary complex of GluR1-SAP97-AKAP has been observed in brain homogenates using a coimmunoprecipitation method, and the assembly of this complex seems to be required to increase phosphorylation of GluR1-S845 upon PKA activation (Colledge et al., 2000). A recent study showed that chemLTD induced by bath application of NMDA moves AKAP-PKA complexes away from synapses (Smith et al., 2006), which may be responsible for the persistent dephosphorylation of GluR1-S845 observed following LTD. The importance of AKAP-PKA complex in regulating AMPA receptor function can be further seen from studies that report a 'run-down' of fast excitatory synaptic transmission (Rosenmund et al., 1994; Snyder et al., 2005), and a reduction in surface GluR1 (Snyder et al., 2005) by distrupting PKA interaction with AKAP. Interestingly, synaptic depression caused by PKA dissociation from AKAP occluded further LTD (Snyder et al., 2005), suggesting that this mechanism is likely utilized for normal LTD expression. Collectively, these results suggest a model where LTD induction leads to a loss of PKA-AKAP complex from synapses, which then results in a persistent dephosphorylation of GluR1-S845 and a loss of surface AMPA receptors. The exact relationship between GluR1-S845 dephosphorylation and AMPA receptor endocytosis is unknown at present.

In addition to dephosphorylation of GluR1-S845, LTD increases phosphorylation of GluR2-S880 (Kim et al., 2001). S880 is part of a PDZ (PDZ: postsynaptic density-95, discs large, and ZO-1) ligand (-SVKI) on the extreme carboxy terminal tail of GluR2, which interacts with two types of PDZ domain–containing proteins, glutamate receptor interacting protein/AMPAR-binding protein (GRIP/ABP) and protein interacting with C-kinase 1 (PICK-1) (reviewed in Song and Huganir, 2002). Phosphorylation of S880 differentially affects GRIP and PICK-1 binding to GluR2 such that it prevents GRIP binding, but not PICK-1 (Matsuda et al., 1999; Chung et al., 2000). One of the current models suggests that activated PKC binds PICK-1, which in turn interacts with GRIP via its BAR (BAR: Bin1, amphyphysin, Rvs167) domain (Lu and Ziff, 2005). This allows PKC to locate in close proximity to GluR2 carboxy-terminal to phosphorylate S880 (Lu and Ziff, 2005). This dissociates GRIP from GluR2 and allows stable interaction with PICK-1 (Chung et al., 2000; Lu and

Ziff, 2005). PICK-1 is then thought to initiate endocytosis of GluR2-containing AMPA receptors by producing membrane curvature via its BAR domain (Lu and Ziff, 2005), which interacts with membrane lipids (Lu and Ziff, 2005; Jin et al., 2006). Recruitment of adaptor protein-2 (AP2) and clathrin coats is then thought to mediate endocytosis of the receptors (Carroll et al., 1999; Man et al., 2000; Lee et al., 2002). Although the role of PKC in cerebellar LTD is well established (Linden and Connor, 1991; De Zeeuw et al., 1998; Xia et al., 2000; Goossens et al., 2001; Chung et al., 2003), hippocampal LTD is not blocked by a PKC inhibitor (Kim et al., 2001). On the contrary, PKC activity is reported to decrease following LTD induction (Hrabetova and Sacktor, 1996; Thiels et al., 2000). Therefore, it is unlikely that PKC is responsible for GluR2-S880 phosphorylation following LTD in hippocampus.

Despite evidence supporting a role of GluR2-S880 in hippocampal LTD (Kim et al., 2001), GluR2 itself seems unnecessary for LTD expression. Knockout mice lacking GluR2 or double knockout of GluR2 and GluR3 still exhibit LTD (Meng et al., 2003), presumably via the remaining GluR1 subunit. The exact relationship between GluR1-dependent and GluR2-dependent LTD mechanisms are unclear at this point. They could be two independent mechanisms that can occur in parallel. If this is the case, while a GluR1-dependent mechanism may be able to support LTD in the absence of GluR2 (Meng et al., 2003), the opposite seems not to be the case when considering the observation that GluR1 phosphorylation site mutants lack LTD despite having GluR2 (Lee et al., 2003). There is some indication that GluR1- and GluR2-dependent mechanisms may occur in parallel. For example, NMDA receptor–dependent activation of Rab5 leads specifically to GluR1-S845 dephosphorylation and AMPA receptor endocytosis without changes in GluR2-S880 phosphorylation (Brown et al., 2005). An alternative possibility is that GluR1- and GluR2-dependent mechanisms of LTD occur in series, such that GluR1 dephosphorylation is required for GluR2-dependent endocytosis mechanisms to proceed, or GluR1 dephosphorylation stabilizes AMPA receptors that are endocytosed by GluR2-dependent machinery (for a more detailed discussion see Lee, 2006a). However, if this is the case, it is a bit more difficult to explain how a GluR1-dependent LTD mechanism can be sufficient in the absence of GluR2 subunits (Meng et al., 2003). **Figure 3** summarizes the proposed roles of both GluR1 and GluR2

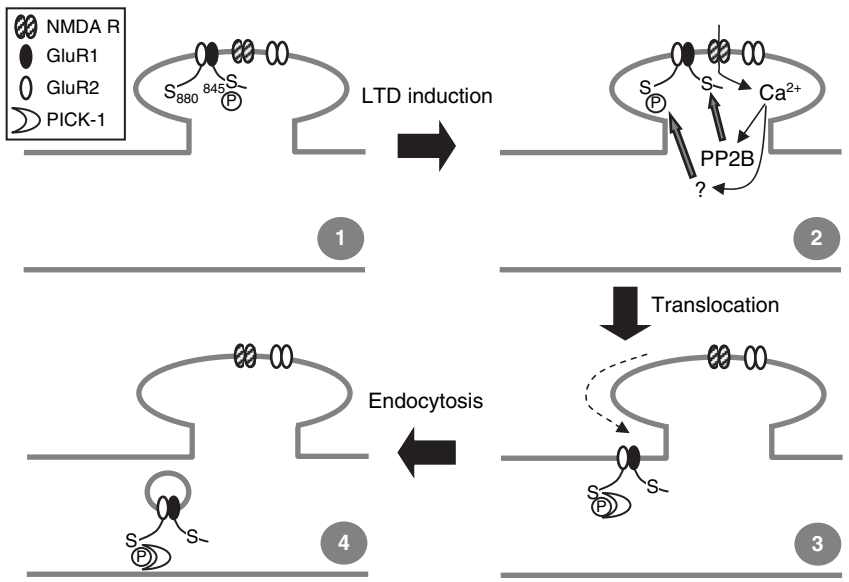


Figure 3 A model of AMPA receptor regulation during LTD. Activation of NMDA receptors by LTD-inducing stimuli activates calcineurin (PP2B), which dephosphorylates GluR1-S845. NMDA receptor activation also leads to phosphorylation of GluR2-S880 via an unknown protein kinase. Changes in phosphorylation of GluR1 or/and GluR2 lead to synaptic removal of the receptor, which occurs first by translocation to extrasynaptic sites followed by endocytosis. Binding of PICK-1 to the GluR2 subunit is thought to phosphorylate GluR2-S880 and initiate endocytosis. However, the exact interaction between GluR1 and GluR2 in mediating LTD is unclear. GluR1 dephosphorylation alone may be sufficient for LTD expression.

phosphorylation sites in LTD. How regulation of GluR1 and GluR2 phosphorylation coordinates AMPA receptor endocytosis during LTD awaits further investigation.

4.31.5 How Is LTP Different from LTD Reversal (Dedepression)?

HFS can result in synaptic potentiation whether it is from a naive state (as in the case of LTP) or from a previously depressed state (which is referred to as LTD reversal or DeD). There is emerging evidence that LTP and DeD may be expressed by different mechanisms (Abeliovich et al., 1993; Daw et al., 2000; Heynen et al., 2000; Lee et al., 2000) and can be affected differentially in some transgenic mouse lines (Abeliovich et al., 1993; Laird et al., 2005). This is quite interesting in light of the fact that both LTP and DeD are often induced by identical stimulation protocols, suggesting that prior history of synaptic activity can alter the mechanisms underlying subsequent synaptic potentiation.

While both LTP (Collingridge et al., 1983; Coan et al., 1987; Tsien et al., 1996) and DeD (Lee et al., 2000) in the CA1 region depend on NMDA receptor activation, distinct signaling cascades may be activated downstream. Although CaMKII is critical for LTP (Malenka et al., 1989; Malinow et al., 1989; Silva et al., 1992; Otmakhov et al., 1997; Hinds et al., 1998; Frankland et al., 2001; Elgersma et al., 2002), inhibition of CaMKII does not affect DeD (Daw et al., 2000; Lee et al., 2000). In addition, knockouts of PKCgamma display reduced LTP, but DeD was unchanged (Abeliovich et al., 1993). These results suggest that while CaMKII/PKC signaling is important for LTP, it is not critical for DeD. On the other hand, DeD is preferentially affected by inhibition of PKA (Lee et al., 2000). The observed differences in protein kinase requirement for LTP and DeD are consistent with distinct changes in GluR1 phosphorylation sites following the two forms of potentiation (Lee et al., 2000). Unlike LTP, which increases phosphorylation of GluR1-S831 (Barria et al., 1997b; Lee et al., 2000) and GluR1-S818 (Boehm et al., 2006), DeD is correlated with phosphorylation of GluR1-S845 (Lee et al., 2000). Collectively, these findings suggest that a CaMKII/PKC-dependent pathway in LTP, and a PKA-dependent signaling in DeD. One of the downstream events for LTP and DeD is distinct regulation of GluR1 phosphorylation sites (**Figure 4**).

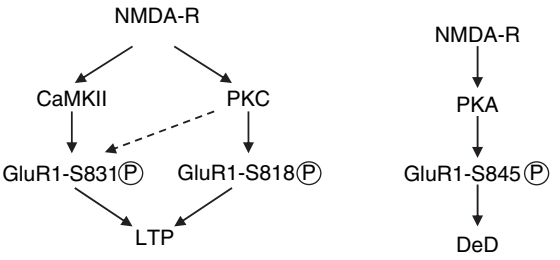


Figure 4 Comparison of AMPA receptor regulation underlying LTP and dedepression (DeD). While both LTP and DeD are induced by activation of NMDA receptors (R), they differ in their downstream signaling. LTP involves activation of CaMKII and PKC, which results in phosphorylation of GluR1 on S818 and S831. These two phosphorylation sites are involved in synaptic insertion of GluR1-containing AMPA receptors and increase in channel conductance of homomeric GluR1s as depicted in **Figure 1**. On the other hand, DeD is not dependent on activation of CaMKII or PKC, at least the gamma isoform of PKC, but requires PKA activity. PKA is then thought to increase GluR1-S845 phosphorylation to mediate DeD. Whether GluR1-S818 increases with DeD is currently unknown. How GluR1-S845 phosphorylation alone (if GluR1-S818 is not phosphorylated) leads to synaptic potentiation is unclear.

Despite the differences, there are also similarities between LTP and DeD, in that both can occur in the absence of GluR2 and GluR3 (Meng et al., 2003), which implies that GluR1 plays a dominant role in both forms of synaptic strengthening. In addition, DeD, like LTP, involves an increase in synaptic content of AMPA receptors without changes in total amount of these receptors (Heynen et al., 2000). (Differences with the findings of Nayak et al. (1998), who reported increases in newly synthesized AMPA receptors following LTP, will be discussed later.) These results suggest that, despite the differences in protein kinase requirement and regulation of distinct GluR1 phosphorylation sites, both LTP and DeD likely involve activity-dependent trafficking of existing AMPA receptors to synaptic loci mediated by GluR1. It is unclear at this point why LTP and DeD are mediated by regulating distinct GluR1 phosphorylation sites (**Figure 4**), if one of the downstream events is identical (i.e., more synaptic AMPA receptors). However, it may be that LTP and DeD have other downstream effectors that need differential regulation. In support of this view, while LTP does not increase synaptic content of an obligatory NMDA receptor subunit NR1, DeD does (Heynen et al., 2000).

4.31.6 How is LTD Different from LTP Reversal (Depotentiation)?

It is perhaps no surprise that LTD in naive synapses and DeP (i.e., synaptic depression following LTP) are also mediated by different molecular mechanisms. It has been recognized that LTD and DeP show distinct features (**Table 1**). One of the earliest indications that LTD and DeP may differ came from observations that stimulation protocols that fail to reliably induce LTD

Table 1 Comparison of long-term depression and depotentiation

	Long-term depression	*Depotentiation*
Induction protocols	1–3 Hz, 900 pulses (Dudek and Bear, 1992; Mulkey and Malenka, 1992).	1–5 Hz, 900–1200 pulses (Dudek and Bear, 1993; Bashir and Collingridge, 1994; O'Dell and Kandel 1994; Wagner and Alger, 1995; Huang et al., 1999; Heynen et al., 2000; Milner et al., 2004). TPS (theta pulse stimulation: 5 Hz, 1 min) (Larson et al., 1993; Staubli and Chun, 1996a,b).Strong intensity TBS (theta burst stimulation) (Barr et al., 1995). 1 Hz, 100 pulses (Staubli and Lynch, 1990).
Age dependence	Magnitude is reduced with age (Dudek and Bear, 1993; Wagner and Alger, 1995).	Reliably induced in adults (Staubli and Lynch, 1990; O'Dell and Kandel, 1994; Wagner and Alger, 1995; Heynen et al., 2000; Milner et al., 2004).
Time dependence	–	Depends on induction protocol. Only within 20 min post-LTP: TPS (Larson et al., 1993; Staubli and Chun, 1996b), 5 Hz (900 pulses) (O'Dell and Kandel, 1994), 2 Hz (1200 pulses) (Huang et al., 1999). Even after 30 min post-LTP: 1 Hz (900–1000 pulses) (Fujii et al., 1991; Dudek and Bear, 1993), 2 Hz (900 pulses) (Bashir and Collingridge, 1994), strong intensity TBS (Barr et al., 1995).
Induction mechanisms	NMDA-R dependent (Dudek and Bear, 1992; Mulkey and Malenka, 1992). Not blocked by group 1 mGluR antagonist, MCPG (Selig et al., 1995).	Depends on induction protocol. 1 or 5 Hz, 900 pulses: NMDA-R dependent (Fujii et al., 1991; O'Dell and Kandel, 1994; Wagner and Alger, 1995; Milner et al., 2004). TPS: Not dependent on NMDA-R (Staubli and Chun, 1996a), but on A1-adenosine R (Staubli and Chun, 1996a). 2 Hz, 900 pulses: Not dependent on NMDA-R, but on group 1 mGluR (Bashir and Collingridge, 1994). 2 Hz, 1200 pulses: Dependent on NMDA-R and A1-adenosine R, but not on group 1 mGluR (Huang et al., 2001). Strong intensity TBS: NMDA-R dependent (Barr et al., 1995).
Intracellular signaling	Blocked by inhibitors of PP1 and PP2A (Mulkey et al., 1993). Depend on calcineurin (Mulkey et al. 1994; Zeng et al., 2001). Reversed by PKA activators (Kameyama et al., 1998).	Blocked by PP1 inhibitors (O'Dell and Kandel, 1994; Huang et al., 2001). Does not require calcineurin (Lu et al., 1996; Zhuo et al., 1999; Zeng et al., 2001), except 5 Hz (900 pulses)-induced DeP (Zhuo et al., 1999). Dephosphorylation of CaMKII-T286 (Huang et al., 2001).
Regulation of AMPA receptors	Dephosphorylation of GluR1 S845 without changes in S831 (Lee et al., 2000). Increase in GluR2 S880 phosphorylation (Kim et al., 2001). Decrease in synaptic GluR1 and GluR2 (Heynen et al., 2000).	Dephosphorylation of GluR1 S831 without changes in S845 (Lee et al., 2000; Huang et al., 2001). Decrease in synaptic GluR1 and GluR2 (Heynen et al., 2000).

are still able to depress previously potentiated synapses (Staubli and Lynch, 1990; Larson et al., 1993; Bashir and Collingridge, 1994; O'Dell and Kandel, 1994; Barr et al., 1995; Staubli and Chun, 1996a; Huang et al., 1999). In addition, while the magnitude of LTD is diminished as animals mature (Dudek and Bear, 1993; Milner et al., 2004), several laboratories reported robust DeP in adults (Staubli and Lynch, 1990; O'Dell and Kandel, 1994; Wagner and Alger, 1995; Heynen et al., 2000; Milner et al., 2004).

Understanding the mechanisms of DeP has been complicated by the fact that the characteristics of DeP itself often seem to differ across laboratories (**Table 1**). For instance, there are contradictory reports as to whether DeP is NMDA receptor–dependent (Fujii et al., 1991; Bashir and Collingridge, 1994; O'Dell and Kandel, 1994; Barr et al., 1995; Wagner and Alger, 1995; Staubli and Chun, 1996a; Milner et al., 2004), or whether it can be elicited past a critical time window ($\leq$20 min) after LTP induction (Fujii et al., 1991; Dudek and Bear, 1993; Larson et al., 1993; Bashir and Collingridge, 1994; O'Dell and Kandel, 1994; Barr et al., 1995; Staubli and Chun, 1996b; Huang et al., 1999). Interestingly, the apparent contradiction seems to stem from differences in the induction protocols used for each study. If the induction protocol for DeP is also effective at producing LTD, it is likely NMDA receptor dependent (Fujii et al., 1991; Wagner and Alger, 1995; Staubli and Chun, 1996a; Milner et al., 2004) and it can be induced even 30–60 min after LTP induction (Fujii et al., 1991; Dudek and Bear, 1993; Wagner and Alger, 1995) (for exceptions refer to **Table 1**). However, if the induction protocol is ineffective at producing LTD, DeP is often induced in a manner independent of NMDA receptor activation (Larson et al., 1993; Bashir and Collingridge, 1994; O'Dell and Kandel, 1994; Staubli and Chun, 1996a; Huang et al., 1999) and is limited to a short critical time window ($\leq$20 min) after LTP induction (Larson et al., 1993; Staubli and Chun, 1996b; Huang et al., 1999) (for exceptions refer to **Table 1**). These differences in various DeP protocols led to a notion that they may not trigger the same process. Consequently, LTP reversal that can only happen in a narrow time window after LTP was coined as deconsolidation (Bear and Abraham, 1996), to distinguish it from DeP, which is not critically dependent on the time after LTP induction (**Figures 1(d)** and **1(e)**). As will be discussed, our knowledge on the molecular mechanisms of LTP reversal suggest that deconsolidation and depotentiation may be two distinct processes.

The observation that stimuli that do not produce LTD by themselves can still reverse LTP (i.e., deconsolidation) when delivered immediately after LTP induction raises an important point. This suggests that there is a critical window of time when LTP is vulnerable. The reason for this vulnerability is unclear at this point, but may have to do with some of the properties of LTP. As discussed in a previous section, LTP is thought to entail transient synaptic expression of GluR1 homomeric AMPA receptors, which are then replaced by GluR2-containing receptors over time (Plant et al., 2006). It will be of interest to know whether the vulnerable time window of LTP is due to the transient increase in calcium-permeable GluR1 homomeric AMPA receptors at synapses following LTP. If this is the case, it may be that once newly inserted calcium-permeable AMPA receptors are replaced by GluR2-containing receptors, LTP can only be reversed by stimuli that are more robust at inducing synaptic depression.

While there are data that DeP is blocked by inhibitors of protein phosphatase 1 (PP1) (O'Dell and Kandel, 1994; Huang et al., 2001), it is not affected by calcineurin (PP2B) inhibitors (Lu et al., 1996; Huang et al., 2001). This suggests that, unlike LTD, which is calcineurin dependent (Mulkey et al., 1994), DeP may be independent of this enzyme. This is further confirmed by the observation that DeP is normal, even though LTD is absent, in a forebrain-specific calcineurin knockout mouse (Zeng et al., 2001). However, the involvement of calcineurin in DeP seems to also depend on the induction protocol used. For instance, 5 Hz (900 pulses)–induced LTP reversal was absent in calcineurin A-alpha knockout mice, but 1 Hz (900 pulses)–induced DeP was intact (Zhuo et al., 1999). Since the 5 Hz (900 pulses) protocol does not reliably induce LTD in naive slices (Zhuo et al., 1999) and only occurs within a critical time window after LTP induction (O'Dell and Kandel, 1994), it may be considered deconsolidation rather than DeP. This again stresses the potential differences in mechanism between the two forms of LTP reversal.

Another difference between DeP and LTD is that they seem to involve dephosphorylation of distinct sites on the GluR1 subunit of AMPA receptors (**Table 1**, **Figure 5**). While LTD results in a dephosphorylation of GluR1-S845 (Lee et al., 2000), DeP correlates with a dephosphorylation on GluR1-S831 (Lee et al., 2000; Huang et al., 2001). These differences were interpreted on the basis that DeP is a reversal of LTP, which increases GluR1-S831 phosphorylation (Lee et al., 2000). The difference in

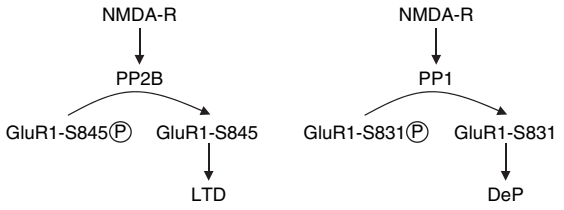


Figure 5 Differences in AMPA receptor regulation during LTD and depotentiation (DeP). NMDA receptor activation during LTD induction activates calcineurin (PP2B), which dephosphorylates GluR1-S845. This eventually leads to endocytosis of AMPA receptors to express LTD. On the other hand, DeP is independent of PP2B, but depends on protein phosphatase 1 (PP1). Activation of PP1 is thought to either directly or indirectly dephosphorylate GluR1-S831. How dephosphorylation of this residue leads to synaptic depression is currently unknown, but may involve changes in AMPA receptor conductance.

GluR1 phosphorylation sites affected by DeP could be due to two possibilities, which are not mutually exclusive. First, LTP induction may 'mask' the GluR1-S845 phosphorylation site; hence it is no longer accessible for dephosphorylation. Second, DeP may recruit a distinct signaling pathway, which may selectively target S831. For instance, if calcineurin selectively targets GluR1-S845, while PP1 targets S831, this may explain the differential dephosphorylation following LTD and DeP.

Interestingly, DeP not only reverses increases in GluR1 and GluR2 following LTP, but is also associated with a decrease in synaptic NMDA-type glutamate receptor subunit (NR1) (Heynen et al., 2000). This suggests that there may be an asymmetry between mechanisms of LTP and DeP. The decrease in NR1 observed following DeP was similar to what was observed following LTD (Heynen et al., 2000), suggesting that synaptic removal of AMPA receptors may be coupled to NMDA receptor downregulation. Functional relevance for the observed downregulation in NMDA receptor function following synaptic depression is unclear at this point. In addition, whether the decrease in synaptic AMPA receptors is due to dephosphorylation of GluR1-S831 is unknown.

4.31.7 Is There a Difference between LTP Decay and LTP Reversal (Depotentiation)?

In addition to activity-dependent reversal of LTP by DeP, it is known that LTP can decay over time (i.e., fail to stabilize) under certain conditions (**Figure 1(f)**). This raises a question as to whether LTP decay and DeP are mediated by the same molecular mechanisms. Another way to pose the question is whether LTP decay is an activity-dependent process that resembles DeP. There are conflicting data as to whether the magnitude of LTP is dependent on basal stimulation after LTP induction (Kauer et al., 1988; Volianskis and Jensen, 2003; Fonseca et al., 2006; Plant et al., 2006). Results from awake freely moving animals suggest that LTP decay is an active process requiring NMDA receptor activation (Villarreal et al., 2002). However, it is important to note that synaptic activity in freely moving animals may be high enough to trigger deconsolidation or DeP, which depends on NMDA receptors. Therefore, trying to tease apart LTP decay from LTP reversal is quite difficult. To start the discussion, it is prudent to define our use of the term 'LTP decay.' We will use this term to describe a reduction in synaptic strength by basal stimulation *in vitro* (which presumably has less endogenous synaptic activity compared to *in vivo* awake situations) after LTP without active stimulation at frequencies that are known to elicit DeP. Here we will discuss three types of manipulations that facilitate LTP decay to gain insights into the mechanism: (1) reagents that affect protein kinase or protein phosphatase activity, (2) pharmacological blockade of GluR2-lacking AMPA receptors, and (3) inhibition of protein transcription or translation. Keeping in line with the focus of this review, we will only discuss how these manipulations may affect AMPA receptor regulation. The evidence presented is consistent with a model of LTP decay as a failure to consolidate LTP.

It is known that application of various protein kinase inhibitors, especially PKC and PKA inhibitors, prevents stable maintenance of LTP (Lovinger et al., 1987; Reymann et al., 1988; Malinow et al., 1989; Klann et al., 1991; Matthies and Reymann, 1993; Huang and Kandel, 1994; Qi et al., 1996; Abel et al., 1997; Otmakhova et al., 2000; Ling et al., 2002; Duffy and Nguyen, 2003; Serrano et al., 2005). CaMKII inhibitors, on the other hand, do not affect LTP maintenance (Otmakhov et al., 1997). In addition, there is evidence that protein phosphatases, especially calcineurin, negatively regulate LTP maintenance (Winder et al., 1998; Malleret et al., 2001). These observations suggest that a balance between ongoing protein kinase (likely PKC and/or PKA) and protein phosphatase activity may be critical in determining the stability of LTP. The

critical substrates of the protein kinases required to stabilize LTP can be numerous, including molecules involved in controlling gene transcription (Impey et al., 1998; Pittenger and Kandel, 2003), translation (reviewed in Kelleher et al., 2004), and many of the synaptic proteins (reviewed in Lee, 2006b). In terms of AMPA receptor regulation, an interesting observation is made from mice lacking two of the GluR1 phosphorylation sites (S831 and S845). These GluR1 double phosphomutants show LTP, which then decays faster than that in wild-type littermates (Lee et al., 2003; Lee, 2006a). This suggests that one of the targets of the protein kinases implicated in LTP maintenance may be GluR1. How GluR1 phosphorylation stabilizes LTP is unclear at this point, but may have to do with either enhancing synaptic incorporation of AMPA receptors or promoting exchange with GluR2-containing receptors (refer to **Figure 2**).

Another manipulation that leads to faster LTP decay is application of a polyamine toxin, which selectively blocks GluR2-lacking AMPA receptors and prevents their exchange with GluR2-containing receptors (Plant et al., 2006). These results suggest that replacement of newly inserted AMPA receptors with GluR2-containing receptors may be critical in preventing LTP decay. Whether this process is dependent on ongoing protein kinase activity or is an independent mechanism for ensuring LTP stability is currently unclear. However, if the exchange of newly inserted GluR2-lacking receptors occurs via similar mechanisms as the activity-dependent AMPA receptor subunit exchange (referred to as CARP) described in cerebellar stellate cells (Liu and Cull-Candy, 2000, 2002; Gardner et al., 2005), it would require PICK-1 interaction with GluR2 (Gardner et al., 2005; Liu and Cull-Candy, 2005) and, hence, phosphorylation of GluR2-S880.

A third manipulation that leads to LTP decay is blockade of protein synthesis (Stanton and Sarvey, 1984; Frey et al., 1988; Huang and Kandel, 1994; Nguyen et al., 1994) or transcription (Nguyen et al., 1994; Frey et al., 1996; Vickers et al., 2005). Interestingly, a recent study found that LTP decay by protein synthesis inhibitor itself is dependent on activation of NMDA receptors following LTP induction (Fonseca et al., 2006). The interpretation of this finding was that synaptic stimulation following LTP may increase protein turnover rate via NMDA receptor activation and hence make LTP more susceptible to protein synthesis inhibitors (Fonseca et al., 2006). It is important to stress that inhibition of protein synthesis or transcription can affect a vast array of proteins (for a list of some of the genes regulated by LTP see Abraham and Williams, 2003), but we will restrict the discussion to regulation of AMPA receptor synthesis. Nayak et al. (1998) reported an increase in newly synthesized GluR1 and GluR2 measured 3 h following LTP induction. However, there is also a report that the total amount of GluR1 and GluR2 are not changed 3–6 h after LTP induction *in vivo* (Heynen et al., 2000). The discrepancy between the two studies may be due to differences in experimental methods. Nayak et al. (1998) measured newly synthesized AMPA receptors by ^{35}S-methionine labeling, while the Heynen et al. (2000) detected AMPA receptor subunits in total hippocampal homogenate, which will include not only newly synthesized receptors but also preexisting receptors. In any case, the former result suggests that increasing synthesis of AMPA receptor subunits may be one mechanism to stabilize LTP. Interestingly, inhibitors of PKA or transcription prevented the increase in newly synthesized AMPA receptors following LTP, suggesting that PKA signaling and transcriptional upregulation may be involved (Nayak et al., 1998).

From the standpoint of AMPA receptor regulation, it seems LTP decay and DeP differ only in how they affect LTP maintenance mechanisms. In the case of DeP, active stimulation of either NMDA receptors or other receptors (e.g., A1-adenosine receptors or mGluRs) results in a signaling cascade that ultimately downregulates synaptic AMPA receptors. As for LTP decay, whether it is an active (i.e., dependent on stimulation or NMDA receptor activation) or a passive (i.e., independent of synaptic activation) process, it ultimately seems to result from failure to maintain LTP-induced upregulation of AMPA receptor function.

4.31.8 Concluding Remarks

Reversible regulation of AMPA receptors has surfaced as one of the core mechanisms mediating bidirectional synaptic plasticity in the central nervous system. Studies on the mechanisms of LTP, LTD, and their reversal counterparts reveal that the molecular changes associated with synaptic potentiation and depression depend critically on the history of prior experience of the synapse. For example, synaptic potentiation in naive synapses recruits distinct signaling and downstream effectors when compared to synapses that have undergone prior LTD. These

results suggest that synapses have distinct states dependent on prior synaptic changes. Interpreting results of distinct GluR1 phosphorylation sites regulated by LTP, LTD, DeP, and DeD in hippocampal CA1 region, we have suggested that synaptic AMPA receptors may exist in at least three distinct states (Lee et al., 2000). As our understanding of the molecular events following bidirectional synaptic plasticity has expanded, we have come to gain more insights into the molecular mechanisms involved in transiting between different synaptic states by synaptic activity. Discrete synaptic states that depend on the history of synaptic activity have also been demonstrated in CA3-to-CA3 recurrent connections (Montgomery and Madison, 2002, 2004). While the details of the transition between distinct synaptic states at CA3-to-CA3 synapses differ from those observed in Schaffer collateral-to-CA1 synapses, these findings nonetheless indicate that this may be a common property of diverse sets of synapses.

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4.32 The Role of the Postsynaptic Density and the Spine Cytoskeleton in Synaptic Plasticity

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4.32.1 Introduction

The regulatory machinery that orchestrates induction, expression, and stabilization of long-term potentiation (LTP) and long-term depression (LTD) at excitatory synapses in the forebrain is located in specialized structures called synaptic spines. Spines are tiny structures, usually less than 1 μm wide and a few micrometers long (Harris, 1999). They have a specialized cytoskeletal structure that consists of an elaborate submembrane scaffold beneath synaptic receptors, termed postsynaptic density (PSD) (Kennedy, 2000), and an actin-based skeleton that maintains their mushroom-like shape (Matus, 2000). The PSD comprises an arrangement of scaffold proteins that organize signaling enzymes that respond to Ca^{2+} influx through activated *N*-methyl-D-aspartate (NMDA)-type glutamate receptors (NMDARS) or metabotropic glutamate receptors. The signaling machinery in the PSD, together with cytoskeletal regulatory proteins in the spine, work together to alter the structure of the spine to produce LTP or LTD.

To understand the intricate biology of the PSD and spine, it is important to understand (1) the protein components of the PSD, (2) the physical interactions among these proteins, and (3) the composition,

function, and regulation of the subdomains of the protein network formed by these interactions. Studies from our laboratory and many others over the past 15 years have identified the most prominent and ubiquitous PSD proteins (Kennedy et al., 1983; Cho et al., 1992; Kistner et al., 1993; Kornau et al., 1995; Muller et al., 1996; Niethammer et al., 1996; Kennedy, 1997; Kim et al., 1997, 1998; Chen et al., 1998; Boeckers et al., 1999a; Lim et al., 1999; Tu et al., 1999; Sheng and Kim, 2000). More recently, continuing proteomic analyses (Husi et al., 2000; Walikonis et al., 2000; Li et al., 2004; Peng et al., 2004) have resulted in a catalog of putative PSD-associated proteins that comprises a few hundred individual proteins. Although a few of these studies have begun to address the crucial question of the relative abundance of each protein and their stoichiometric ratios in the PSD fraction (Li et al., 2004, 2005; Peng et al., 2004), we are still in the early stages of understanding which core structures are common to most excitatory synapses, and which proteins are found in a subset of such synapses, perhaps conferring specialized properties.

One useful way to envision the molecular architecture of the PSD is to imagine it as a top-down hierarchy starting with the three major classes of glutamate receptors (NMDAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid [AMPA]-type glutamate receptor [AMPAR], and mGluR) and continuing with scaffold proteins that bind directly to these receptors; for example, PSD-95, TARPs, and homer, respectively (**Figure 1**). These scaffolds, in turn, link to more distal scaffold proteins such as GKAP/SAPAP and shank/ProSAP, which form links between the protein complexes associated with each class of glutamate receptor. At every level (receptors, proximal scaffolds, and distal scaffolds), other effector proteins, regulatory enzymes, and adaptors are recruited into specific signal transduction modules that enable specialized adaptive functions.

4.32.2 Protein Complexes Associated With the NMDAR

The NMDAR (*See* Chapter 4.20) is a ligand-gated Ca^{2+}-permeable ion channel that plays a critical role in the initiation of several forms of synaptic plasticity, including long-term potentiation (LTP; *See* Chapters 4.16, 4.30) and long-term depression (*See* Chapters 4.17, 4.31). The predominant composition of the receptor is a tetramer containing a pair of NR1 subunits that contain a glycine-binding site and are essential for formation of the ion channel pore, and a pair of NR2 subunits that contain the glutamate-binding site and have long cytosolic tails of 500–600

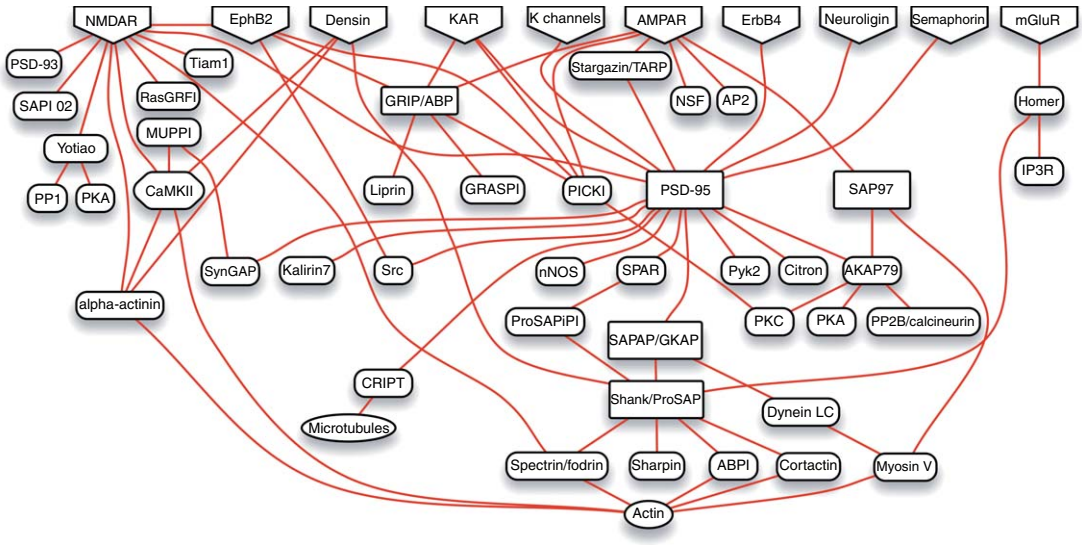


Figure 1 Protein–protein interaction map of postsynaptic density (PSD) and cytoskeletal proteins. The diagram represents protein–protein interactions documented in the literature and discussed in this chapter. The white boxes represent individual proteins, and the red lines represent binary physical interactions between two proteins. This representation does not imply that a series of interconnected proteins form an obligatory complex. PSD-95 is included as a representative of the related proteins PSD-93 and SAP-102, each of which interacts with many of the same proteins as PSD-95. As discussed in the text, these three proteins have partially redundant functions.

residues that extend into the cytosol and interact with scaffold proteins and signaling enzymes. Four different NR2 subunits are encoded on distinct genes, NR2A–D. NR2A and NR2B are the most abundant NR2 subunits in forebrain neurons. Individual receptors can contain two copies of either NR2A or NR2B, or they can contain one copy of each (Hollmann and Heinemann, 1994; Dingledine et al., 1999; Mayer and Armstrong, 2004; Mayer, 2005).

The most commonly studied forms of LTP (Bliss and Collingridge, 1993; Malenka and Bear, 2004) require activation of NMDARs (Morris et al., 1986), elevation of postsynaptic calcium concentration (Sabatini et al., 2001), and subsequent activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Giese et al., 1998). Remarkably, a smaller increase in Ca^{2+} through these same receptors has been shown to trigger the opposite change in synaptic strength – long-term depression or LTD (Cummings et al., 1996). Thus, differences in the timing and extent of activation of NMDARs and the consequent influx of Ca^{2+} into the spine determine whether activation of a synapse will strengthen or weaken it (Zucker, 1999; Franks and Sejnowski, 2002). This delicate balance between distinct Ca^{2+}-activated processes is believed to depend on two characteristics of postsynaptic signaling proteins: their affinities for binding and activation by Ca^{2+} and their proximity to the source of Ca^{2+} influx, in this case the NMDAR pore. The spatial arrangement of signaling proteins is important because the influx of Ca^{2+} through the channel pores produces a transient rise and fall in Ca^{2+} concentration that is spatially restricted to the activated postsynaptic spine (Koester and Sakmann, 1998; Sabatini et al., 2002). Indeed, because Ca^{2+}-ATPases and exchangers rapidly pump Ca^{2+} back across the postsynaptic membrane, it is likely that Ca^{2+} influx produces a gradient with the highest Ca^{2+} concentrations reached near the mouth of receptor pores (Franks and Sejnowski, 2002). Thus, the precise location of individual Ca^{2+}-sensitive enzymes will help to determine how well they compete with others for binding and activation by Ca^{2+} moving through the NMDAR. A series of scaffold proteins and enzyme binding sites in the spine create highly organized signaling machinery near the NMDARs (Kennedy, 2000) (**Figure 2**). This machinery makes up a major portion of the PSD that is seen in the electron microscope.

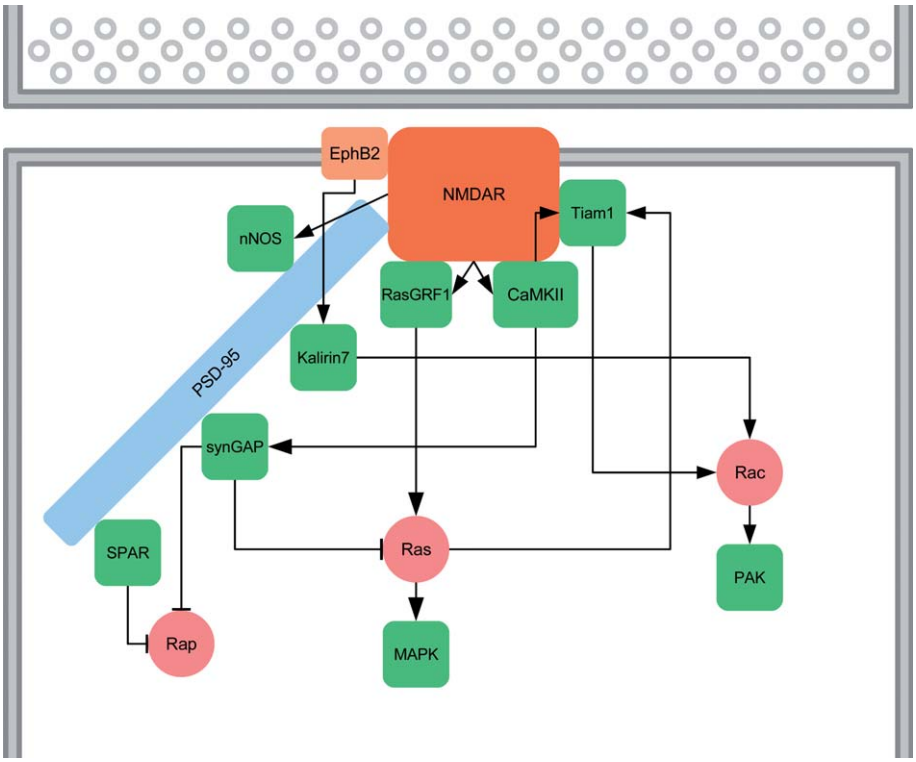


Figure 2 *N*-methyl-D-aspartate-receptor-associated complex. A schematic diagram of the major proteins of the NMDAR-associated complex. Binary physical interactions between two proteins are represented by their juxtaposition. Arrows represent regulatory actions (→ stimulatory, ⊣ inhibitory).

4.32.2.1 PSD-95

The first scaffold protein identified in the PSD was PSD-95 (also known as SAP90) (Cho et al., 1992; Kistner et al., 1993). Its sequence contains three PDZ domains, an SH3 domain, and a GUK (guanylate kinase-like) domain, all of which can act as specific protein-binding sites. Indeed, PDZ domains were first defined in PSD-95 (Cho et al., 1992; Kornau et al., 1995). The first and second PDZ domains of PSD-95 bind to the NMDAR primarily through association with an SXV motif at the C-terminus of the NR2A and NR2B subunits (Kornau et al., 1995). PSD-95 and its more recently identified homologs, including synaptic proteins SAP97 (Lue et al., 1994; Muller et al., 1995), PSD93/Chapsyn-110 (Kim et al., 1996; Brenman et al., 1996b), and SAP102 (Lau et al., 1996; Muller et al., 1996) have been termed MAGUKs (membrane-associated guanylate kinases).

The MAGUK proteins can induce clustering of NMDARs when both are overexpressed in heterologous cells (Kim et al., 1996). Studies on NMDARs expressed together with PSD-95 in *Xenopus* oocytes suggest that PSD-95 is capable of modulating their surface expression and biophysical properties (Yamada et al., 1999; Lin et al., 2006). Such findings were originally interpreted to mean that MAGUKs might be important for the surface expression and postsynaptic clustering of NMDARs. However, mutant mice with a mutation in the PSD-95 gene that deletes the first and second PDZ domains of PSD-95 have normal levels of synaptic NMDARs but display abnormal synaptic plasticity (Migaud et al., 1998). PSD-95, as well as other MAGUKs have been shown to play a role in targeting of AMPA receptors to the synapse in hippocampal neurons (Leonard et al., 1998; Chen et al., 2000; Schnell et al., 2002) (see also the section titled 'Stargazin binding to PSD-95'). Deletion from mice of both PSD-95 and PSD-93 alters AMPA-receptor trafficking in many synapses but does not reduce the number of NMDARs at synapses (Beique et al., 2006; Elias et al., 2006). Thus, the interaction between the NMDAR and MAGUK proteins is important for proper alignment and interaction of signaling proteins within the spine and for control of receptor trafficking rather than for receptor clustering at the synapse.

An important role that we emphasize in this chapter is the recruitment and organization of downstream signaling proteins that are regulated by Ca^{2+} flux through the NMDAR pore. For example, PSD-95 can bind to neuronal nitric oxide synthase (nNOS) through an atypical interaction with the first and second PDZ domains, linking it to the NMDAR (Brenman et al., 1996a; Christopherson et al., 1999). Similarly, synGAP, an abundant Ras-GTPase activating protein in the PSD fraction, binds to the third PDZ domain of PSD-95 (Chen et al., 1998; Kim et al., 1998). Both nNOS and synGAP are regulated by Ca^{2+} entry through the NMDAR (Christopherson et al., 1999; Oh et al., 2004). Thus, a principal role for PSD-95 may be to act as a scaffold for these proteins, placing them in proximity to a source of activating Ca^{2+} to form a signaling module whose activity is spatially and temporally restricted (Kennedy, 2000; Franks et al., 2006).

4.32.2.1.1 PSD-95 and regulation of small GTPases at the synapse

PSD-95 has been reported to bind to several regulators of small GTPase proteins, including synGAP (Chen et al., 1998; Kim et al., 1998), kalirin-7 (Penzes et al., 2000), SPAR (Pak et al., 2001), and citron (Zhang et al., 1999), suggesting that it may play a particularly important role in the orchestration of GTPase signaling in the postsynaptic spine.

Kalirin-7 is a guanine nucleotide exchange factor (GEF) for the Rho family of small GTPases that regulates the formation and maintenance of dendrites and spines (Penzes et al., 2000, 2001; Ma et al., 2003; Rabiner et al., 2005). When kalirin-7 is activated by binding of ephrin to the EphB2 receptor, it activates Rac and PAK1, leading to formation of mature dendritic spines (Penzes et al., 2003). We discuss the role of kalirin in actin dynamics in the section titled 'Kalirin.' Interestingly, activation of EphB2 promotes association between EphB2 itself and the NR1 subunit of the NMDAR (Dalva et al., 2000), as well as with postsynaptic PDZ domain-containing scaffold proteins such as GRIP/ABP (Irie et al., 2005) and PICK1 (Torres et al., 1998). In this way, activated EphB2 is recruited to the postsynaptic membrane. There it recruits and activates members of the Src family of nonreceptor tyrosine kinases, which phosphorylate NR2 subunits of the NMDAR, resulting in an increase in its ability to conduct Ca^{2+} upon glutamate stimulation (Wang and Salter, 1994; Yu et al., 1997; Takasu et al., 2002). By virtue of its association with kalirin-7, PSD-95 may help to coordinate regulation of NMDAR current and spine morphology by EphB2.

SynGAP is a Ras and Rap GTPase that is concentrated in the PSD, presumably by virtue of its interaction with the third PDZ domain of PSD-95 (Chen et al., 1998). Its GAP activity would be expected to increase the rate of inactivation of Ras that has been activated by RasGRF or by postsynaptic

TrkB receptors stimulated by BDNF. SynGAP's activity is enhanced by phosphorylation by CaMKII that is activated by stimulation of NMDARs (Oh et al., 2004). SynGAP appears to be a crucial synaptic regulatory protein because deletion of the synGAP gene is lethal in mice a few days after birth (Komiyama et al., 2002; Vazquez et al., 2004; Knuesel et al., 2005). Mice heterozygous for the synGAP deletion have learning deficits and defects in generation of LTP (Komiyama et al., 2002). Neurons cultured from mouse embryos homozygous for the synGAP deletion show derangements in cytoskeletal regulation (Vazquez et al., 2004) and in AMPA receptor trafficking (Krapivinsky et al., 2004; Rumbaugh et al., 2006).

The GUK domain of PSD-95 can bind directly to a spine-associated Rap-Gap, termed SPAR, and recruit it into the NMDAR complex. SPAR binds to actin and contributes to regulation of the actin cytoskeleton of the spine by downregulating the activity of Rap (Pak et al., 2001). SPAR also acts as a scaffold by associating with the master scaffold protein Shank/ProSAP (see the section titled 'Shank ProSAP scaffold of scaffolds') via an intermediate protein (ProSAPiP1) (Wendholt et al., 2006).

Finally, the third PDZ domain of PSD-95 associates with citron-N, a splice variant of citron kinase that lacks the kinase domain and is situated in neuronal cell bodies, as well as concentrated at glutamatergic synapses in inhibitory neurons in the hippocampus and cortex and excitatory neurons in the thalamus and basal ganglia (Furuyashiki et al., 1999; Zhang et al., 1999; Zhang and Benson, 2006). Citron-N is a target of activated Rho and participates in organization of the Golgi apparatus in neurons (Camera et al., 2003). It binds to the Golgi apparatus and controls actin filaments locally by assembling together the Rho effector ROCK-II and the neuron-specific actin-binding protein profilin IIa. The precise role of citron-N at synapses is still unknown. However, mutations in the gene encoding citron were recently implicated in genetic predisposition toward affective disorders (Lyons-Warren et al., 2005).

4.32.2.1.2 The GUK domain and GKAP

In addition to the PDZ domains, the C-terminal GUK domain of PSD-95 and other MAGUKs acts as a specific protein interaction site. A family of proteins called GK-associated proteins (GKAPs) bind to the GUK domain and are enriched in the PSD fraction (Kim et al., 1997; Satoh et al., 1997; Takeuchi et al., 1997). GKAPs in turn bind to members of the shank/ProSAP family (Naisbitt et al., 1999; Boeckers et al., 1999b). Shank proteins, like PSD-95, appear to function mainly as scaffold proteins because they contain a series of protein interaction motifs and lack identified catalytic domains. The single PDZ domain of shank binds to the C-terminus of GKAP. As we discuss in the section titled 'Shank ProSAP scaffold of scaffolds,' shank proteins interact with a series of other scaffold proteins, linking together protein complexes associated with the AMPA and metabotropic glutamate receptors.

4.32.2.1.3 Other membrane proteins that associate directly with PSD-95

A variety of other proteins have been reported to associate directly with PSD-95, including AKAP-79/150, which binds to the SH3 and GUK domains and acts as a scaffold for cAMP-dependent protein kinase (Colledge et al., 2000). The postsynaptic adhesion protein neuroligin binds to the third PDZ domain of PSD-95 (Irie et al., 1997; Song et al., 1999) and regulates synapse development (Prange et al., 2004) and retrograde signaling (Futai et al., 2007). PSD-95 – like EphB2 – can bind to Src kinases and thus may localize and/or regulate them in the PSD (Tezuka et al., 1999; Kalia and Salter, 2003; Kalia et al., 2006). The cytosolic C termini of both the receptor tyrosine kinase ErbB4 (Garcia et al., 2000; Huang et al., 2000) and semaphorin adhesion proteins (Schultze et al., 2001; Burkhardt et al., 2005) can bind to the first and second PDZ domains of PSD-95, as can the tails of shaker-type voltage-gated K^+ channels (Kim et al., 1995) and several inwardly rectifying potassium channels (Nehring et al., 2000). Finally, as we discuss below, PSD-95 and its homologs, including SAP-97, associate with complexes surrounding AMPA receptors. How and when interactions with each of these proteins occur at different synapses, how they are regulated, and how they alter synaptic function are subjects for future study.

4.32.2.1.4 Dynamics of association of PSD-95 with the PSD

Because PSD-95 and the other MAGUK proteins are capable of a large number of high-affinity associations with synaptic proteins (**Figure 1**), it is easy to imagine that individual PSD-95 proteins remain stably bound within a particular PSD once they arrive there. However, studies of tagged PSD-95 proteins *in vivo* reveal quite a different situation. Although the size of individual PSDs is generally stable, individual PSD-95 proteins exist in a highly dynamic equilibrium, moving rapidly among neighboring spines, with an average retention time in a single spine of 1–2 h (Gray et al.,

2006). This behavior suggests that individual spines compete for limiting amounts of PSD-95 and that larger PSDs contain more PSD-95 binding sites. This rapid dynamic equilibrium may permit correspondingly rapid remodeling of individual PSDs in response to biochemical changes in PSD-95-binding proteins triggered by synaptic activity.

Structural studies of PSD-95 itself hint at possible regulatory mechanisms that could control the availability of its binding sites. The SH3 domain of PSD-95 interacts intimately with the GK domain such that a portion of the GK domain is necessary to complete the SH3 fold (McGee and Bredt, 1999; McGee et al., 2001; Tavares et al., 2001). Point mutations that disrupt the interaction between the two domains interfere with the ability of PSD-95 to cluster potassium channels in heterologous cells, suggesting an alteration in availability of binding sites (Shin et al., 2000). Furthermore, patch analysis of the tertiary structure of PSD-95 suggests that it can exist in two distinct stable conformations: one that obscures the binding cleft of the third PDZ domain and another that exposes it (Korkin et al., 2006). Future studies of regulation of the conformation of PSD-95 may reveal new mechanisms that contribute to regulation of synaptic size.

4.32.2.2 Direct Association of Signaling Proteins with the Tails of NMDAR Subunits

Among several unusual specializations of the NMDAR are the extended C termini of the NR2 subunits. These tails comprise approximately 600 residues and extend into the cytosol, where they interact with PSD-95 (Kornau et al., 1995) but also bind directly to the structural protein; α-actinin (Wyszynski et al., 1997); the scaffold protein yotiao, which binds protein phosphatase 1 (PP1) and protein kinase A (PKA) (Westphal et al., 1999); and signaling enzymes CaMKII (Bayer et al., 2001) and RasGRFI (Krapivinsky et al., 2003).

4.32.2.2.1 CaMKII

Among the proteins that directly interact with NMDARs in the PSD, one of the best studied is Ca^{2+}/CaMKII (Rosenberg et al., 2005) (*See* Chapter 4.25). It is one of several critical determinants of synaptic plasticity that are activated by influx of Ca^{2+} through the NMDAR (Lisman et al., 2002; Kennedy et al., 2005). Its positioning near the NMDAR would place it in an ideal location to influence synaptic plasticity. Indeed, it appears that the level of CaMKII in the PSD correlates with the frequency threshold for induction of LTP (Elgersma et al., 2002).

To understand how CaMKII associates with the NMDAR (and other proteins in the PSD), it is necessary to understand a bit about its structure. CaMKII is activated by the Ca^{2+}-bound form of calmodulin (Ca^{2+}/CaM), which binds to a CaM-binding domain within the catalytic subunits and in so doing opens up the catalytic site so that it is able to interact with substrate proteins (Bennett et al., 1983; Bennett and Kennedy, 1987). The catalytic subunits are situated in a large holoenzyme made up of 12 individual catalytic units held together by interactions among association domains in the carboxyl third of their sequence (Bennett et al., 1983; Rosenberg et al., 2005, 2006). The holoenzymes are randomly assembled from two closely related catalytic subunits termed α and β. In the forebrain, α-subunits predominate over β-subunits by a ratio of 3 to 1. When all of the subunits are in an inactive state, the holoenzyme is actually composed of six dimers because individual pairs of catalytic subunits bind to each other by a coiled coil interaction between their calmodulin binding domains (Rosenberg et al., 2005). Activation of subunits is accompanied by autophosphorylation of a key residue in each subunit, threonine 286. Phosphorylation of this site prevents the catalytic site from closing when CaM dissociates; thus, it holds the subunit in an active conformation (Miller and Kennedy, 1986). In order for this autophosphorylation to occur, Ca^{2+}/CaM must be bound to two neighboring subunits, one that acts as the kinase and a second that acts as the substrate (Hanson et al., 1994). Based on their crystal structure, Rosenberg et al. (2005) proposed that binding of Ca^{2+}/CaM dissociates the members of an inactive dimer pair, exposing their active sites and permitting autophosphorylation to occur between them.

CaMKII is a prominent constituent of the subcellular fraction enriched in PSDs (Kennedy et al., 1983). Because the holoenzyme is so large ($\sim 20 \times 10$ nm), it contains many possible sites that could bind to docking proteins in the PSD. Furthermore, it is an abundant protein in the brain, constituting approximately 1% to 2% of the total protein content (Erondu and Kennedy, 1985), and is, therefore, likely to be present in excess of its individual binding sites in the PSD. At least three proteins in the PSD have been identified as potential docking sites for CaMKII in the PSD: the NR1 subunit of the NMDAR, the tails of the NR2 subunits of the NMDAR, and densin, a sialylated transmembrane protein that has a short cytosolic domain and is highly enriched in the PSD (Apperson et al., 1996). Three

other proteins located near the PSD also bind CaMKII. These are α-actinin, which forms a stable ternary complex with CaMKII and densin (Walikonis et al., 2001) and with CaMKII and NR2B (Leonard et al., 2002); F-actin, which binds the less abundant β-subunit of CaMKII, but not the more abundant α-subunit (Ohta et al., 1986; Shen et al., 1998); and the PDZ-domain-containing scaffold protein MUPP-1 (Krapivinsky et al., 2004).

The two most prominent NR2 subunits in the forebrain, NR2A and NR2B, both bind CaMKII at sites located in their long cytosolic C termini (Gardoni et al., 1998; Strack and Colbran, 1998; Leonard et al., 1999). Binding to either one requires activation of CaMKII by Ca^{2+}/CaM; however, the binding sites in NR2A and NR2B are not identical. The tail of NR2B binds CaMKII with high affinity at two distinct sites (Strack et al., 2000; Bayer et al., 2001): a distal site (residues 1259–1310) that requires binding of Ca^{2+}/CaM to CaMKII and a more proximal site (residues 829–1120) that only binds CaMKII after autophosphorylation at threonine 286 (Bayer et al., 2001; Schulman, 2004). Binding to the distal site stabilizes the activated form of CaMKII (Bayer et al., 2001). However, phosphorylation by CaMKII of serine 1303 on NR2B reduces the affinity of the distal site and leads to slow dissociation of the kinase from NR2B (Strack et al., 2000). The tail of NR2A binds CaMKII with a lower affinity than NR2B. Binding to a site in NR2A between residues 1244 and 1464 is enhanced by Ca^{2+}/CaM and by autophosphorylation (Gardoni et al., 1999). Phosphorylation by protein kinase C of serine 1416 in NR2A inhibits binding of CaMKII and promotes dissociation of the CaMKII/NR2A complex, providing a mechanism by which activation of mGluRs decreases binding of CaMKII to the NMDAR (Gardoni et al., 2001).

The short cytosolic tail of the NR1 subunit of the NMDAR can bind CaMKII in the 30-residue membrane proximal region termed C0 (Leonard et al., 2002). Like the binding to the membrane proximal region of NR2B, this binding requires autophosphorylation of CaMKII at threonine 286. Binding of CaMKII is competitive with binding of Ca^{2+}/CaM and α-actinin to NR1 (Leonard et al., 2002). Because binding of α-actinin increases the open probability of the NMDAR, the competitive binding of autophosphorylated CaMKII may contribute to Ca^{2+}-dependent inactivation of NMDARs (Krupp et al., 1999).

Differences in the requirements for the binding of CaMKII to different proteins in the spine and PSD suggest ways that translocation of CaMKII may be orchestrated in the spine under different conditions of stimulation. For example, binding of Ca^{2+}/CaM to CaMKII causes its release from F-actin (Ohta et al., 1986) and from the scaffold protein MUPP1 (Krapivinsky et al., 2004). In contrast, binding of Ca^{2+}/CaM and autophosphorylation dramatically increase its binding to all subunits of the NMDAR. Thus, Ca^{2+} influx into the spine would be expected to promote release of CaMKII from the spine cytoskeleton and subsequent binding to the NMDAR (Merrill et al., 2005; Ahmed et al., 2006). As another example, binding of CaMKII to both the NMDAR and to densin are facilitated by concomitant binding to α-actinin (Walikonis et al., 2001; Leonard et al., 2002). However, densin has a much greater affinity for the α-subunit than for the β-subunit (Walikonis et al., 2001), whereas the NMDAR does not show this selectivity. Since the α-subunit, but not the β-subunit, is synthesized from mRNA in dendrites in response to stimuli that induce potentiation (Burgin et al., 1990; Ouyang et al., 1999), densin may function to preferentially concentrate at the PSD holoenzymes that are newly synthesized in the dendrite.

Conditions that produce translocation of CaMKII into spines have been studied with the use of GFP-labeled CaMKII transfected into cultured neurons. Stimulation of NMDARs in cultured hippocampal neurons produced a rapid and transient redistribution of CaMKII from the actin cytoskeleton to synaptic sites (Shen and Meyer, 1999; Shen et al., 2000), whereas chemical induction of LTP in hippocampal slices by application of forskolin in medium containing no Mg^{2+} caused significant translocation of CaMKII into spines (Otmakhov et al., 2004). Because hypoxia can also cause accumulation of CaMKII in PSDs (Suzuki et al., 1994; Dosemeci et al., 2001), there has been some confusion about whether physiological stimulation *in vivo* produces translocation of CaMKII to the PSD. However, translocation under normal physiological circumstances *in vivo* was reported in living zebrafish (Gleason et al., 2003). Repeated sensory stimulation produced reproducible and reversible translocation of GFP-CaMKII to the PSD in an identified interneuron within the sensorimotor circuit.

The complex regulation of localization of CaMKII in the spine and PSD illustrates how scaffold proteins help to dynamically configure the location of regulatory proteins in the spine.

4.32.2.2.2 RasGRF1

Ras activation plays critical roles in synaptic plasticity both via regulation of insertion and removal of

AMPA receptors in the spine (Zhu et al., 2002) and via downstream activation of ERK1/2 (Sweatt, 2004). RasGRF1, a Ras-specific GDP/GTP exchange factor (GEF) mediates activation of Ras and the ERK1/2 pathway by NMDAR activation (Farnsworth et al., 1995; Hardingham et al., 2001; Krapivinsky et al., 2003). RasGRF1 is activated by Ca^{2+}/CaM (Farnsworth et al., 1995) and binds directly to the cytosolic tail of the NR2B subunit of the NMDAR (Krapivinsky et al., 2003). Location near the mouth of the NMDAR would render it able to efficiently sense a local rise in Ca^{2+} concentration around the activated receptor. As predicted, disruption of the interaction by introduction into neurons of a peptide blocker disrupts activation of the ERK1/2 pathway by NMDA (Krapivinsky et al., 2003). Moreover, learning and memory are impaired in RasGRF1 knockout mice (Giese et al., 2001).

The finding that RasGRF1 is associated directly with the NMDAR raises some interesting questions. The identified binding sites for RasGRF1 and CaMKII on NR2B overlap extensively; however, it is not yet known whether the two compete for binding to the NMDAR. The answer to this question will have implications for the orchestration of Ca^{2+} signaling in the spine. SynGAP, a Ras inactivating GTPase that binds to PSD-95, is stimulated by phosphorylation of CaMKII that is activated by Ca^{2+} flux through the NMDAR. Presumably the relative timing of activation of RasGRF and synGAP by Ca^{2+} flux would determine the time course of Ras activation. It will be interesting to learn how the PSD scaffold helps to regulate this timing.

4.32.3 Protein Complexes Associated with the AMPA Receptor

AMPARs are the principal mediators of rapid excitatory synaptic transmission in the brain. They are composed of four homologous subunits (GluR1–4 or GluRA–D) that assemble in combinations to form particular AMPAR subtypes (Hollmann and Heinemann, 1994; Dingledine et al., 1999; Mayer and Armstrong, 2004; Mayer, 2005). Regulation of trafficking, insertion, and removal of AMPARs to and from the postsynaptic membrane is a major mechanism underlying activity-dependent changes in synaptic strength such as LTP and LTD (Barry and Ziff, 2002; Malinow and Malenka, 2002; Song and Huganir, 2002; Bredt and Nicoll, 2003; Park et al., 2004). For example, activity-dependent recruitment of AMPARs to the postsynaptic membrane underlies some forms of LTP during development and in adult synapses (Isaac et al., 1995; Liao et al., 1995; Shi et al., 1999, 2001; Hayashi et al., 2000; Malinow, 2003; Matsuzaki et al., 2004; Bagal et al., 2005). In pyramidal neurons of the hippocampus, where LTP and LTD are most often studied, AMPARs are primarily heteroligomers of GluR1 and 2 subunits (GluR1/2) or of GluR2 and 3 subunits (GluR2/3). GluR2/3 AMPARs cycle constitutively in and out of the postsynaptic membrane, whereas GluR1/2 AMPARs are inserted into the postsynaptic membrane upon synaptic stimulation (Shi et al., 2001). AMPAR trafficking is thoroughly discussed in Chapter 4.30. Here we concentrate on interactions of GluR subunits with scaffold proteins that contribute to their localization at the postsynaptic membrane.

Five scaffold proteins interact directly with GluR subunits and participate in regulation of AMPAR trafficking: Stargazin, GRIP (GluR-interacting protein)/ABP (AMPAR-binding protein), PICK1 (protein interacting with C kinase 1), and NSF (N-ethylmaleimide-sensitive factor). In addition, PSD-95 binds directly to the GluR6 and KA2 subunits of kainate receptors (KARs; Garcia et al., 1998; Mehta et al., 2001).

4.32.3.1 Stargazin Binding to PSD-95

Stargazin/γ-2 and three structurally and functionally homologous proteins (γ-3, γ-4, and 8) are transmembrane auxiliary subunits of AMPARs and are collectively referred to as TARPs (Tomita et al., 2003; Nicoll et al., 2006; Osten and Stern-Bach, 2006). Stargazin/γ-2 is structurally related to the γ-1 subunit of the voltage-dependent calcium channel (VDCC) and was initially believed to be a calcium channel subunit (Letts et al., 1998). It was soon recognized that the TARPs are not part of the VDCC; instead, they bind to subunits of the AMPA receptor (Chen et al., 2000; Tomita et al., 2004) and influence their trafficking, targeting, and biophysical properties (Fukata et al., 2005; Nakagawa et al., 2005; Vandenberghe et al., 2005).

The C termini of the TARPs contain a consensus PDZ-domain-binding motif that binds selectively to the first two PDZ domains of PSD-95 (Fukata et al., 2005), and this interaction is required for activity-dependent translocation of AMPA receptors to postsynaptic sites (Schnell et al., 2002). Overexpression of PSD-95 increases the number of synaptic AMPARs (Schnell et al., 2002), whereas disruption of the synaptic localization of PSD-95 dramatically reduces it (El-Husseini Ael et al., 2002). The enhancement of AMPAR-mediated synaptic transmission that follows

PSD-95 overexpression occludes LTP and enhances LTD (Stein et al., 2003). Thus, the interaction of AMPAR/TARP complexes with PSD-95 may function to recruit extrasynaptic AMPAR/TARP complexes to postsynaptic sites in order to increase synaptic strength (Ehrlich and Malinow, 2004). Therefore, the scaffold protein PSD-95 appears to play a role in regulation of AMPAR location as well as in the formation of signaling complexes associated with the NMDAR.

4.32.3.2 GRIP/ABP, PICK1, and NSF

A distinct set of scaffold proteins bind selectively to GluR2/3 AMPARs and are believed to mediate regulation of their insertion and removal from synaptic sites (Lu and Ziff, 2005). The homologous PSD scaffold proteins termed GRIP (GRIP1) (Dong et al., 1997) and ABP (GRIP2) (Srivastava et al., 1998; Dong et al., 1999; Wyszynski et al., 1999) contain a total of six to seven PDZ domains and no obvious catalytic domain. The cytosolic tail of the GluR2 subunit of the AMPAR can bind to the fourth or fifth PDZ domain of either protein. GRIP/APBs exist in a palmitoylated form that is targeted to the postsynaptic membrane and a nonpalmitoylated form that is targeted to intracellular membranes (Yamazaki et al., 2001; DeSouza et al., 2002) and are thus thought to anchor AMPARs to either of these subcellular compartments in a regulated fashion to modulate AMPAR trafficking (Daw et al., 2000; Osten et al., 2000; Kim et al., 2001; Braithwaite et al., 2002; DeSouza et al., 2002; Fu et al., 2003; Hirbec et al., 2003; Seidenman et al., 2003). However, disruption of the interaction between the AMPAR and GRIP/ABP interferes most severely with the synaptic localization of the receptors (Dong et al., 1997).

PICK1 is a scaffold protein that contains a PDZ domain that binds selectively to protein kinase C-α (PKC-α) (Staudinger et al., 1997) and a BAR domain that that preferentially binds to curved membranes (Peter et al., 2004). GRIP/ABP also associates with the cytosolic tail of the GluR2 and GluR3 subunits of the AMPA receptor via the single PDZ domain (Dev et al., 1999; Xia et al., 1999). Because PICK1 forms homomultimers, even when the PDZ domain is occupied, PICK1 is believed to bring PKC-α in proximity to the tail of GluR2 (Chung et al., 2000; Perez et al., 2001), where it can promote phosphorylation of GluR2 on serine 880 by PKC-α (Matsuda et al., 1999; Perez et al., 2001). Phosphorylation of GluR2 on serine 880 releases GluR2 from GRIP/APB but does not alter its association with PICK1.

PICK1 and GRIP/ABP appear to work together to regulate trafficking and surface expression of GluR2/3 receptors. Since GRIP/ABP serves as an anchor for GluR2 subunit-containing AMPARs on both postsynaptic and intracellular membrane compartments, dissociation of GluR2 from GRIP/ABP is required for both insertion and removal of AMPARs at synaptic sites (Daw et al., 2000; Osten et al., 2000; Kim et al., 2001; Braithwaite et al., 2002; Fu et al., 2003; Hirbec et al., 2003; Seidenman et al., 2003). PICK1 appears to promote dissociation of GluR2 from GRIP/ABP when it binds to the GRIP/ABP/GluR2 complex and induces phosphorylation of GluR2 on serine 880 by PKC-α. As GRIP/ABP unbinds from the phosphorylated form of GluR2, PDZ domains on PICK1 multimers can bind to GluR2. The BAR domain on PICK1 then promotes GluR2 association with budding endo- and exocytotic vesicles that mediate the trafficking of AMPARs to and from the postsynaptic membrane. Thus, PICK1 paradoxically promotes both internalization and recycling of GluR2 (Lu and Ziff, 2005). As predicted from these findings, disruption of interaction of GluR2/3 with PICK1 or GRIP/ABP impairs expression of LTD (Kim et al., 2001; Seidenman et al., 2003).

NSF (*N*-ethylmaleimide-sensitive fusion protein) and the clathrin adaptor AP2 interact with the cytosolic tail of the GluR2 subunit of the AMPAR in a membrane proximal region distinct from the C-terminal PDZ-binding domain that interacts with GRIP/ABP and PICK1. NSF and AP2 are also required for correct regulation of AMPAR trafficking and surface expression and consequently play important roles in synaptic plasticity, particularly LTD (Nishimune et al., 1998; Osten et al., 1998; Song et al., 1998; Luscher et al., 1999; Lee et al., 2002).

In addition to the AMPAR, GRIP/ABP binds to other synaptic proteins including the receptor tyrosine kinase EphB2 (Hoogenraad et al., 2005), the scaffold protein liprin-α (Wyszynski et al., 2002), and the neuronal Ras-GEF GRASP-1 (Ye et al., 2000). Interactions with these proteins likely help to regulate other, less well understood, aspects of AMPAR function.

4.32.4 Protein Complexes Associated with the Metabotropic Glutamate Receptor

The excitatory neurotransmitter glutamate acts upon two major classes of postsynaptic receptors: ionotropic (NMDARs, AMPARs, and KARs) and metabotropic

(mGluRs). The G-protein-coupled mGluRs modulate neuronal excitability and synaptic strength by regulating the production of second messengers.

4.32.4.1 Homer

Group 1 mGluRs, which include mGluR1 and mGluR5, are linked to phospholipase C, which produces the second messengers IP3 and diacylglycerol. Group I mGluRs are present at the periphery of the PSD (Baude et al., 1993; Nusser et al., 1994; Lujan et al., 1997) and play an important role in synaptic plasticity (Bortolotto et al., 1999). Their linkage to the PSD is mediated by the scaffold protein homer (Brakeman et al., 1997; Xiao et al., 1998). Homer is actually a family of proteins comprising homer 1, 2, and 3, each of which is alternatively spliced into long and short isoforms (termed 'a' isoforms) (Kato et al., 1998; Xiao et al., 1998). All of the homers contain an N-terminal EVH1 domain, which binds to a characteristic proline-rich motif (PPXXF) in homer-interacting proteins (Beneken et al., 2000). In addition, the long homer isoforms contain a C-terminal coiled-coil (CC) domain, which promotes their self-association into tetramers (Tadokoro et al., 1999; Hayashi et al., 2006). The short isoforms do not contain the CC domain and thus are monomeric (Kato et al., 1998). Moreover, short homers, for example homer1a, were originally characterized as 'immediate early genes' whose expression is rapidly induced by synaptic activity. In contrast, long homers, for example, homer1b/c, are constitutively expressed (Xiao et al., 2000; Fagni et al., 2002).

4.32.4.2 Linkage to the IP3R

In addition to group 1 mGluRs, several other proteins bind to the EVH1 domain of Homer via the characteristic PPXXF motif (Xiao et al., 2000; Fagni et al., 2002). Two such proteins are the type 1 inositol trisphosphate receptor (IP3R) (Tu et al., 1998) and shank, a PSD scaffold protein (Tu et al., 1999). Because of their ability to self-associate, long homers function in the PSD as hubs that crosslink homer-interacting proteins (Tu et al., 1998, 1999). On the other hand, short monomeric homers antagonize this function and thus act as endogeneous 'dominant negatives' that are rapidly and transiently expressed upon synaptic stimulation (Brakeman et al., 1997; Kato et al., 1997).

An interesting example of the structural and functional coupling provided by long homers is the link between group 1 mGluRs and the IP3 receptor (IP3R) (Tu et al., 1998) (**Figure 3**). The IP3R is an IP3-sensitive Ca^{2+} channel localized to the smooth endoplasmic reticulum (SER). Activation of group 1 mGluRs by glutamate results in production of the second messenger IP3. IP3 then binds to the IP3R, causing it to open and release Ca^{2+} from the SER, which functions as an intracellular calcium store. The mGluR/homer/IP3R ternary complex thus couples synaptic activation of group 1 mGluRs with the release of calcium from SER compartments located in a subset of dendritic spines (Spacek and Harris, 1997; Sala et al., 2005). Remarkably, the SER of dendritic spines is juxtaposed with the postsynaptic membrane at the periphery of the PSD, exactly where group I mGluRs and homer are located (Xiao et al., 1998). Activity-induced expression of short isoforms of homer can apparently exert a dominant negative effect on the coupling between mGluRs and the IP3R, because overexpression of a short homer impairs release of intracellular calcium evoked by quisqualate, an mGluR agonist (Tu et al., 1998).

A second layer of regulation of mGluRs is provided by the ability of homer 1a to induce constitutive activation of mGluR1a and mGluR5 when it binds to their intracellular domains (Ango et al., 2001). The long homers can then disrupt this interaction and antagonize the constitutive activity conferred by homer 1a (Ango et al., 2001).

The regulatory properties of homer proteins may play a role in mechanisms of addiction (Szumlinski et al., 2004, 2005) and in susceptibility to schizophrenia (Szumlinski, 2005), as evidenced by the phenotypes of mice with deletions of homer 1 or homer 2.

4.32.5 Shank/ProSAP Scaffold of Scaffolds

A laminar organization of the PSD scaffold is suggested by the localization of PSD scaffolds as measured by immunoelectron microscopy (Valtschanoff and Weinberg, 2001; Petersen et al., 2003). Peak concentrations of PSD-95, and GKAP-shank were detected in the PSD at an average distance of 12 and 24–26 nm, respectively, from the plasma membrane. Cript and dynein, proteins that may be involved in linking PSD proteins to cytoskeletal elements, lay even farther away, 29–31 nm, from the plasma membrane. The shank/ProSAP scaffold proteins (Lim et al., 1999; Naisbitt et al., 1999; Tu et al., 1999; Yao et al., 1999;

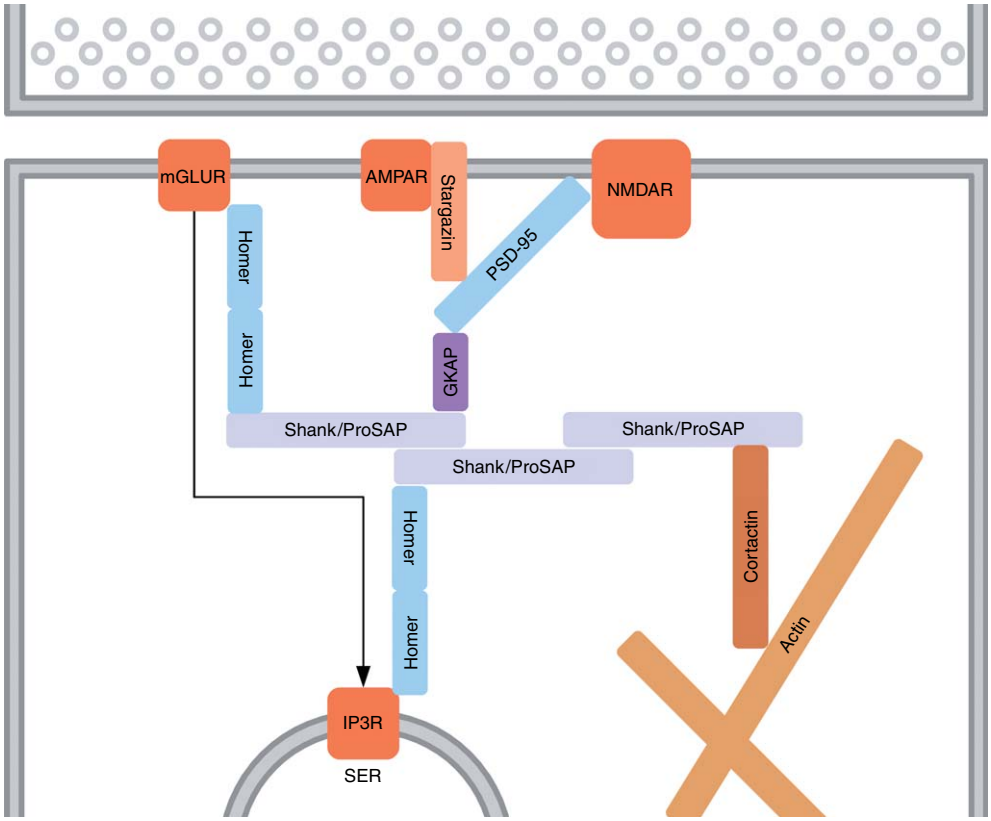


Figure 3 ProSAP/shank forms a master scaffold linking NMDA, AMPA, and metabotropic glutamate receptor-associated complexes in the postsynaplic density. Self-association of ProSAP/Shank scaffold proteins forms a lattice linking *N*-methyl-D-aspartate, AMPA, and metabotropic glutamate receptor-associated complexes together. The scaffold forms additional links with the actin cytoskeleton and the smooth endoplasmic reticulum (SER). Binary physical interactions between two proteins are represented by their juxtaposition. Arrows represent regulatory actions (→ stimulatory, ⊣ inhibitory).

Boeckers et al., 1999a, 1999b) act as master scaffolding proteins linking together PSD-95-associated proteins, homer-associated proteins, and the actin cytoskeleton (Sheng and Kim, 2000; Boeckers et al., 2002). Shank forms homomultimers through interactions of its SAM domains with each other and with sharpin (Naisbitt et al., 1999; Lim et al., 2001). Its single PDZ domain can also dimerize in an antiparallel configuration, contributing to multimerization (Im et al., 2003). These interactions suggest that shank can form a two-dimensional protein lattice that, by virtue of its rich collection of protein-interaction domains (6–7 ankyrin repeats, an SH3 domain, a PDZ domain, a proline-rich region), can accommodate several ligands including homer and GKAP/SAPAP (Baron et al., 2006). The shank complex can thus be thought of as a platform that cross-links the NMDA and metabotropic glutamate receptor complexes (**Figure 3**). It may also link to AMPAR complexes either via direct binding to the GluR1 subunit (Uchino et al., 2006) or through interactions between the TARP proteins and PSD-95 (Nicoll et al., 2006).

The physical interaction between group 1 mGluRs and the shank scaffold is mediated by the long isoforms of homer (Ehlers, 1999). This structural coupling may underlie functional coupling between group 1 mGluRs and the NMDAR (O'Connor et al., 1994), in which mGluR1 can upregulate NMDAR function through proline-rich tyrosine kinase 2 (Pyk2) and Src-family kinases that are part of the NMDAR complex (Heidinger et al., 2002). The long isoforms of homer and shank also appear to cooperate in morphogenesis of dendritic spines. Accumulation of shank in synapses depends on its ability to bind to homer (Sala et al., 2003) and also to GKAP/SAPAP (Naisbitt et al., 1999). When overexpressed alone or in combination, the long isoforms

of homer and shank synergistically increase the number and size of spines (Sala et al., 2001). As expected, overexpression of the short isoforms of homer have the opposite effect on spine morphogenesis, reducing synaptic targeting of shank and other PSD proteins and inhibiting synaptic transmission through their dominant-negative function (Sala et al., 2003).

Through its interactions with cortactin, Abp1, and spectrin/fodrin, the shank scaffold mediates binding of the PSD to the underlying spine actin cytoskeleton (Naisbitt et al., 1999; Bockers et al., 2001; Qualmann et al., 2004) and thus facilitates a functional connection between activation of postsynaptic receptors and changes in the spine cytoskeleton.

4.32.6 Attachment of the Cytoskeleton to the PSD by Actin-Associated Proteins

Dendritic spines contain a higher density of actin filaments than any other neuronal structure, and it is the arrangement of these filaments that gives spines their characteristic shape (Fifkova and Delay, 1982; Markham and Fifkova, 1986). The narrow neck contains longitudinally bundled filaments, whereas the head contains a lattice-like mesh that forms the bulbous head (Landis and Reese, 1983). The section titled 'Attachment of the cytoskeleton to the PSD by actin-associated proteins' discusses three actin-binding proteins that physically connect the cytoskeleton to the PSD: α-actinin, cortactin, and spectrin. Functional analysis of these proteins indicates that attachment of F-actin to the PSD immobilizes receptors at the synapse and regulates their function.

4.32.6.1 α-Actinin

α-Actinin is an actin-binding protein with filament crosslinking activity (Meyer and Aebi, 1990). The α-actinin family consists of four members (α-actinin1–4), of which isoforms 1, 2, and 4 are expressed in the brain (Wyszynski et al., 1998; Walikonis et al., 2000, 2001). The α-actinin family is characterized by an actin-binding domain at its N terminus, four spectrin repeats in the central region (Meyer and Aebi, 1990), and a Ca^{2+}-binding EF hand motif (Witke et al., 1993) and PDZ domain-binding sequence at the C terminus (Walikonis et al., 2001). α-Actinin self-associates in antiparallel dimers that can bind actin on both ends, giving it the ability to crosslink F-actin. Consistent with this function, overexpression of α-actinin-2 increases the length and density of dendritic protrusions in cultured hippocampal neurons (Nakagawa et al., 2004).

A number of studies indicate that α-actinin plays an important role in the localization and function of NMDARs. α-Actinin2 colocalizes with NMDARs in spines and binds directly to both the NR1 and NR2 subunits (Wyszynski et al., 1997; Dunah et al., 2000). This interaction is antagonized by both calcium-bound CaM and autophosphorylated CaMKII (Wyszynski et al., 1997; Leonard et al., 2002). Interestingly, dissociation of the NMDAR from F-actin causes rapid inactivation of the channel (Wyszynski et al., 1997; Zhang et al., 1998), while association with the cytoskeleton enhances NMDAR activity (Rosenmund and Westbrook, 1993). Thus, NMDARs are probably tethered to F-actin under resting conditions but dissociate and become inactivated in response to calcium influx through the channel.

Densin-180 is another point of attachment for F-actin within the PSD. The conserved C-terminal region of α-actinin-4 binds to the PDZ domain of densin. Densin also binds to CaMKII, which itself directly binds to α-actinin-4, forming a ternary complex. Since autophosphorylated CaMKII has an increased affinity for densin, this entire complex is probably dependent on intracellular calcium levels (Walikonis et al., 2001). Further studies will shed light on the functional significance of this complex.

4.32.6.2 Cortactin

Cortactin drives *de novo* actin nucleation and creates branch points on preexisting filaments by activating the ARP2/3 complex. In addition to promoting filament extension, cortactin binding also stabilizes F-actin. Structurally, cortactin contains an acidic domain at its N terminus similar to other actin nucleation-promoting factors such as WASP, N-WASP, and SCAR/WAVE; all proteins that interact with the ARP2/3 complex (Wu and Parsons, 1993; Weed et al., 2000; Uruno et al., 2001; Weaver et al., 2001). The central region contains 6.5 tandem copies of a 37–amino acid repeat that mediate its interaction with F-actin (Wu and Parsons, 1993). The C terminus contains a helical domain, a proline-rich domain, and a SH3 domain (Daly, 2004). The SH3 domain of cortactin interacts with shank, physically linking the PSD to the cytoskeleton (Du et al., 1998; Naisbitt et al., 1999).

Cortactin is highly enriched in spines where it colocalizes with F-actin (Hering and Sheng, 2003;

Racz and Weinberg, 2004). Electron microscopic studies of imunogold-labeled cortactin show that approximately 10% of cortactin in the spine is located in the PSD, whereas the bulk of the staining occurs within the spine core, 100–150 nm away from the PSD (Racz and Weinberg, 2004). Thus, two pools of cortactin exist in the spine, with only a small fraction interacting with the PSD. Interestingly, NMDAR activation induces translocation of cortactin from spines into the dendrite (Hering and Sheng, 2003), suggesting a role for cortactin in activity-dependent modification of the cytoskeleton.

As predicted by its ability to activate the Arp2/3 complex, cortactin is a strong determinant of spine morphology in cultured hippocampal neurons. Knockdown of endogenous cortactin in mature cultured hippocampal neurons by siRNA drastically reduces the total number of dendritic protrusions (Hering and Sheng, 2003). Thus, cortactin is required for the maintenance of mature spines, perhaps by stabilizing filaments or promoting growth of the actin network. Spines on cultured neurons overexpressing cortactin are abnormally long, similar to those observed in overexpression studies of α-actinin, and this elongation requires the N-terminal region of cortactin that interacts with F-actin and the Arp2/3 complex (Hering and Sheng, 2003).

4.32.6.3 Spectrin

Spectrin is the main component of the cell membrane skeleton. It was first identified in erythrocytes, where it forms a filamentous network required for red blood cells to maintain their shape and elasticity (Marchesi and Steers, 1968). The spectrin family of proteins includes α- and β-spectrin and α- and β-fodrin (also referred to as α- and β-spectrin II, or brain spectrin). In the following discussion, the term spectrin will refer to the brain form of spectrin. Several unique domains differentiate the α and β subunits: the α subunit contains 21 triple helical repeats with a segment containing an SH3 domain in the tenth repeat, and the β subunit has a shorter triple helical repeat region (17 repeat segments) and does not contain SH3 or EF hand domains. The β subunit also contains an actin-binding region at its N terminus, and some β subunits contain a PH domain. Within the membrane skeleton, the α and β subunits typically form heterotetramers by forming antiparallel heterodimers that interact head to head to form a tetramer. By interacting with transmembrane proteins such as ankryin, spectrin connects the actin cytoskeleton to cell membranes (Goodman et al., 1995; Czogalla and Sikorski, 2005).

In addition to interacting with the cell membrane, spectrin is also present in the PSD, where it binds directly to NR1, NR2A, and NR2B subunits of NMDARs in an activity-dependent manner (Carlin et al., 1983; LeVine and Sahyoun, 1986; Wechsler and Teichberg, 1998). CaM inhibits spectrin-NR1 interaction, and Ca^{2+} antagonizes the spectrin-NR2B binding (Wechsler and Teichberg, 1998). Spectrin binding is also inhibited by phosphorylation of the NR1 C-terminal domain by PKA and PKC and of the NR2A C-terminal domain by fyn (Wechsler and Teichberg, 1998). In the neuromuscular junction, removal of actin and spectrin from myotube membranes results in dispersal of AChRs (Bloch, 1986). By analogy, spectrin may be important for immobilizing receptors at the synapse (Adam and Matus, 1996). The calcium and kinase dependence of the spectrin-NMDAR interaction provides a potential mechanism by which NMDAR motility in the membrane may be regulated by synaptic activity.

In mature synapses, the actin cytoskeleton immobilizes some, but not all, components of the PSD. For example, treatment of cultured neurons with latrunculin A, a toxin that causes depolymerization of actin by sequestering G-actin, results in a decrease in AMPA and NMDARs, α-actinin-2, drebrin (an actin-binding protein that can cluster actin), and CaMKII from spines. However, PSD-95 remains in spines even after filament depolymerization (Allison et al., 1998, 2000), suggesting that PSD-95 may form a stable core scaffold that does not require the cytoskeleton for its aggregation.

4.32.7 Regulation of the Spine Actin Cytoskeleton by Signaling Complexes in the PSD

Real-time live imaging of fluorescently labeled spines of cultured neurons reveals that they are surprisingly motile structures (for reviews, see Yuste and Bonhoeffer, 2004; Oertner and Matus, 2005). When monitored continuously, one sees that spines exhibit a 'twitching' motion resulting from the continuous turnover of the actin cytosleteton even under resting conditions. Even more dramatic is the observation that certain patterns of synaptic activation cause spines to grow or shrink. Many investigators have documented that the volume of a spine head is proportional to its synaptic strength (Harris and Stevens,

1989; Nusser et al., 1998; Schikorski and Stevens, 1999). One likely mechanism proposed for LTP is the addition of new AMPA receptors at the postsynaptic site (Shi et al., 2001), with a corresponding enlargement of the spine head. This notion is supported by the observation that stable LTP cannot be induced if actin polymerization is inhibited pharmacologically (Kim and Lisman, 1999; Krucker et al., 2000). Likewise, there is evidence that smaller spines preferentially undergo LTP, whereas larger, mature spines are less plastic (Matsuzaki et al., 2004). A study that measured actin polymerization with a fluorescence resonance energy transfer (FRET) technique shows that tetanic stimulation that typically induces LTP increased formation of F-actin in spines. Conversely, low-frequency stimulation that typically induces LTD results in depolymerization of spine action (Okamoto et al., 2004). This dynamic property of spines, with its implications for cellular mechanisms of learning and memory, has provoked intense interest in identifying the signaling complexes that coordinate synaptic activity and regulation of the underlying actin cytoskeleton.

4.32.7.1 Mechanisms of Regulation of Actin Polymerization

Because actin monomers are continuously treadmilling on and off of filaments, regulation of the overall structure of the cytoskeleton in spines occurs at many different levels: growth by addition of monomers, attrition by actin depolymerization, capping of filaments to block addition of new monomers, sequestration of monomers to limit growth of filaments, severing capped filaments to initiate new growth, and filament elaboration by branching. The following is a brief description of some of the major regulators of actin polymerization in spines (for reviews, see Pollard and Borisy, 2003; Dillon and Goda, 2005).

Actin filaments are polar structures that appear to have a barbed end and a pointed end. Because actin-ATP has a greater affinity for the barbed end, the filament grows three times faster from this end than the pointed end (Pollard, 1976). Over time, ATP hydolyzes to ADP on the older end of the filament. ADF and cofilin are actin-severing proteins that bind to actin monomers with a higher affinity for actin-ADP than actin-ATP. Binding of ADF/cofilin to actin-ADP changes the twist in the actin helix and promotes severing (McGough et al., 1997; Maciver et al., 1998; Blanchoin and Pollard, 1999). In this manner they catalyze disassembly of the older, pointed ends of actin filaments. Phosphorylation of ADF/cofilin at serine 3 inhibits its ability to bind to actin (Agnew et al., 1995). Thus, depolymerization of actin can be regulated by cytosolic signaling pathways. Profilin, on the other hand, promotes the elongation of actin filaments by catalyzing exchange of ADP for ATP on actin monomers, maintaining a pool of actin-ATP monomers that can be added to the growing end of filaments (Wolven et al., 2000). The rate of filament growth is also controlled by capping protein and gelsolin, which bind to the barbed end of filaments, blocking addition of actin monomers (McGough et al., 2003; Wear et al., 2003). Last, *de novo* filament nucleation and branching are both achieved through activation of the Arp2/3 complex, which acts as a template for filament assembly. Thus, growth and maintenance of the actin cytoskeleton is a finely tuned process that has many points of regulation.

4.32.7.2 Regulation of Rac in the PSD

Identification of signaling pathways that link synaptic activity to cytoskeletal regulation in spines is an active field of research. In recent years, a clear role for Rho family GTPases, and Rac in particular, in spine morphogenesis has emerged. Neurons expressing a constitutively active form of Rac form ruffle-like protrusions, composed of many small spines (Luo et al., 1996; Nakayama et al., 2000; Tashiro et al., 2000; Pilpel and Segal, 2004). Conversely, expression of dominant negative Rac in neurons reduces spine density and causes significant lengthening of spines (Nakayama et al., 2000; Tashiro et al., 2000; Pilpel and Segal, 2004; Tashiro and Yuste, 2004). Blocking Rac1 activity in mature spines leads to reduction in spine head width, indicating that Rac1 plays a role in maintaining mature spine morphology (Tashiro and Yuste, 2004). Rac, like all members of the small GTPase superfamily, functions as a binary switch by cycling between the active GTP-bound and inactive GDP-bound states. To activate Rac-GDP, a Rac GEF must release GDP, allowing the more abundant GTP to bind. This section summarizes our current understanding of postsynaptic Rac activation by the GEFs, kalirin and Tiam1.

4.32.7.2.1 Kalirin

Activation of Rac signaling by kalirin is crucial for development and maintenance of dendritic spines. Kalirin is encoded by a single gene, but several isoforms are generated by alternative splicing

and different promoters (Johnson et al., 2000; McPherson et al., 2002, 2004). Kalarin-7, -9, and -12 are the most abundant isoforms in the CNS. Kalirin-9 and -12 are prevalent early in development, whereas kalirin-7 expression appears at the time of synapse formation and remains high throughout adulthood (Hansel et al., 2001; Ma et al., 2001, 2003). Of these three isoforms, only kalirin-7 contains a PDZ-binding motif that mediates its interaction with PSD-95. Indeed, kalirin-7 is enriched in PSD fractions of rat cerebral cortex and is labeled immunohistochemically in discrete puncta along dendritic processes of cultured cortical neurons (Penzes et al., 2000). When kalirin-7 is overexpressed in cortical neurons, the size and number of spines increases. Overexpression of a mutant lacking the PDZ-binding motif results in mislocalization of kalirin and formation of filopodia on the soma instead of on dendrites (Penzes et al., 2001). Thus, proper targeting of kalirin to the PSD is necessary for normal spinogenesis.

Kalirin-dependent spine formation is induced by EphB activation. EphB receptors are protein tyrosine kinase-linked receptors located on in the postsynaptic membrane (McPherson et al., 2004). Mutant mice with a triple knockout of EphB receptors (EphB1-3) fail to form dendritic spines in cultured hippocampal neurons (Henkemeyer et al., 2003). Ephrin B1 activation of EphB receptors induces rapid formation of mature spines along the dendrites of 10-day-old cultured hippocampal neurons by recruiting kalirin-7 into clusters along the dedritic shaft and in spines (Penzes et al., 2003). Furthermore, kalirin's effects are dependent on downstream Rac activation since expression of dominant negative Rac blocks ephrin-induced spine formation.

4.32.7.2.2 Tiam1

Tiam1 is a Rac GEF located just below the postsynaptic membrane and is expressed in the brain during development and throughout adulthood (Ehler et al., 1997; Tolias et al., 2005). RNAi knockdown of Tiam1 in cultured neurons results in a severe reduction in the number of dendritic spines, and the spines that remain are longer and more filopodial in shape, much like the spine phenotype induced by dominant negative Rac. Furthermore, knockdown of Tiam1 blocks NMDAR-mediated spine outgrowth, indicating that it plays a crucial role in NMDAR-dependent morphological plasticity (Tolias et al., 2005).

Tiam1 interacts with NR1 subunits and is activated in response to NMDAR stimulation, resulting in elevation of GTP-bound Rac (Tolias et al., 2005). Several signaling mechanisms might mediate NMDAR-dependent activation of Rac by Tiam1 (**Figure 2**). CaMKII can phosphorylate and activate Tiam1 (Fleming et al., 1999). Thus, CaMKII autophosphorylation in response to synaptic stimuli that activate NMDARs may in turn activate Rac through Tiam1. Tiam1 can also be activated by direct binding of Ras-GTP to its Ras binding domain (Lambert et al., 2002), raising the possibility that NMDAR-induced Ras activation through RasGRF1 may stimulate Rac signaling pathways. It is intriguing that knockout of synGAP, a suppressor of Ras activity, increases the number and size of spines in hippocampal cultures (Vazquez et al., 2004), suggesting that crosstalk between the Ras and Rac pathways in spines might coordinate synaptic strength and morphological plasticity.

4.32.7.3 Reshaping the Spine Cytoskeleton Through the PAK/LIMK/Cofilin Pathway

Spine morphogenesis by either kalirin- or Tiam1-mediated Rac activation is accompanied by increased PAK phosphorylation (Penzes et al., 2003; Tolias et al., 2005). PAKs are a family of closely related serine/threonine protein kinases that were discovered relatively recently in a screen for Rho-GTP binding partners (Manser et al., 1994; Bokoch, 2003). In the inactive state, PAKs form homodimers in which the regulatory domain of one PAK molecule inhibits the catalytic domain of another. Binding of GTP-bound Cdc42 or Rac1 to PAK causes conformational changes that destabilize folding of the inhibitory switch domain and facilitate catalytic activation (Bokoch, 2003). Phosphorylation of threonine 423 in the PAK catalytic domain is required to suppress autoinhibition and achieve full catalytic function.

It is clear that PAKs have a crucial role in regulation of spine formation. PAKs phosphorylated at threonine 423 (pPAKs), in particular pPAK1 and pPAK3, are concentrated in dendritic spines and enriched in the PSD fraction (Penzes et al., 2003; Hayashi et al., 2004). Treatment of cultured hippocampal neurons with an inhibitory peptide consisting of the PAK1 inhibitory domain fused to the cell-penetrating peptide TAT reduces the effect of ephrin-B ligands on spine formation (Penzes et al., 2003). Transgenic mice expressing the peptide inhibitor of PAK1-3 in forebrain neurons under the control of the α-CaMKII promoter show a significant reduction in spine density and increase in spine head size in cortical neurons by 4 weeks of age (Hayashi et al., 2004). When PAK3 is specifically inhibited by siRNA

in hippocampal organotypic cultures, spines become thin and elongated, and the total number of synapses decreases (Boda et al., 2004).

How do the PAKs influence spine morphology? PAKs have many potential downstream regulatory targets. One target likely to mediate effects on the actin cytoskeleton is the enzyme LIMK, which phosphorylates and inactivates ADF/cofilin. LIMK1 is a serine/threonine kinase that contains two LIM domains and a PDZ domain (Okano et al., 1995) and that is enriched in hippocampal pyramidal neurons (Foletta et al., 2004). Activated PAK forms a complex with LIMK1 and activates it by phosphorylating threonine 508 in the catalytic domain (Edwards et al., 1999). The activated LIMK1 then phosphorylates serine 3 of ADF/cofilin, inhibiting its activity (Arber et al., 1998; Yang et al., 1998). Golgi staining of neurons in brain sections from homozygous LIMK1 knockout mice reveals abnormal spine morphology compared with wild-type mice: spine head size is dramatically decreased, and spine necks are thicker. Furthermore, the actin-severing protein, cofilin, is significantly less phosphorylated in the mutant than in wild-type brains. In its dephosphorylated form, cofilin binds F-actin and promotes severing of the filament. This study supports a model in which LIMK1 inactivates cofilin, slowing the turnover of F-actin and permitting formation of larger spine heads.

Immunoelectron microscopy shows that cofilin is located in the PSD and the spine shell but is excluded from the core, providing evidence for the notion that F-actin in the core is relatively stable, whereas actin around the periphery and projecting into the PSD is more motile (Racz and Weinberg, 2006). *In vivo* LTP in the dentate gyrus is associated with an increase in the F-actin content in spines that is NMDAR-dependent and involves inactivation of cofilin (Fukazawa et al., 2003). These changes occur within 20 min of stimulation and last as long as 5 weeks. In acute hippocampal slices from neonatal rats, two-photon time-lapse imaging reveals that induction of LTD by low-frequency stimulation is associated with a shrinkage of spines that is mediated by cofilin activation (Zhou et al., 2004). Thus, cofilin appears to be a key player in the bidirectional morphological plasticity of spines.

4.32.7.4 Actin Dynamics and Mental Retardation

Mental retardation (MR) is defined as a significant impairment of cognitive and adaptive functioning, with the onset occurring before the age of 18 years. Although the underlying causes of MR are heterogeneous, several studies of golgi-impregnated postmortem brains from mentally retarded individuals reveal that dendritic spine abnormalities are a common feature of individuals with cognitive impairment (Huttenlocher, 1970, 1974; Marin-Padilla, 1972; Purpura, 1974). In recent years, a host of genetic forms of mental retardation have been mapped to gene mutations affecting Rho family GTPases and their downstream effectors that control actin dynamics (reviewed in Huttenlocher, 1974; Ramakers, 2002; Newey et al., 2005). Here we highlight recent research on one form of mental retardation, fragile X syndrome (FXS), which shows a compelling link between regulation of spine morphology and normal cognition in humans.

As early as the 1940s, clinicians identified families in which mental retardation segregated in a sex-linked pattern where more males than females inherited the condition (reviewed in Stevenson and Schwartz, 2002). Of the more than 1200 genes on the X chromosome, it is anticipated that mutations in approximately 200 genes give rise to different pedigrees of X-linked mental retardation (XLMR) (Stevenson, 2005). FXS is the most prevalent form of XLMR and is the second most common cause of mental disability, affecting 1 in 4000 males and 1 in 8000 females (Turner et al., 1996). In addition to cognitive deficits, FXS is also associated with other pathologies such as hyperactivity, attention deficit, and autistic-like behavior. Genetic analysis of affected families and studies of animal models have yielded tremendous advances in our understanding of the molecular mechanisms responsible for the cognitive deficits in FXS.

Postmortem analysis of human brain samples shows that pyramidal neurons from the cortex and hippocampus of FSX patients have an increased number of long, thin spines that resemble those normally seen early in development (Hinton et al., 1991; Irwin et al., 2001). In 1991, the gene mutated in FXS was cloned and named *Fmr1* (Verkerk et al., 1991). *Fmr1*-null mice have an increase in long, thin 'immature' spines similar to those observed in humans with FXS (Comery et al., 1997; Nimchinsky et al., 2001; Irwin et al., 2002; McKinney et al., 2005; Grossman et al., 2006). Since no gross anatomical brain abnormalities are associated with FXS, it is hypothesized that altered synaptic function or spine maturation is responsible for the observed cognitive deficits.

The mutation causing FXS is an unstable expansion of CGC repeats that results in transcriptional silencing of *Fmr1*, and consequently the absence of Fragile X mental retardation protein (FMRP) expression.

FMRP is an mRNA-binding protein that can act as a translational suppressor at synapses, suggesting that inappropriate regulation of target mRNAs contributes to the abnormal maturation of spines in FSX patients and FMRP knockout mice (reviewed in Bardoni et al., 2001). FMRP also interacts with two cytoplasmic proteins, CYFIP1 and CYFIP2, which specifically bind only to the GTP-bound form of Rac1 (Schenck et al., 2001). In *Drosophila*, Rac-GTP binding to CYFIP causes dissociation and activation of FMRP (Schenck et al., 2003), suggesting that Rac activation may suppress the translation of some mRNAs. Recent work in murine fibroblasts shows that Rac-induced actin remodeling is enhanced in the absence of FMRP (Castets et al., 2005). Comparison of proteomes of cells expressing FMRP compared with those without, revealed that expression of the catalytic domain of PP2A was enhanced in the absence of FMRP. Since FMRP is a known translational suppressor and binds to pp2acβ mRNA, it is likely that FMRP normally suppresses PP2Ac in these cells. The authors also found that phophorylation of the actin-severing protein, cofilin, was decreased in the FMRP-negative cells. Since PP2A can activate cofilin by dephosphorylating it, the authors propose that the increase in actin remodeling observed in the FMRP-negative fibroblasts is a result of the increased activity of cofilin induced by elevated PP2A expression. It remains to be determined whether FMRP suppresses PP2A translation in neurons. However, it is tempting to speculate that dysregulation of cofilin activity might account at least in part for the immature spine phenotype observed in FXS patients. Overactivation of cofilin could destabilize the F-actin network, causing a decrease in spine head size. Interestingly, Williams syndrome, which results from an autosomal mutation, is associated with a decrease in cofilin phosphorylation by a completely different mechanism. One of the genes deleted in Williams syndrome is LIM kinase. Knocking out LIM kinase in mice causes a decrease in cofilin phosphorylation and a corresponding decrease in spine head size. Furthermore, two forms of nonsyndromic X-linked mental retardation (so called because mental retardation is the only phenotype) are also caused by mutations in Rac signaling: ARHGEF6 (also called αPix or Cool-2), a Rac-GEF, and the downstream effector PAK3 (Kutsche et al., 2000) and (Allen et al., 1998), respectively. Such examples of deficits in human cognition caused by mutations in genes controlling Rac signaling add strength to the hypothesis that activity-dependent morphological plasticity in spines is important for encoding information at the cellular level.

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4.33 Translational Control Mechanisms in Synaptic Plasticity and Memory

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4.33.1 Introduction

It is well accepted that one of the critical events that is required for the conversion of short-term to long-term memory is the requirement for new gene expression. At the cellular level, the conversion of short-lasting to long-lasting synaptic plasticity, processes that in many instances require the same molecular mechanisms as their memory counterparts, also requires new gene expression. Many laboratories have contributed to our understanding of the molecular mechanisms that control gene expression during long-lasting synaptic plasticity and long-term memory. Initially these studies focused on transcriptional control (Kandel, 2001), although it had been known for many years that long-term memory requires protein synthesis (Flexner et al., 1963). Many of the early studies on the requirement of protein synthesis (commonly referred to as translation) for long-term memory relied nearly exclusively on the injection of general protein synthesis inhibitors into the brains of intact animals. These studies provided much information concerning the temporal (when) and spatial (where in the brain) requirements for protein synthesis during long-term memory formation for a particular behavioral task but provided virtually no information about the molecular mechanisms of translational control during memory. Fortunately, there has been an incredible increase in our knowledge of how translation, especially translation initiation, is regulated during long-lasting synaptic plasticity and long-term memory.

Hippocampal long-term potentiation (LTP) was first shown to require new protein synthesis *in vivo* more than 20 years ago (Krug et al., 1984). Later, it was shown that LTP has distinct temporal phases that can be separated on the basis of whether the LTP is transcription and/or translation dependent (Frey et al., 1988; Huang and Kandel, 1994; Nguyen et al., 1994; Nguyen and Kandel, 1997). More mild types of LTP-inducing stimulation induce short-lasting potentiation (1–2 h of potentiated synaptic transmission) that is dependent on neither transcription nor translation. This short-lasting LTP is now commonly referred to as early-phase LTP (E-LTP). In contrast, stronger types of LTP-inducing stimulation that result in long-lasting potentiation (3 or more h of potentiation) that is dependent on transcription and translation is now commonly referred to as

late-phase LTP (L-LTP). Distinct temporal phases similar to those in LTP have been described for long-term facilitation (LTF) in *Aplysia*, another form of long-lasting synaptic plasticity (Kandel, 2001).

Most forms of L-LTP require activation of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor, but protein synthesis–dependent potentiation of synaptic transmission is not exclusively glutamatergic. Translation-dependent synaptic plasticity in hippocampal slices can also be induced with neurotrophic factors (Kang and Schuman, 1996) and facilitated with agonists of dopamine (Huang and Kandel, 1995; Smith et al., 2005) and β-adrenergic receptors (Gelinas and Nguyen, 2005). Moreover, long-lasting protein synthesis–dependent forms of hippocampal long-term depression (LTD) also have been described. LTD, regardless of whether it is induced by NMDA receptors (Kauderer and Kandel, 2000; Sajikumar and Frey, 2004), group I metabotropic glutamate receptors (mGluR; Huber et al., 2000), or insulin receptors (Huang et al., 2004), is blocked by protein synthesis inhibitors. Thus, protein synthesis–dependent LTP and LTD can be induced via activation of multiple receptors in the hippocampus.

The basic temporal phases of synaptic plasticity that can be distinguished by transcription and translation inhibitors appear to have correlates in memory function. An excellent example is the gill-withdrawal reflex in *Aplysia*, which requires LTF. A single sensitizing stimulus (usually an electrical shock) results in a short-term memory that is insensitive to transcription and translation inhibitors. In contrast, multiple, spaced sensitizing electrical shocks result in long-term memory that is sensitive to both transcription and translation inhibitors (Castellucci et al., 1989). Similar observations have been made in rodents, where in general, new mRNA and protein synthesis is required for long-term but not short-term memory (McGaugh, 2000; Kandel, 2001).

4.33.2 Local Protein Synthesis and Synaptic Plasticity

One of the intriguing features of neurons is that dendrites and their spines contain all the components required for synthesizing proteins. Polyribosomes (Steward and Levy, 1982), translation factors (Tang et al., 2002), and mRNA that can be translated into protein are all present at synapses (Crino and Eberwine, 1996). Thus, neurons presumably are able to synthesize proteins without engaging transcription in the nucleus and without mRNA transport to their dendrites.

Does local protein synthesis occur in dendrites? Earlier studies addressing this question relied on reduced biochemical preparations of pre- and postsynaptic components termed synaptoneurosome preparations. Membrane depolarization (Bagni et al., 2000), brain-derived neurotrophic factor (BDNF) (Yin et al., 2002), and glutamate receptor agonists (Weiler et al., 1997; Bagni et al., 2000) all have been shown to translate new proteins in synaptoneurosomes. More direct evidence demonstrating that protein synthesis occurs in dendrites has been provided with imaging studies utilizing fluorescent reporters. Application of BDNF to neurons and manipulations that result in the blockade of neuronal activity have been shown to trigger protein synthesis in dendrites (Aakulu et al., 2001; Ju et al., 2004; Sutton et al., 2004, 2006). Is local protein synthesis required for long-lasting synaptic plasticity? Protein synthesis inhibitors block both BDNF-induced LTP (Kang and Schuman, 1996) and mGluR-LTD (Huber et al., 2000) when the cell bodies of hippocampal neurons are severed from their dendrites. Thus, these types of synaptic plasticity require local protein synthesis independent of a requirement for transcription. Local protein synthesis also is required for LTF in *Aplysia* sensory neurons and plasticity at neuromuscular synapses in *Xenopus* (Martin et al., 1997; Zhang and Poo, 2002). Thus, local protein synthesis is required for numerous types of synaptic plasticity.

An obvious question is whether local protein synthesis is required for hippocampal L-LTP. The answer to this question has been difficult to ascertain because L-LTP requires both transcription and translation. However, translation inhibitors block L-LTP at earlier times than the transcription inhibitors (Kelleher et al., 2004a; Banko et al., 2005), suggesting that L-LTP consists of an early translation–dependent phase that is independent of transcription, which is followed by a transcription- and translation-dependent phase. The translation-dependent early phase of L-LTP is presumably due to local protein synthesis. Consistent with this notion, local application of a protein synthesis inhibitor to dendrites has been shown to block L-LTP (Bradshaw et al., 2003). Furthermore, translation-dependent, transcription-independent LTP has been shown in isolated dendrites in hippocampal slices (Cracco et al., 2005; Vickers et al., 2005). Finally, β-adrenergic receptor-dependent facilitation of L-LTP also requires local dendritic translation independent of a requirement

for transcription (Gelinas and Nguyen, 2005). Thus, local protein synthesis appears to be required at fairly early temporal windows during L-LTP.

4.33.3 Translational Control of Gene Expression

As described earlier, translational control plays an important role in the regulation of gene expression, which leads to L-LTP and memory consolidation (Steward and Schuman, 2001; Kelleher et al., 2004b; Klann and Dever, 2004). Translational control provides the cell with a faster response to external stimuli than transcription, mRNA processing, and transport. In eukaryotes, translational control operates mainly at the rate-limiting initiation step, during which the small 40S ribosomal subunit is recruited to mRNA and is positioned at the initiation codon (Mathews et al., 2000). Translation initiation entails, first, the binding of the initiator Met – $tRNA_i^{Met}$ to the small 40S ribosomal subunit; second, location of the ribosome complex to the initiation codon; and third, joining of the large ribosomal subunit to generate a translation-competent ribosome (**Figure 1**). Translational control is generally modulated by changes in the phosphorylation status of initiation factors or their regulators. Two major mechanisms of regulation are (i) the phosphorylation of eIF2α, which prevents the exchange of GDP for GTP, and (ii) the modulation of eIF4F assembly.

4.33.4 Regulation by Phosphorylation of eIF2α

The eukaryotic initiator factor eIF2, which consists of three subunits (α, β, and γ), binds the initiator Met – $tRNA_i^{Met}$ and GTP to form the ternary complex. eIF2 then associates with the small ribosomal subunit in its GTP-bound form. GTP is hydrolyzed to release eIF2 from the ribosome in the GDP-bound state. Exchange of GDP for GTP on eIF2 is catalyzed by a five-subunit factor termed eIF2B and is required to reconstitute a functional ternary complex for a new round of translation initiation (Hinnebusch, 2000). By slowing the dissociation of eIF2 from eIF2B, phosphorylation of the α subunit at Ser51 blocks the GDP/GTP-exchange reaction and therefore causes a decrease in general translation initiation (Hinnebusch, 2000; Sonenberg and Dever, 2003) (**Figure 2**). Most cells contain more eIF2 than eIF2B, and consequently phosphorylation of a fraction of the eIF2α is sufficient to inhibit eIF2B and thus block translation.

While phosphorylation of eIF2α leads to a general inhibition of translation, it paradoxically results in translational upregulation of a subset of mRNAs that contain upstream open reading frames (uORFs) (Hinnebusch, 2000; Sonenberg and Dever, 2003) (**Figure 2**). The molecular mechanism underlying this selective translation was extensively studied in the general amino acid control response in the yeast *Saccharomyces cerevisiae* (Hinnebusch, 2000). Amino acid starvation of yeast leads to activation of the Gcn2p eIF2α kinase and inhibition of translation. However, translation of the GCN4 mRNA is stimulated because it contains in its 5′ untranslated region (5′UTR) four short ORFs. When amino acids are available, scanning ribosomes translate these short ORFs but dissociate from the mRNA before reaching the authentic GCN4 start codon. In contrast, when amino acids are scarce, eIF2α is phosphorylated, causing a fraction of the scanning 40S subunits to form active translational complexes only after they bypassed the upstream ORF and allowing initiation at the proper GCN4 start codon (Hinnebusch and Natarajan, 2002).

A similar mechanism was described in mammalian cells where the translation of the mRNA for the transcriptional modulator activating transcription factor 4 (ATF4) is enhanced by eIF2α phosphorylation (Harding et al., 2000; Vattem and Wek, 2004) (**Figure 2**). Therefore, eIF2α phosphorylation regulates both general and gene-specific translation. Importantly, ATF4 and its homologues play important roles as repressors of synaptic plasticity and memory formation in diverse phyla (Bartsch et al., 1995; Abel et al., 1998; Chen et al., 2003). An activity-dependent modulation of eIF2α phosphorylation in neurons is important for sustained alterations in synaptic transmission and learning and memory. In hippocampal slices, the gene expression-dependent L-LTP, induced by either tetanic stimulation or forskolin, is associated with decreased eIF2α phosphorylation (Costa-Mattioli et al., 2005). In hippocampal neurons treated with BDNF, which also induces L-LTP in hippocampal slices (Tang et al., 2002), phosphorylation of eIF2α is reduced (Takei et al., 2001). The evidence for the involvement of eIF2α in synaptic plasticity and memory formation was bolstered by a recent study on mice lacking the eIF2α kinase GCN2: in these mice, the

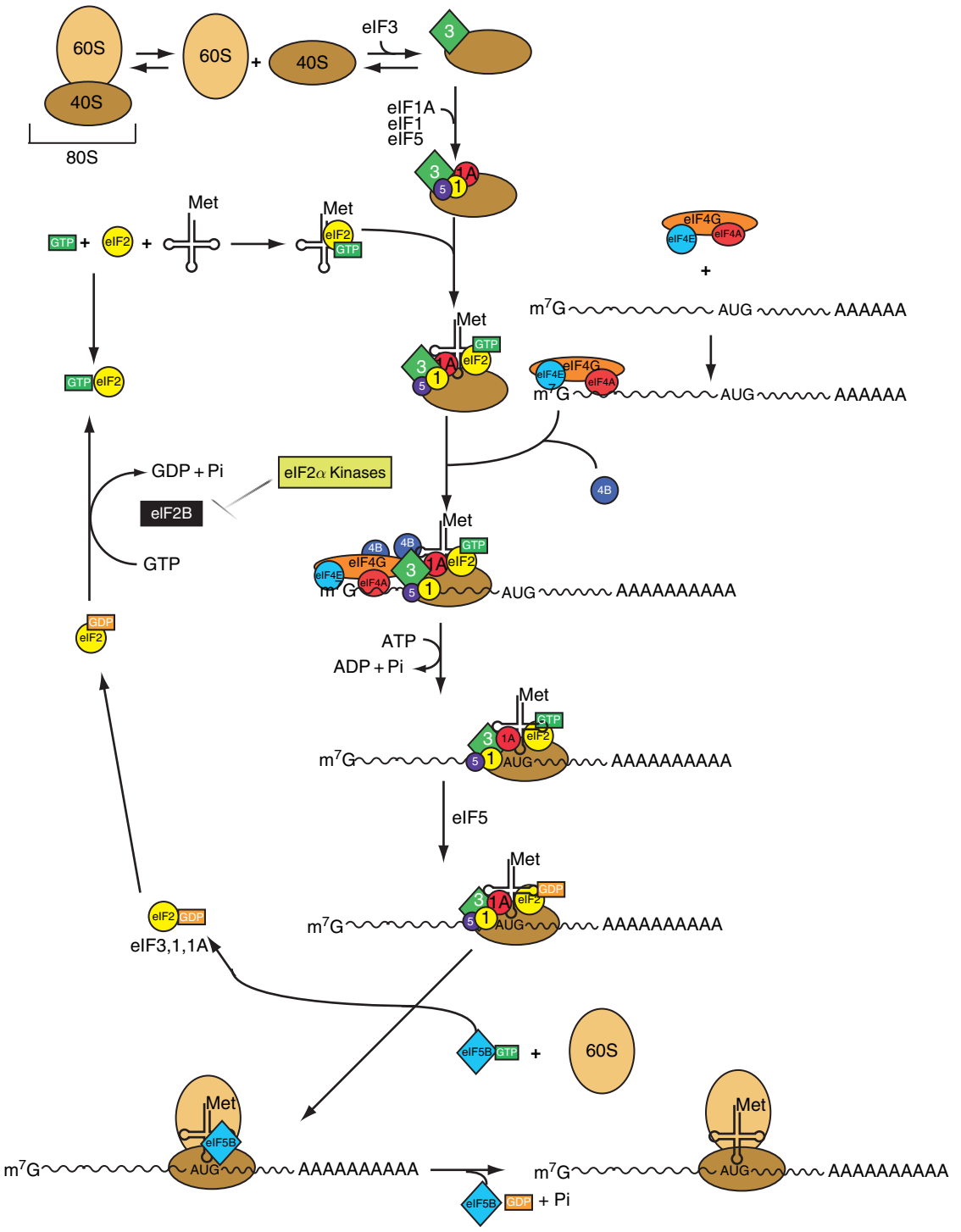


Figure 1 Translation initiation pathway in eukaryotes. Initiation factors are shown as color-coded symbols and appear when first implicated in the pathway.

reduction in phosphorylated eIF2α is associated with altered synaptic plasticity and memory (Costa-Mattioli et al., 2005; see following discussion).

In higher eukaryotes, the status of eIF2α phosphorylation is controlled by at least four known Ser/Thr protein kinases for which eIF2α is the

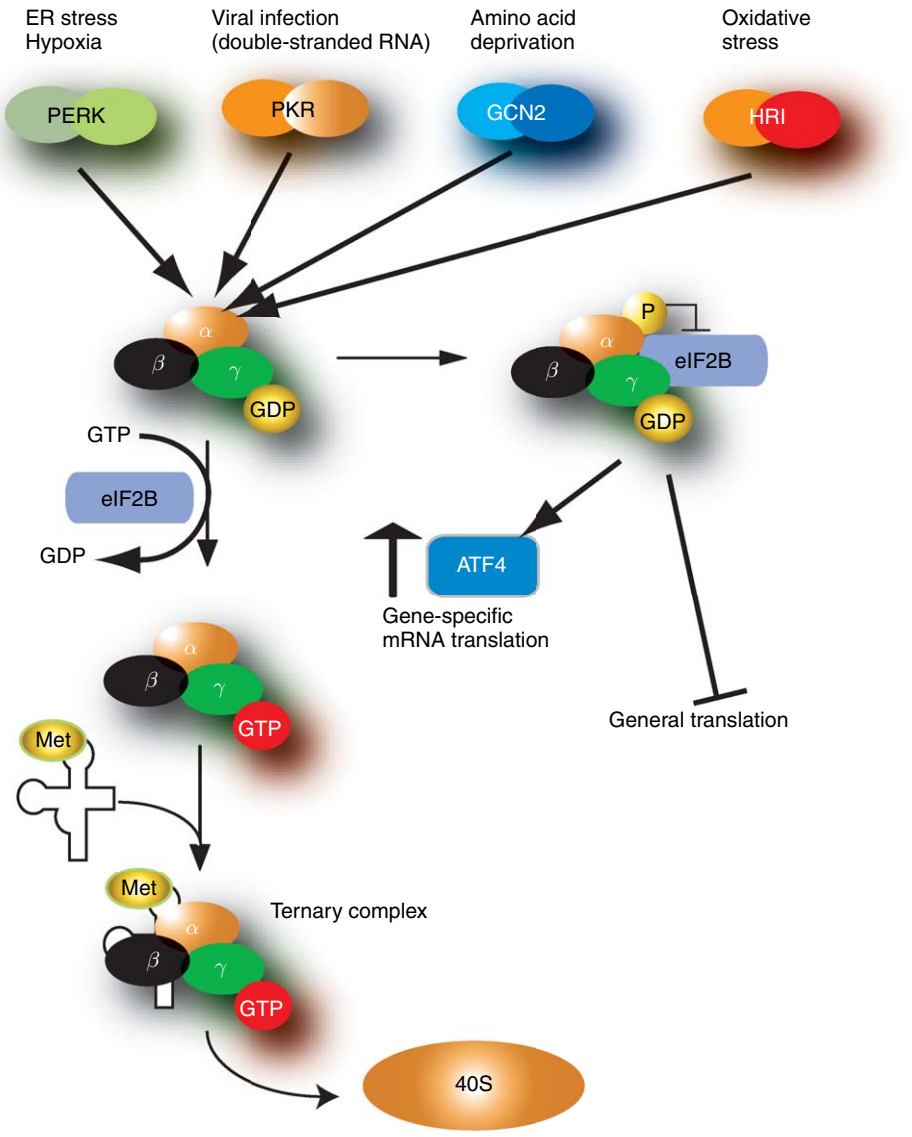


Figure 2 Translational control by the eIF2α kinases. Different stress conditions activate the eIF2α kinases, PERK (RNA-activated protein kinase (PKR)-endoplasmic reticulum–related kinase), PKR, HRI (hemin-regulated inhibitor kinase), and GCN2 (general control nonderepressible kinase), resulting in phosphorylation of α subunit of eIF2. Phosphorylation of eIF2α represses global rate of translation, by sequestering eIF2B, but stimulates translation of ATF4 mRNA. Studies from Costa-Mattioli et al. (2005) indicate that GCN2 and eIF2α phosphorylation are critical for long-lasting synaptic plasticity and memory.

major substrate (**Figure 2**). They include the double-stranded (ds) RNA-activated protein kinase (PKR), the hemin-regulated inhibitor kinase (HRI), the pancreatic eIF2α or the PKR-endoplasmic reticulum (ER)-related kinase (PEK/PERK), and the general control nonderepressible kinase (GCN2). The eIF2α kinases share extensive homology in the kinase catalytic domain (Meurs et al., 1990; Chen et al., 1991; Chong et al., 1992; Shi et al., 1998; Berlanga et al., 1999; Harding et al., 1999; Sood et al., 2000). They are activated in response to external stress, such as viral double-stranded RNA (dsRNA), heme deficiency, misfolded proteins in the ER, and amino acid deprivation, respectively. All four eIF2α kinases are expressed in the brain (but only a small amount of HRI) (Pal et al., 1991; Crosby et al., 1994; Mellor et al., 1994; Berlanga et al., 1999). GCN2 is the ancestral and the only eIF2α kinase that is evolutionarily conserved from yeast to mammals (Santoyo et al., 1997; Olsen et al., 1998; Sattlegger et al.,

1998; Berlanga et al., 1999; Hinnebusch, 2000; Sood et al., 2000). GCN2 is activated under conditions of amino acid deprivation via the accumulation of uncharged tRNA. However, it is also activated by other stimuli such as ultraviolet irradiation (Deng et al., 2002; Narasimhan et al., 2004). The finding that GCN2 mRNA is enriched in the brain of flies (Santoyo et al., 1997) and mammals (Berlanga et al., 1999; Sood et al., 2000), especially in the hippocampus (Costa-Mattioli et al., 2005), is consistent with a role for GCN2 in synaptic plasticity and memory storage.

4.33.5 Regulation of Synaptic Plasticity and Memory Consolidation by GCN2

GCN2 knockout mice exhibit a reduction in eIF2α phosphorylation in the hippocampus, which is accompanied by a decrease in the amount of the memory repressor ATF4 (Costa-Mattioli et al., 2005). Consistent with a decrease in ATF4 protein levels, the threshold for eliciting L-LTP was lower in hippocampal slices from GCN2 knockout mice. Specifically, a protocol that usually elicits a short-lasting E-LTP, which is independent of gene expression (translation and transcription), elicited a typical gene expression-dependent L-LTP (Costa-Mattioli et al., 2005). A comparable phenotype to that of GCN2 knockout mice was observed in mice expressing an inducible inhibitor of ATF4 and CCAAT/enhancer binding proteins (C/EBP) (EGFP-AZIP) (Chen et al., 2003). The *Aplysia* homolog of ATF4, ApCREB2 represses LTF (Bartsch et al., 1995). Upon injection of anti-ApCREB2 antibodies into *Aplysia* sensory neurons, a single pulse of 5-HT, which normally induces only short-term facilitation (lasting minutes), is sufficient to evoke a gene expression–dependent LTF that lasts longer than 1 day.

Surprisingly, in slices from GCN2 knockout mice, strong stimulation that normally induces L-LTP elicited only a short-lasting LTP. This effect of GCN2 deletion is specific to LTP because normal LTD could be induced in slices from the GCN2 knockout mice by low-frequency stimulation (LFS) or by incubation with DHPG, an agonist of group I mGluRs. Therefore, it is possible that either GCN2 does not play any role in protein synthesis–dependent LTD or the pathway that turns off GCN2 activity is not modified by LTD inducing protocols.

To determine whether the GCN2 deletion alters memory, GCN2 knockout mice were subjected to two distinct tasks: fear conditioning and the Morris water maze. Pairing tone presentations with foot shocks in a particular environmental context leads to both auditory and contextual fear conditioning. In the latter, the context, which is represented in the hippocampus and acts as the conditioned stimulus (CS), is associated with the foot shock, the unconditioned stimulus (US). Auditory fear conditioning, which associates a tone (CS) with the foot shock (US), depends on the amygdala but not the hippocampus (Fanselow and LeDoux, 1999; LeDoux, 2000). Both types of fear conditioning require new protein synthesis (Bourtchouladze et al., 1998; Schafe et al., 1999). When tested 24 h and 10 days after training, the GCN2 knockout mice showed deficits in contextual memory, which suggests that long-term memory is disrupted in these mice. Surprisingly, the lack of GCN2 selectively affects hippocampal-dependent memories but not amygdala-dependent auditory fear conditioning.

In agreement with the findings on contextual fear conditioning, the GCN2 knockout mice were deficient in long-term memory when tested in the Morris water maze. Specifically, in a conventional strong training protocol (three trials per day), spatial learning of GCN2 knockout mice was impaired, as indicated by longer escape latencies during training as well as by the increased time spent in the target quadrant and the number of platform crossings. In contrast, when the GCN2 knockout mice were given a weak training consisting of only one trial per day, they exhibited enhanced learning and memory (Costa-Mattioli et al., 2005). These behavioral findings correlate precisely with the electrophysiological findings in hippocampal slices from these animals, where weak stimulation elicited L-LTP instead of E-LTP, but strong stimulation failed to evoke the expected L-LTP. The findings reviewed here represent a significant advance in our understanding of the molecular mechanism underlying synaptic plasticity and memory formation. The levels of active GCN2 may be a determinant of the amount and schedule of training required for long-term memory. In *Aplysia*, *Drosophila*, mice, and rats, the balance between memory repressors and activators appears to be crucial for long-term synaptic plasticity and memory storage (Abel et al., 1998; Kandel, 2001). The GCN2 and 4E-BP2 knockout mice provide the first examples of memory repressor factors operating at the translation level.

4.33.6 Regulation by mTOR and the eIF4E-Binding Proteins

Ribosomal recruitment to the mRNA is facilitated by the 5′-cap structure (m7GpppX, where X is any nucleotide), which is present on all nuclear transcribed eukaryotic mRNAs (Shatkin, 1985). The cap structure is specifically recognized by eukaryotic initiation factor 4F (eIF4F) (Gingras et al., 1999), a complex comprised of three subunits: (1) eIF4E, the cap-binding subunit (Sonenberg et al., 1978); (2) eIF4A, a bidirectional RNA helicase; and (3) eIF4G, a large polypeptide that serves as a bridge between the ribosome and the mRNA (Imataka et al., 1998). eIF4E is limiting among all initiation factors and plays a critical role in translational regulation (Gingras et al., 1999). eIF4E is regulated at multiple levels (Raught and Gingras, 1999) via: (1) transcription, (2) phosphorylation, and (3) interaction with inhibitory proteins, the eIF4E-binding proteins (4E-BPs). In mammals, the 4E-BP family consists of three related small molecular-weight proteins, 4E-BP1, 4E-BP2, and 4E-BP3 (Pause et al., 1994; Poulin et al., 1998), which compete with eIF4G for eIF4E binding (Mader et al., 1995; Marcotrigiano et al., 1999). The 4E-BPs specifically inhibit cap-dependent translation initiation by preventing the assembly of the eIF4F complex and, consequently, ribosome binding to the mRNA (Haghighat et al., 1995; Pause et al., 1994) (**Figure 3**).

The mammalian target of rapamycin (mTOR) is a protein kinase that serves as a critical checkpoint for the integration of signals from the extracellular stimuli, growth factors, nutrient levels, and energy status (Hay and Sonenberg, 2004). mTOR triggers translation initiation by phosphorylating at least two translational modulators, p70 ribosomal S6 kinase 1 and 2 (S6K1/2), and the 4E-BPs (Gingras et al., 2001; Hay and Sonenberg, 2004) (**Figure 3**).

Extracellular stimuli such as growth factors, hormones (insulin), the G-conjugated protein receptor, and mitogens activate the phosphatidylinositol-3 kinase (PI3K) pathway. For instance, insulin activates the insulin receptor tyrosine kinase, which in turn phosphorylates the insulin receptor substrates (IRS) (**Figure 2**). PI3K then binds phosphorylated IRS via its SH2 regulatory domain. This interaction activates the PI3K catalytic subunit, which phosphorylates the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PIP2) to form PIP3, which then recruits Akt to the cell membrane, where it is phosphorylated and activated by PDK1 (Brazil and Hemmings, 2001). Akt activates mTOR through inhibition of the tuberous sclerosis complex (TSC). TSC is a heterodimer that contains TSC1 (hamartin) and TSC2 (tuberin), the latter serving as a GAP (GTPase activating protein) for Rheb, a *ras*-like GTPase. Activated Akt phosphorylates TSC2, decreasing its Rheb-GAP function (Garami et al., 2003; Inoki et al., 2005a,b). Subsequently, GTP-Rheb activates mTOR through a mechanism that is not well understood at this time.

One of the best characterized functions of mTOR is regulation of translation. mTOR exists in two distinct complexes (**Figure 3**). One complex contains raptor and is sensitive to the drug rapamycin (a macrolide that binds to the immunophilin FKBP12 to form a gain of function complex). The other complex contains rictor, is rapamycin insensitive, and phosphorylates Akt/PKB (Hara et al., 2002; Kim et al., 2002; Loewith et al., 2002; Hay and Sonenberg, 2004; Sarbassov et al., 2004). Raptor serves as an adaptor protein that recruits two downstream effectors: S6K1 and S6K2 and 4E-BPs (Beugnet et al., 2003; Choi et al., 2003; Schalm et al., 2003). Rapamycin disrupts the mTOR–raptor interaction, preventing mTOR from phosphorylating S6K1/2 and 4E-BP (Kim et al., 2002; Oshiro et al., 2004).

4E-BP1 (and the other 4E-BPs) binding to eIF4E is regulated by its phosphorylation status (Pause et al., 1994). Hypophosphorylated 4E-BP1 binds with high affinity to eIF4E, and hyperphosphorylation of 4E-BP1 prevents this interaction (Pause et al., 1994; Beretta et al., 1996). Upon cell stimulation with serum, growth factors, or hormones (e.g., insulin), 4E-BP1 becomes phosphorylated through an mTOR-dependent pathway and dissociates from eIF4E to relieve translational inhibition (Pause et al., 1994; Gingras et al., 2001) (**Figure 3**).

A large body of evidence implicates the mTOR signaling pathway in long-lasting synaptic plasticity and memory formation. First, rapamycin suppresses synaptic plasticity in invertebrates, including LTF in *Aplysia* sensory neurons (Casadio et al., 1999) and rodent hippocampal slices (Tang et al., 2002; Hou and Klann, 2004). It also blocks long-term spatial memory formation in mammals (Tischmeyer et al., 2003; Dash et al., 2006). Second, in hippocampal slices, several forms of synaptic plasticity are associated with the activation of mTOR and its downstream targets (Tang et al., 2002; Hou and Klann, 2004; Banko et al., 2005, 2006; Chen et al.,

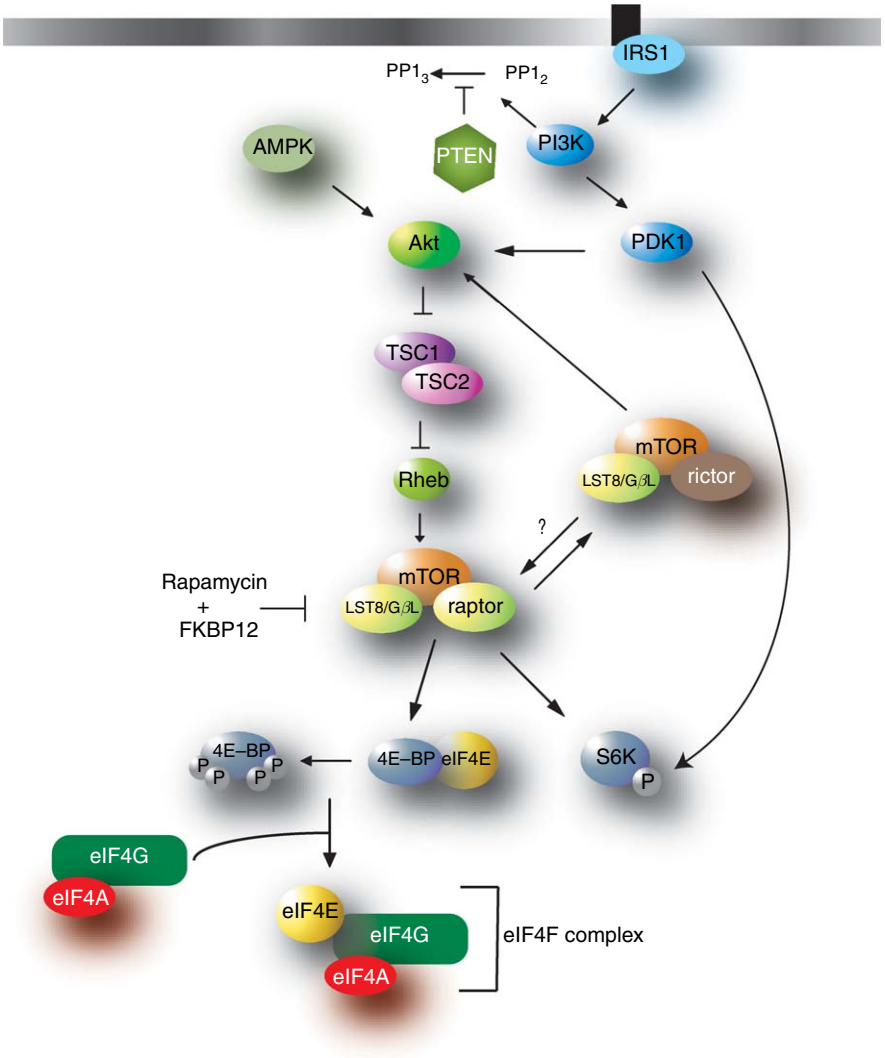


Figure 3 mTOR signaling to translation initiation. The regulation mTOR (mammalian target of rapamycin) activity is mediated by the PI3K/Akt signaling pathway leading to phosphorylation and inhibition of TSC2 by Akt and to the subsequent activation of Rheb, which activates mTOR by an as yet unknown mechanism. The raptor/GβL/mTOR complex is sensitive to rapamycin and mediates the phosphorylation of 4E-BP (eIF4E-binding proteins) and p70S6K. The rictor/GβL complex is rapamycin insensitive and phosphorylates Akt/PKB. The raptor/GβL/mTOR complex mediates phosphorylation of 4E-BPs and p70S6K. Hyperphosphorylation of 4E-BPs causes their release from eIF4E, allowing to eIF4E to form the ternary complex. A number of recent studies indicate that mTOR signaling and 4E-BP phosphorylation are critical for protein synthesis–dependent forms of long-lasting synaptic plasticity and memory.

2005; Tsokas et al., 2005). Third, the PI3K signaling pathway is also required for synaptic plasticity in different areas of the brain (Lin et al., 2001; Man et al., 2003; Opazo et al., 2003; Hou and Klann, 2004). In addition, PI3K inhibitors block memory consolidation in the hippocampus and amygdala (Lin et al., 2001; Chen et al., 2005). Fourth, the mTOR downstream target 4E-BP2 is critical for long-term synaptic plasticity and memory.

4.33.7 Regulation of Synaptic Plasticity and Memory Consolidation by 4E-BPs

Substantial evidence indicates that long-term synaptic plasticity and memory requires new mRNA translation. Significantly, in wild-type hippocampal slices, both LTD- and LTP-inducing stimulation are associated with an increase in the phosphorylation of

4E-BP2, the 4E-BP family member preferentially expressed in the mammalian hippocampus (Banko et al., 2005, 2006). The increase in 4E-BP2 phosphorylation during both LTP and mGluR-LTD is associated with an increase in eIF4F complex formation. To study the biological significance of these changes, 4E-BP2 knockout mice were recently used (Banko et al., 2005). The function of 4E-BP2 in synaptic plasticity has been most intensively studied at Schaffer collateral-CA1 pyramidal neuron synapses in the adult hippocampus. In accordance with an increase in general translation in mice lacking the translational repressor 4E-BP2, the amount of eIF4F complex was increased compared to wild-type mice (Banko et al., 2005). These data nicely correlate with an enhanced mGluR-LTD observed in these mice (Banko et al., 2006). Interestingly, rapamycin did not block the enhanced mGluR-LTD in slices from the 4E-BP2 knockout mice. Therefore, these data strongly argue that 4E-BP2 is the only mTOR downstream target involved in mGluR-dependent-LTD. In addition, as was the case for the GCN2 knockout mice in slices from the 4E-BP2 knockout mice, a standard stimulation paradigm that normally elicits protein synthesis–independent E-LTP in wild-type slices is converted to protein synthesis–dependent L-LTP (Banko et al., 2005). Taken together, these data indicate that the increase in eIF4F complex formation, a hallmark of ongoing translation, correlates with an enhanced LTP and LTD in slices from 4E-BP2 knockout mice. Strikingly, when LTP was elicited by a stronger stimulation protocol (four trains of HFS) that produced L-LTP in wild-type slices, the potentiation evoked in the slices from the 4E-BP2 knockout mice decayed to baseline in less than 2 h (Banko et al., 2005). Thus, there must be limits to the extent to which the threshold for LTP induction can be manipulated. Consequently, enhanced eIF4F complex formation and possibly excessive translation may be detrimental to L-LTP (Banko et al., 2005). 4E-BP2 knockout mice were studied in hippocampus-dependent learning and memory tasks. In correlation to what has been found with strong-LTP inducing protocols, 4E-BP2 knockout mice are impaired in spatial learning in the Morris water maze, as determined by both time spent in the target quadrant and the number of platform crossings (Banko et al., 2005). It would be interesting to determine whether this impairment can be rescued after intensive training, as was the case for other knockout mice implicated in learning and memory (Elgersma et al., 2002; Silva et al., 1992a,b). Consistent with these data, 4E-BP2 knockout mice are impaired in both auditory and contextual fear conditioning (Banko et al., 2005). These indicate that proper translational control is critical for long-term memory formation.

4.33.8 Translational Control by eIF4E Phosphorylation

In mammals, eIF4E is phosphorylated at a single site, Ser209 (Joshi et al., 1995; Whalen et al., 1996), by MAPK signal-integrating kinase/MAPK-interacting kinase 1 and 2 (Mnk1/2) (Fukunaga and Hunter, 1997; Pyronnet et al., 1999; Waskiewicz et al., 1999). Mnk1/2 physically interacts with the C-terminal region of eIF4G, which serves as a docking site to phosphorylate eIF4E. It is thought that eIF4E phosphorylation enhances general translation (Sonenberg and Dever, 2003). However, there has been debate as to the physiological function of phosphorylation of eIF4E at Ser209 (Scheper and Proud, 2002). In *Drosophila*, mutation at eIF4E–S251A, the equivalent Ser209 site, results in a delay in development and smaller flies (Lachance et al., 2002), indicating that eIF4E phosphorylation is important for normal development. In addition, *Drosophila* lacking Lk6, the functional homolog of the mammalian Mnk kinases, exhibit reduced viability, slower development, and reduced adult size under nutrient-deficient conditions (Arquier et al., 2005; Reiling et al., 2005). In apparent contrast to these findings, overexpression of the Mnks in mammalian cells and *Drosophila* results in a reduction in translation and/or growth rates (Knauf et al., 2001). These data are consistent with those in *Aplysia*, where overexpression of *Aplysia* Mnk resulted in a decrease in cap-dependent translation (Ross et al., 2006). Thus, it is possible that either too much eIF4E phosphorylation or other targets of Mnk1/2 are detrimental to cap-dependent translation. In mammals, Mnk1/2 double-knockout mice, in which eIF4E phosphorylation is abrogated, are fertile, develop normally, and have global translation rates that are unaltered (Ueda et al., 2004).

Multiple forms of neuronal activity and application of L-LTP induction protocols alter the phosphorylation status of eIF4E. For example, *Aplysia* neurons treated with serotonin, which elicits protein synthesis–dependent LTF, exhibited a p38-dependent increase in eIF4E phosphorylation (Dyer and Sossin, 2000). In mammals, NMDA receptor activation results in an extracellular signal-regulated kinase

(ERK)-dependent phosphorylation of eIF4E in hippocampal area CA1 (Banko et al., 2004), and BDNF treatment of cultured neurons elicits phosphorylation of eIF4E (Takei et al., 2001). In addition, forskolin treatment induces eIF4E phosphorylation, and NMDA-induced eIF4E phosphorylation requires PKA activity (Banko et al., 2004). Recently, Kelleher and collaborators demonstrated that both L-LTP and fear conditioning elicit an ERK-dependent increase in the phosphorylation of eIF4E (Kelleher et al., 2004a). In conclusion, several studies indicate that eIF4E phosphorylation correlates with protein synthesis–dependent synaptic plasticity and memory. Genetic evidence supporting the role of eIF4E phosphorylation in cap-dependent translation and long-term synaptic plasticity and memory remains to be provided.

4.33.9 Translational Control by CPEB

Cytoplasmic polyadenylation is another mechanism by which mRNA translation is regulated upon synaptic stimulation (Steward and Schuman, 2001). The molecular mechanisms underlying cytoplasmic polyadenylation have been extensively characterized in *Xenopus* oocytes, where a subset of dormant mRNAs have small poly(A) tails (usually ~20–40 nucleotides in length) (Mendez and Richter, 2001). In response to developmental cues, the poly(A) tails of the dormant mRNAs are lengthened, and translation ensues. Polyadenylation is controlled by two cis elements in the 3′ UTR of the mRNA: the cytoplasmic polyadenylation element (CPE, sequence of UUUUUAU) and the hexanucleotide AAUAAA, which is also important for nuclear pre-mRNA cleavage and polyadenylation (Fox et al., 1989; McGrew et al., 1989). Cytoplasmic polyadenylation element (CPE) binding factor (CPEB), which contains an RNA recognition motif (RRM) and zinc finger domain, binds with strong affinity the CPE (Hake and Richter, 1994; Hake et al., 1998) and interacts with a number of other regulatory factors. These factors include (i) symplekin, a scaffold-like protein; (ii) cleavage and polyadenylation specificity factor (CPSF), a group of four proteins that binds the hexanucleotide AAUAAA; and (iii) Gld2 (Barnard et al., 2004), an unusual poly(A) polymerase first discovered in yeast and *Caenorhabditis elegans* (Read et al., 2002; Saitoh et al., 2002; Wang et al., 2002). Polyadenylation is initiated when aurora (Eg2) phosphorylates CPEB (Mendez et al., 2000a), which induces CPEB to interact and possibly stabilize CPSF on the hexanucleotide AAUAAA (Mendez et al., 2000b). This process is likely necessary for the recruitment of poly(A) polymerase. One mechanism by which polyadenylation is believed to stimulate translation involves maskin, a protein that interacts with both CPEB and eIF4E (Stebbins-Boaz et al., 1999). The maskin-eIF4E interaction inhibits translation by precluding the eIF4E-eIF4G interaction and formation of the eF4F complex and ribosomal recruitment to the mRNA (Cao and Richter, 2002). Polyadenylation leads to the recruitment of the poly(A) binding protein (PABP), which binds to eIF4G (Wakiyama et al., 2000), a process that enhances the eIF4G-eIF4E association and promotes the dissociation of maskin from eIF4E (Cao and Richter, 2002).

4.33.10 Regulation of Synaptic Plasticity and Memory Consolidation by CPEB

Neuroguidin (Ngd), a maskin-like protein, was recently described in mammalian and *Drosophila* neurons (Jung et al., 2006). Ngd is similar to maskin in that it interacts with both eIF4E and CPEB. It is present as puncta in axons and dendrites and in growth cones and filopodia in mammalian neurons. In *Xenopus* embryos, Ngd is found both in the neural tube and neural crest cells and plays a role in neural development. The identity of the mRNAs whose translation is regulated by Ngd and its role in local translation remain to be established.

CPEB and several of its associated factors are localized postsynaptically, where local translation most likely occurs (Huang and Richter, 2004). Importantly, synaptic stimulation results in polyadenylation and translation of the α subunit of calcium-/calmodulin-dependent protein kinase II (αCaMKII) mRNA, which contains a CPE in the 3′UTR (Wu et al., 1998; Wells et al., 2001; Huang et al., 2002). Mutant mice with a deletion of the dendritic localization signal within the 3′ UTR of the αCaMKII mRNA exhibit a defect in dendritic protein synthesis of αCaMKII and an impairment in protein synthesis–dependent phase of LTP and memory (Miller et al., 2002).

Further evidence supports a role for CPEB in synaptic plasticity and memory. In CPEB knockout mice, LTP elicited by a single train of 100 Hz or one theta-burst stimulation was impaired. In contrast to GCN2 and 4E-BP2 knockout mice, gene-expression-dependent L-LTP or four trains of

theta-burst stimulation was not altered in slices from CPEB knockout mice. LTD evoked by 1 Hz stimulation appears to be moderately facilitated in CPEB knockout mice (Alarcon et al., 2004). These data indicate that CPEB is involved in specific forms of LTP.

A battery of behavioral tests was performed in CPEB knockout mice. Although no differences were found in the Morris water maze and fear conditioning, CPEB knockout mice exhibited a deficit in a form of memory known as extinction (Berger-Sweeney et al., 2006), an active and dynamic learning process that results in the formation of new memories. Extinction does not reflect forgetting but, rather, relearning, in which the new association of the CS with the absence of the original reinforcer comes to control behavior (Bouton and Nelson, 1994; Berman and Dudai, 2001).

Studies in *Aplysia* have provided additional evidence supporting a role for CPEB in synaptic plasticity and memory. In *Aplysia* sensory neurons, 5-HT treatment increased the content of CPEB four- to fivefold in a transcription-independent manner. Moreover, when CPEB mRNA levels were decreased by an antisense oligonucleotide, LTF was impaired (Si et al., 2003a), suggesting that CPEB-mediated translation could result in the establishment of a tag at synapses.

Curiously, the form of CPEB in *Aplysia* and other invertebrate neurons differs from that in vertebrate neurons in that it contains a long stretch of glutamines, which is often found in proteins that have characteristics of a prion (Si et al., 2003b). In fact, *in vitro* studies have established that *Aplysia* CPEB exhibits features of a prion protein. In these studies it was also demonstrated that CPEB could assume two forms; an aggregated form (i.e., prion-like) and a nonaggregated form (Si et al., 2003b). Only the aggregated form was able to bind RNA *in vitro*, and remarkably, the prion-like form of the protein could convert the nonaggregated form into the aggregated form. It was speculated by the authors of these studies that synaptic stimulation causes CPEB to assume a prion-like form that either stimulates the translation of some mRNAs, causing it to alter its substrate specificity, or releases some mRNAs from an inhibited state. These tantalizing findings support a dynamic model that would explain how memories are stored and maintained for long time periods. Whether polyglutamine-lacking CPEB in mammalian neurons plays a similar role to its *Aplysia* counterpart is a matter of current investigation.

4.33.11 Regulation of 5′TOP Translation

As mentioned earlier, several protein kinases and translation factors act as downstream effectors of mTOR and ERK to regulate cap-dependent translation initiation. Both of these kinases also may regulate translation via an additional downstream effector, p70S6 kinase (also known as S6K or p70S6K). Both mTOR and ERK, as well as phosphoinositide-dependent kinase 1 (PDK-1) and protein kinase Cζ, phosphorylate and regulate p70S6K activity (Dufner and Thomas, 1999). One substrate of p70S6K is ribosomal protein S6, a subunit of the 40S ribosome, whose phosphorylation usually coincides with the translation of mRNA species that contain a terminal 5′ oligopyrimidine tract (5′TOP). A unique feature of 5′TOP mRNAs is that they encode for translation factors and all vertebrate ribosomal protein subunits (Meyuhas, 2000). Because localization and the ongoing synthesis of functional ribosomes is energetically costly for neurons to maintain near synaptic sites, it might be advantageous to position polyribosomes at locations where they could be efficiently utilized and dynamically modulated to influence translational competence in response to the appropriate synaptic signals. Activation of S6 and concomitant 5′TOP synthesis represents one possible signal transduction pathway that may promote such events in neurons.

There is evidence that p70S6K and S6 are regulated during long-term synaptic plasticity and long-term memory. Protein synthesis–dependent forms of LTP are associated with increased phosphorylation, and presumably increased activation, of p70S6K (Cammalleri et al., 2003; Tsokas et al., 2005). Increased S6 phosphorylation has been reported in *Aplysia* synaptosome preparations treated with serotonin, a facsimile of protein synthesis–dependent LTF (Khan et al., 2001). Moreover, increased S6 phosphorylation also has been observed to be enhanced after the induction of LTP, following training in a hippocampus-dependent memory task (Kelleher et al., 2004a). Interestingly, the levels of S6, whose mRNA contains a 5′TOP, are increased by serotonin in *Aplysia* synaptosomes (Khan et al., 2001). These findings suggest that activation of p70S6K, and the subsequent phosphorylation and synthesis of ribosomal protein S6, may play a role in long-lasting synaptic plasticity and memory.

Another protein whose mRNA contains a 5′TOP sequence that may be crucial for protein synthesis–dependent synaptic plasticity is eEF1A, a critical translation factor that mediates peptide elongation by promoting GTP-dependent binding of aminoacyl tRNA to the ribosome. eEF1A synthesis has been reported to be elevated during the protein synthesis–dependent form of LTP (Tsokas et al., 2005), which was accompanied by an activation of p70S6K. Transport of EF1A mRNA from the soma to dendrites was first identified in *Aplysia*, where it was found to be synthesized during the maintenance phase of serotonin-induced LTF (Giustetto et al., 2003). The primary function of eEF1A is as an elongation factor, but noncanonical functions of EF1A are known to exist. One such function for eEF1A is to promote the assembly of actin monomers into F-actin (Liu et al., 2002). Intriguingly, eEF1A protein has been found to migrate toward activated synapses during *in vivo* LTP, an event that was blocked with the F-actin inhibitor lantruculin B (Huang et al., 2005). Regardless of its function, the involvement of eEF1A in multiple forms of synaptic plasticity strongly suggests that eEF1A is an essential molecule associated with the formation of long-term changes in synaptic strength.

It should be noted that in nonneuronal cells, there are reports that p70S6K is required for neither S6 phosphorylation nor synthesis of 5′TOP encoded proteins (Tang et al., 2001; Pende et al., 2004; Stolovich et al., 2005). Additional substrates and binding partners of p70S6K (for a review, see (Ruvinsky and Meyuhas, 2006) include neurabin (Oliver et al., 2002) and eukaryotic initiation factor 4B (Raught et al., 2004; Holz et al., 2004). Future studies on these potential effector molecules of p70S6K, as well as studies with p70S6K mutant mice, will be required for a fuller understanding of the role of this kinase in the expression of protein synthesis–dependent synaptic plasticity and memory.

Interestingly, at synapses, the protein synthetic machinery, which would largely consist of proteins that contain 5′TOP sequences in their mRNA, appears to be quite limited (for a review, see Schuman et al., 2006). The observation that there are relatively few polyribosomes at synapses is somewhat surprising, although there is evidence that polyribosomes move from dendritic shafts to dendritic spines following the induction of LTP (Ostroff et al., 2002). One way to increase the translational capacity would be make new ribosomes in response to synaptic stimulation. It has been reported that 5′TOP mRNAs that encode ribosomal proteins are present in *Aplysia* neurites (Moccia et al., 2003) and mammalian dendrites (Zhong et al., 2006). These findings suggest that ribosomal proteins can be synthesized and potentially assembled into ribosomes in neurons at locations quite distant from the nucleolus, where ribosomes are typically assembled in eukaryotic cells. Whether long-lasting synaptic plasticity and/or memory formation triggers the synthesis of ribosomal proteins and the subsequent assembly of these proteins into a translationally competent ribosome in dendrites remains to be determined.

4.33.12 Regulation of Eukaryotic Elongation Factor 2

Eukaryotic elongation factor 2 (eEF2) is a GTP-binding protein that mediates the translocation of peptidyl-tRNA from the A site to the P site on the ribosome (Moldave, 1985). eEF2 kinase, a calcium-/calmodulin-dependent kinase sometimes referred to as calcium-/calmodulin-dependent kinase III, specifically phosphorylates and inactivates eEF2 to decrease the translocation of the peptidyl-tRNA on the ribosome, thereby resulting in a general decrease in protein synthesis (Ryazanov and Davydova, 1989; Carlberg et al., 1990; Redpath et al., 1993). Interestingly, eEF2 and elongation also are regulated indirectly by mTOR. eEF2 kinase can be phosphorylated by mTOR near the calmodulin-binding site; this phosphorylation event decreases the ability of calmodulin to bind eEF2 and thus decreases eEF2 kinase activity (Browne and Proud, 2004). Thus, one might predict that mTOR activation associated with the various forms of protein synthesis–dependent synaptic plasticity and memory would result in increased translation initiation and increased translation elongation. The observation that serotonin stimulates an mTOR-dependent dephosphorylation of eEF2 in *Aplysia* synaptosomes is consistent with this notion (Carroll et al., 2004). However, NMDA receptor activation increases the phosphorylation of eEF2 in several types of reduced experimental preparations (Marin et al., 1997; Scheetz et al., 1997, 2000). Similar findings have been reported for chemically induced LTP (Chotiner et al., 2003). Although the increase in eEF2 phosphorylation was correlated with a decrease in overall protein synthesis, as would be expected, an increase in the synthesis of specific proteins such α-CaMKII

(calcium-calmodulin-dependent protein kinase II), Arc, and Fos was observed (Scheetz et al., 2000; Chotiner et al., 2003). These findings raise the intriguing possibility that the phosphorylation of eEF2, though decreasing overall protein synthesis, triggers the translation of specific mRNAs during protein synthesis–dependent synaptic plasticity.

A similar mechanism of regulation of translation elongation appears to occur during taste memory consolidation. Following novel taste learning, increased eEF2 phosphorylation was observed in synaptoneurosomes prepared from the insular cortex, but not the hippocampus (Belelovsky et al., 2005). Moreover, the learning-induced increase in eEF2 phosphorylation was associated with increased levels of αCaMKII in the same synaptoneurosomal fraction. These studies are remarkably consistent with the *in vitro* studies described in the previous paragraph and suggest that when coupled with increases in translation initiation, translation elongation is the rate-limiting step for new protein synthesis during memory consolidation (Belelovsky et al., 2005). Further investigations will be required to determine whether decreased elongation is a core mechanism required for protein synthesis–dependent forms of learning and memory.

4.33.13 Regulation of mRNA-Binding Proteins during Synaptic Plasticity and Memory

In addition to the regulatory mechanisms that regulate translation initiation and elongation during synaptic plasticity and memory, it is clear that proper protein synthesis also requires regulation of mRNA-binding proteins for long-lasting changes in synaptic strength. As discussed in a recent review (Wells, 2006), dendritic protein synthesis requires that mRNAs be identified after transcription, packaged in a way that keeps them from being translated, and transported to the appropriate dendritic location, where synaptic signals can initiate translation.

One mRNA-binding protein that is involved in synaptic plasticity and memory is CPEB. As discussed earlier, CPEB binds to specific sequences called CPEs in the 3′ UTR of mRNAs that suppresses their translation. Mice that lack CPEB-1, one mammalian isoform of CPEB, exhibit deficits in protein synthesis–dependent LTP induced by a theta-burst stimulation paradigm (Alarcon et al., 2004). The CPEB-1 knockout mice also exhibit a reduction in the extinction of two types of hippocampus-dependent memory (Berger-Sweeney et al., 2006). Similarly, elimination of CPEB results in impaired LTF in *Aplysia* (Si et al., 2003a). Taken together, these data suggest that CPEB might specifically regulate the synthesis of proteins encoded by mRNAs that bind to CPEB during synaptic plasticity and memory. One mRNA that is very likely to be regulated during these processes is that of α-CaMKII, which contains two CPEs in its 3′ UTR (Wu et al., 1998). The identities of additional CPEB-binding mRNAs, whose translation might be regulated during synaptic plasticity and memory in the hippocampus, has been provided in a microarray analysis of the CPEB-1 knockout mice (Berger-Sweeney et al., 2006). Whether these mRNAs are translated into protein during either long-lasting synaptic plasticity or memory formation remains to be determined.

Another mRNA-binding protein that is involved in synaptic plasticity and memory is fragile X mental retardation protein (FMRP). Genetic deletion of FMRP results in fragile X mental retardation, which is the most common inherited disease causing mental retardation, affecting approximately 1:4000 males and 1:8000 females (Turner et al., 1996). FMRP is an mRNA-binding protein thought to regulate translation of specific mRNAs, including its own mRNA (Feng et al., 1997; Brown et al., 2001; Darnell et al., 2001; Zhang et al., 2001; Miyashiro et al., 2003) via binding to KH domains (with homology to the RNA-binding region of hnRNP K) and an RGG (arginine-glycine-glycine) box. Previous studies have shown that FMRP is colocalized with polyribosomes in the neuronal soma as well as in dendritic spines, which suggests the possibility that FMRP is involved in local dendritic protein synthesis (Stefani et al., 2004). FMRP is critically involved in the biochemical regulation of translation during mGluR-LTD. For example, mGluR-LTD is augmented in mice that lack FMRP (Huber et al., 2002), and FMRP is translated in response to stimulation of group I mGluRs (Weiler et al., 1997; Todd et al., 2003a,b; Antar et al., 2004; Hou et al., 2006). In addition to FMRP, mGluR-LTD is associated with increased synthesis of proteins encoded by FMRP-binding mRNAs such as microtubule-associated protein-1B (MAP1B) and α-CaMKII (Hou et al., 2006). Interestingly, the augmented mGluR-LTD in mice that lack FMRP is not protein synthesis dependent (Nosyreva and Huber, 2006; Hou et al., 2006), consistent with studies demonstrating that protein synthesis is regulated improperly in the

FMRP-deficient mice (Weiler et al., 2004; Aschrafi et al., 2005; Qin et al., 2005; Hou et al., 2006). The FMRP-deficient mice also exhibit mild behavioral phenotypes that are consistent with fragile X mental retardation (Kooy, 2003). However, these phenotypes, including contextual fear conditioning, are exaggerated in a mouse model where both FMRP and a paralog, FXR2P, are absent (Spencer et al., 2006). The *Drosophila* model for fragile X mental retardation also exhibits cognitive deficits that can be reversed with mGluR antagonists (McBride et al., 2005). All together the findings described earlier indicate that FMRP plays a critical role in translational control during synaptic plasticity and memory, especially those that are dependent on mGluRs (Bear et al., 2004).

Other mRNA-binding proteins have been implicated in synaptic plasticity and memory. In *Drosophila*, the mRNA for the mRNA-binding proteins pumilio, staufen, and orb (the *Drosophila* homolog of CPEB) is upregulated during memory formation, and disruption of the pumilio and staufen genes disrupts long-term memory (Dubnau et al., 2003). Whether genetic disruption of the mammalian homologs of pumilio and staufen result in synaptic plasticity and memory abnormalities has yet to be determined. Genetic deletion of translin (also known as testis-brain RNA-binding protein), an mRNA-binding protein that has several characteristics similar to FMRP, results in impaired spatial learning in the Morris water maze (Stein et al., 2006). It seems quite likely that removal of any of a number of dendritically localized mRNA-binding proteins will alter protein synthesis–dependent forms of synaptic plasticity and memory.

4.33.14 Summary

Despite the enormous amount of progress that has been made in the last 5 years, a critical issue that remains for researchers studying translational control and how it contributes to synaptic plasticity and memory is identifying the proteins that are synthesized in response to either a particular pattern of synaptic stimulation or learning a memory task. Further complicating this issue, it appears that the signal transduction cascades that are required to initiate cap-dependent translation, at least with respect to the regulation of the joining of the mRNA to the ribosome, are similar, regardless of whether the plasticity induced results in either LTP or LTD. Several ideas have been recently advanced to address this quandary. One idea is that it is not the synthesis of specific proteins but, rather, the creation of specific synaptic tags, one for LTP and another for LTD, that permits the capture of distinct proteins from a large, homogeneous pool of newly synthesized plasticity-related proteins (Kelleher et al., 2004b; Govindarajan et al., 2006). If this idea is correct, one would predict that a similar set of proteins are synthesized after the induction of both LTP and LTD, and these plasticity-related proteins could enable bidirectional plasticity. For example, the induction of LTP would permit long-lasting LTD, and the induction of LTD would permit long-lasting LTP. Interestingly, this type of heterosynaptic associativity between LTP and LTD has been demonstrated (Sajikumar and Frey, 2004), suggesting that it is the synaptic tag, rather than the specific proteins that are made, that differ between LTP and LTD. However, there is evidence to the contrary. For example, protein kinase M ζ (PKMζ), a constitutive active isoform of protein kinase C, is required for the maintenance of L-LTP, but not L-LTD (Sajikumar et al., 2005), suggesting that PKMζ is an LTP-specific protein. Thus, the idea that LTP and LTD result in the synthesis of similar sets of proteins remains to be determined, perhaps with either microarray studies of polysome fractions or proteomic analysis. In regard to the latter, a recent technical advance that permits the identification of newly synthesized proteins in neurons bodes well for a proteomic approach to this critical question (Dieterich et al, 2006).

Does overall protein synthesis, particularly in dendrites, actually increase during either LTP or LTD? It has been reported that global protein synthesis is increased in response to LTP-inducing stimulation (Kelleher et al., 2004a), and treatment of synaptoneurosomes with metabotropic glutamate receptor agonists increased the number of polyribosomes (Weiler and Greenough, 1993) and the translation of a reporter mRNA (Job and Eberwine, 2001). On the other hand, *in vivo* application of the group I mGluR agonist DHPG to the hippocampus did not trigger a detectable increase in overall protein synthesis, though it did result in an increase in the synthesis of eEF1A (Huang et al., 2005). As mentioned earlier, glutamate receptor activation in cultured neurons and NMDA receptor activation in the tadpole tectum results in an overall decrease in protein synthesis that coincides with specific increases in α-CaMKII. Thus, the question of whether overall protein synthesis increases,

particularly in dendrites, or whether gene-specific translation via the specific regulation of mRNA-binding proteins is augmented during either LTP or LTD awaits further investigation.

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4.34 Activity-Dependent Structural Plasticity of Dendritic Spines

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4.34.1 Introduction

Neuroscientists have long been fascinated by the problem of how memory is formed, stored, and recalled, not only because learning and remembering are at the core of our human experience, but also for the evolutionary significance to adapt behaviorally by learning about and making predictions in response to our surroundings. As early as the later part of the nineteenth century and around the time when the cellular basis of brain structure was still hotly debated, Cajal and Tanzi speculated that the improvement of existing skills and the acquisition of new ones required structural changes in nerve cells. Current cellular and molecular models of learning and memory are deeply rooted in these pioneering ideas, whereby biochemical modifications and morphological remodeling of existing synaptic junctions, as well as the formation of new ones, lead to enduring functional changes in neuronal networks. This chapter will review the experimental evidence in support of the activity-dependent structural plasticity of dendritic spines, the small processes extending from the surface of dendrites where most excitatory synapses of the central nervous system (CNS) are formed. We will focus on the morphological consequences of environmental enrichment and behavioral learning of associative tasks, as well as *in vitro* manipulations of neuronal activity resulting in long-term synaptic plasticity in the hippocampus, a brain region critical for memory formation. We will review the actions of neurotrophins and hormones on dendritic spines in the context of hippocampal-dependent learning and memory. Since several developmental disorders associated with mental retardation present with abnormal dendritic spines, we will also discuss the potential implications of such structural differences in cognition as well as in learning and memory.

4.34.2 Brief Historical Perspective

The cellular and molecular mechanisms of memory acquisition, consolidation, and subsequent recall have been intensely studied by neuroscientists for more

than a century. The first ideas about the possibility that experience can modify the structure of the brain, including its comprising nerve cells and their junctions, can be traced back to the later part of the nineteenth century (Bain, 1872; James, 1890; Cajal, 1893; Tanzi, 1893; Foster and Sherringhton, 1897). Eugenio Tanzi and Santiago Ramón y Cajal postulated that the improvement of learned habits and skills by 'mental exercise' arises from the growth of existing synapses, whereas learning new ones requires the formation of new nerve cell connections. Despite the appeal of these remarkably intuitive ideas, it was later realized that the mature nervous system might not be as plastic as it is during development and that neuronal growth may be too slow to account for learning. Later, Donald Hebb proposed a 'dual trace mechanism' to address the discrepancy between the rapidity of learning and the time required for structural growth, whereby

> . . . a reverbatory trace might cooperate with the structural change, and carry the memory until the growth change is made . . . (Hebb, 1949).

Hebb's more famous contribution is a cellular mechanism for associative learning postulating that coincident activity at given synaptic junctions modifies the properties of those synapses, thereby increasing their efficiency (Hebb, 1949). Together with the pioneering ideas of Tanzi and Cajal, this 'Hebbian' principle dominates the current thinking of how use-dependent changes at synapses occur during learning.

Research into the mechanisms underlying learning has thus primarily focused on the computational unit of the nervous system, the synapse. It is now well known that synapses are not static: the cellular mechanisms of learning and memory, triggered by experience, involve molecular modifications and morphological remodeling of existing synapses, as well as genesis of new synapses, resulting in activity-dependent functional changes. The vast majority of the studies on the mechanisms underlying changes in synaptic strength as a consequence of behavioral experience have focused on one of the brain regions most relevant to learning and memory, the hippocampus. The study of synaptic plasticity in the hippocampus and its relationship to learning and memory has uncovered a labyrinth of molecular, cellular, and biophysical mechanisms that have been extensively reviewed (Martin et al., 2000; Abel and Lattal, 2001) (*See* Chapters 4.02, 4.16).

4.34.3 The Structure and Function of Dendritic Spines

The acquisition, consolidation, and storage of information are dynamic processes that involve constant modification of synapses. A dynamic structure that makes up one half of the synapse long thought to be critical for learning and memory is the dendritic spine (**Figure 1**). Spines were first described by Cajal as small protrusions extending from the surface of dendrites (Cajal, 1891), likely representing the points of reception of nerve impulses (Cajal, 1909). It is now well known that these micron-long dendritic projections are the main postsynaptic site of excitatory synapses in the CNS, whereby approximately 90% of spines are innervated by a single

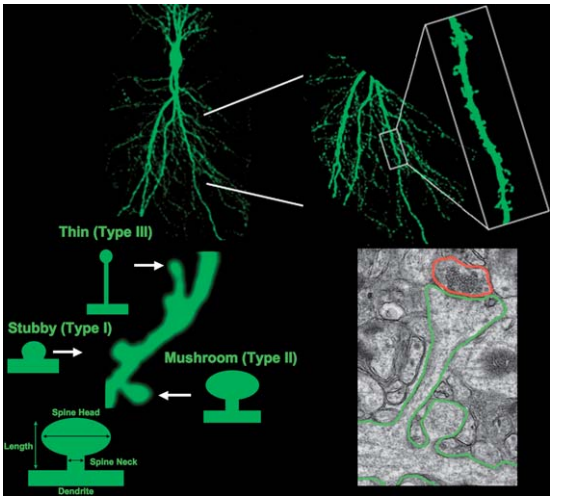


Figure 1 The structure of dendritic spines of hippocampal pyramidal neurons. Using particle-mediated gene transfer (a.k.a. gene gun), organotypic slice cultures were transfected with cDNA coding for enhanced yellow fluorescent protein. *Top panels:* Laser-scanning confocal microscopy images of a pyramidal neuron in area CA1 are shown at different magnifications to illustrate the complexity of their dendritic arbor and the abundance of dendritic spines in secondary and tertiary branches. *Bottom left panel:* A maximum-intensity projection of *z*-stacks shows a dendritic segment studded with the most common spine morphologies (i.e., stubby, mushroom, and thin). The cartoon illustrates the geometrical dimensions measured in individual spines to categorize them. *Bottom right panel:* A mushroom dendritic spine (outlined in green) forms an asymmetric synapse with a single presynaptic terminal (outlined in red) in *stratum radiatum* of area CA1 in organotypic slice culture. Bottom left panel adapted from Tyler WJ and Pozzo-Miller L (2003) Miniature synaptic transmission and BDNF modulate dendritic spine growth and form in rat CA1 neurones. *J. Physiol.* 553: 497–509, with permission.

presynaptic terminal (Gray, 1959b). Furthermore, 'naked' spines lacking a presynaptic partner are hardly observed in serial electron microscopy (EM) reconstructions of the hippocampus (Harris and Stevens, 1989). In some occasions, two or more spines from the same dendrite share the same presynaptic terminal, the so-called multiple synapse bouton. Structurally, a spine consists of a spherical head connected by a neck to its parent dendrite. In the hippocampus, the morphology of individual spines varies widely due to differences in their length, head shape, and neck diameter (Sorra and Harris, 2000). Simple spines can be characterized into three major types: (1) stubby, or Type-I spines, which lack obvious necks; (2) mushroom, Type-II spines, which have large heads and short narrow necks; and (3) thin, Type-III spines, which have small heads and long narrow necks (Peters and Kaiserman-Abramof, 1970). More complex morphological variations have also been observed, including bifurcated spines with multiple heads sharing the same neck (Harris, 1999). Time-lapse imaging of live spines has vividly demonstrated that dendritic spines are not static structures, but rather motile processes, especially during development (Matus, 2000; Dunaevsky and Mason, 2003). Thus, the aforementioned morphological types need to be considered as arbitrary snapshots of an underlying continuous distribution of possible geometrical forms, likely morphing from one another (Parnass et al., 2000). In the following sections, we will further discuss the intriguing possibility that spine geometry is modulated by synaptic activity, and how this structural modification may have functional consequences for synaptic transmission and plasticity.'

At the most distal region of the spine head facing the active zone (i.e., release site) of the apposing presynaptic terminal, a noticeable electron dense thickening is observed in EM micrographs, termed the postsynaptic density (PSD) (Kennedy, 1997; Ziff, 1997; Sheng and Sala, 2001). The PSD contains cytoskeletal components, scaffolding, and regulatory proteins, as well as neurotransmitter receptors. Simple dendritic spines do not contain mitochondria or microtubules, and the main cytoskeletal components are F-actin microfilaments (Peters et al., 1991). Notably, some spines will exhibit polyribosomes at the base of their necks (Spacek, 1985), suggesting that protein synthesis can occur locally near synapses. Approximately half of the spines of hippocampal pyramidal neurons contain one or several cisterns of smooth endoplasmic reticulum (SER) within their heads, a structure sometimes called the spine apparatus (Spacek and Harris, 1997; Cooney et al., 2002). Intriguingly, spine SER cisterns are not static but can dynamically interact with those within the parent dendrite (Toresson and Grant, 2005). Together with the role of SER as a Ca^{2+} source and sink (Berridge, 1998; Pozzo-Miller et al., 2000), the presence of Ca^{2+}-permeable ligand and voltage-gated channels in the spine membrane supports one of the most recognized functions of dendritic spines, i.e., a biochemical compartment for intracellular Ca^{2+} signaling (Muller and Connor, 1991; Petrozzino et al., 1995; Yuste and Denk, 1995; reviewed by Connor et al., 1994; Yuste et al., 2000; Sabatini et al., 2001).

It is becoming increasingly clear that the particular morphology of a dendritic spine may play an important role in determining its function, a concept that has been extensively reviewed (Shepherd, 1996; Yuste and Majewska, 2001; Nimchinsky et al., 2002). Two morphological features of dendritic spines seem to be critical for their role as biochemical compartments: the geometrical dimensions of the head and neck. If one formally considers a dendritic spine in terms of an electrical resistor and relates Ohm's law to diffusional resistance, the electrical coupling between the spine head and the dendritic shaft can be estimated from the diffusion of small fluorescence molecules from the spine to the parent dendrite. Using this approach, it was initially shown that diffusional coupling depends on the length and diameter of the neck (Svoboda et al., 1996). Furthermore, different levels of synaptic activity are able to regulate the diffusional isolation of individual spines (Bloodgood and Sabatini, 2005). Chronic blockade of excitatory synaptic transmission enhanced the diffusion of a photoactivatable green fluorescent protein (GFP) from the spine into the parent dendrite, while increasing excitatory transmission enhanced spine diffusional isolation. Notably, patterns of coincident synaptic activation and postsynaptic action potentials known to induce long-term potentiation (LTP) rapidly restricted diffusion across the spine neck. However, no correlation was found between spine geometry and diffusional isolation. These changes may be relevant for the induction and input specificity of Ca^{2+}-dependent forms of synaptic plasticity (i.e., long-term potentiation), because the diffusional coupling of individual spines affects the spatiotemporal profile of Ca^{2+} signals within spine heads (Korkotian and Segal, 2000; Majewska et al., 2000a). Indeed, a few experimental and modeling studies

have shown that the spine neck can function as a diffusional barrier for Ca^{2+} ions flowing between the spine head and the parent dendrite, whereby short spines facilitated the Ca^{2+} flux from the head to the dendrite and long spines prevented it (Volfovsky et al., 1999; Korkotian et al., 2004). Using two-photon uncaging of methoxy nitroindolino glutamate (MNI-glutamate) to activate *N*-methyl-D-aspartate-type glutamate receptors (NMDARs) in individual spines, it has been shown that larger spines permitted greater efflux of Ca^{2+} into the dendritic shaft, whereas smaller spines manifested a larger increase in Ca^{2+} within the spine compartment as a result of a smaller Ca^{2+} flux through the neck (Noguchi et al., 2005). However, this view has been challenged based on the observations that the spatiotemporal profile of intracellular Ca^{2+} signals within spine heads is primarily determined by sequestration and extrusion, likely within the head or neck (Nimchinsky et al., 2002; Sabatini et al., 2002). Additional confounding factors include the perturbation of the intrinsic Ca^{2+} buffering capacity by the Ca^{2+} indicator dyes, the apparent increased diffusion of Ca^{2+} ions bound to the highly mobile indicator dyes, and the temperature dependence of the sequestration and extrusion processes.

Despite the difficulty of determining the role of spine morphology in the coupling of spine Ca^{2+} signals with the parent dendrite (Majewska et al., 2000a; Nimchinsky et al., 2002), recent evidence supports an early suggestion that spine morphology and size correlate with synaptic strength (Pierce and Lewin, 1994). For example, the volume of the spine head is directly proportional to the number of docked vesicles at the active zone of presynaptic terminals (Harris and Sultan, 1995; Boyer et al., 1998; Schikorski and Stevens, 1999) and to the number of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate-type glutamate receptors (AMPARs) (Nusser et al., 1998). These observations indicate that larger spines bear larger synapses, which are more reliable and stronger in terms of probability of release and postsynaptic sensitivity, respectively (Murthy et al., 2001). Another measure of synaptic strength is the expression of AMPARs at individual excitatory spine synapses, as observed during brain development and after induction of LTP, the most studied cellular model of learning and memory (Malinow and Malenka, 2002). The expression of AMPARs within individual spines of different morphologies was mapped by two-photon uncaging of MNI-glutamate, revealing that mushroom (Type-II) spines show larger AMPAR-mediated responses than thin (Type-III) spines (Matsuzaki et al., 2001). These studies provide functional evidence that larger spines represent stronger synapses, as defined by their expression of AMPARs (Kasai et al., 2003). As we will discuss later, it has been shown that the induction of LTP leads to an increase in spine head size, while long-term depression (LTD) causes spine shrinkage and retraction.

Other functions beyond that of compartmentalization of Ca^{2+} signals have been proposed for dendritic spines (Shepherd, 1996). These include (1) providing additional neuronal surface area to increase synapse density, (2) isolation of cytoplasmic biochemical/molecular signals to activated synaptic inputs, (3) neuroprotection by isolating large Ca^{2+} elevations from parent dendrites, (4) amplification of synaptic potentials, and (5) enhancing the spatial spread of back-propagating action potentials. Because some of these functional properties have been observed in nonspiny neurons, it is still currently unclear what, if any, is the functional advantage of producing and maintaining dendritic spines. Besides the function/structure relationship of dendritic spines, a number of physiological, pathophysiological, and experimental conditions modify spine density and morphology, including behavioral learning, hormonal state, levels of excitatory synaptic activity, growth factors (including neurotrophins), aging, malnutrition, poisoning, and several neurological disorders, such as schizophrenia and mental retardation (Fiala et al., 2002). Before we consider each of the physiological factors that modify dendritic spine number and form, we begin discussing the development of dendritic spines.

4.34.4 The Development of Dendritic Spines

The dendrites of pyramidal neurons in neonatal mammals are initially smooth, devoid of dendritic spines (Cajal, 1909; Marin-Padilla, 1972a; Purpura, 1975b). Spine density in pyramidal neurons seems to follow a developmental pattern similar to synapse formation, where an initial overproduction is followed by an activity-dependent pruning of excess synapses. In addition, spine morphology also changes during brain development. The majority of spines in the developing hippocampus are stubby, although the most frequent dendritic processes are filopodia. Because dendritic spines represent the postsynaptic

compartment of the vast majority of excitatory synapses in the CNS, the study of their formation and evolution during brain development has been recently reviewed (Sorra and Harris, 2000; Yuste and Bonhoeffer, 2004).

Spine synapses were initially thought to originate from established synapses formed first on the dendritic shaft by the extension and narrowing of a neck and the appearance of a spine head (Miller and Peters, 1981). Alternatively, and due to their predominance and motility in the developing brain, dendritic filopodia have been considered as precursors of mature dendritic spines. Considering that approximately 90% of spines have a presynaptic partner (Gray, 1959a; Harris and Kater, 1994), it has been speculated that dendritic filopodia actively search for presynaptic axon terminals to form synapses, giving rise to spines with more mature morphologies after the initial contact (Jontes and Smith, 2000; Yuste and Bonhoeffer, 2004). This model was initially supported by studies showing that dendritic filopodia lacking presynaptic partners populate the dendrites of 1-week-old neurons, which later acquire shorter spines always associated with a presynaptic terminal (Papa et al., 1995). Time-lapse observations in organotypic cultures of hippocampal slices later demonstrated that filopodia are extremely dynamic, extending and retracting in short periods of time (Dailey and Smith, 1996). Furthermore, some filopodia of cultured neurons were shown to make contact with axons at their tips, and the number of filopodia decreased in parallel with an increase in the number of spines (Ziv and Smith, 1996). These observations led to the proposal that the initial contact with axons triggers the formation of spines from filopodia. Serial EM reconstructions in the developing hippocampus *in situ* also support the role of filopodia as the site of initial axon contact, but only after their complete retraction and morphing into a shaft synapse, which will then serve as the emerging site of a mature spine with a synapse on its head (Fiala et al., 1998). Lastly, longitudinal *in vivo* multiphoton imaging of dendritic spines in the neocortex of developing and even adult animals supports the view that spines constantly form by seeking out presynaptic partners in the surrounding neuropil and stabilizing into functional spines of 3-varied morphology, a process driven by sensory experience (Lendvai et al., 2000; Trachtenberg et al., 2002; Holtmaat et al., 2005; Knott et al., 2006).

Regardless of the specific model of spine synapse formation, the highly dynamic behavior of filopodia during synatogenesis and the continuous rapid motility of dendritic spines require a careful orchestration and modulation of the dendritic cytoskeleton (Matus, 2000). The mechanism responsible for filopodia and spine motility is based on actin polymerization (Fischer et al., 1998; Dunaevsky et al., 1999; Korkotian and Segal, 2001; Zito et al., 2004) and seems to involve the Rho family of small GTPases (GTP: guanosine triphosphate) (Nakayama et al., 2000; Tashiro et al., 2000; Hering and Sheng, 2001). Moreover, spine motility is not only modulated by intracellular Ca^{2+} levels (Oertner and Matus, 2005), but may also affect Ca^{2+} compartmentalization within individual spines (Majewska et al., 2000b). Actin-based rapid spine motility, whereby the spine head and neck are in a seemingly constant process of movement, is not only critical for spine formation and synaptogenesis during brain development, but has also been proposed to be fundamental throughout lifetime (Matus, 2005; Harms and Dunaevsky, 2006). As we shall discuss in the following sections, actin-based motility allows dendritic spines to be malleable structures sensitive to ongoing levels of neuronal activity, underlying activity-dependent structural plasticity during experience-driven behavioral learning.

4.34.5 Structural Plasticity of Dendritic Spines Induced by Synaptic Activity: Homeostatic Plasticity, LTP, and LTD

Despite speculation by Cajal and Tanzi more than 100 years ago that the improvement of learned skills by 'mental exercise' arises from the growth of existing synapses, whereas acquiring new ones requires the formation of new neuronal connections, it was not until the 1960s that such structural plasticity was observed in response to the amount of afferent synaptic activity. In these early studies, dendritic spines of pyramidal neurons in the visual cortex were lost when afferent input was deprived by either surgical lesions (Globus and Scheibel, 1966) or by rearing mice in total darkness (Valverde, 1967). Moreover, deafferentation-induced spine loss was reversible and not pathological, as spine density could recover after only a few days of exposure to normal lighting conditions (Valverde, 1971). On the other hand, rearing rats in continuous illumination led to an increase in spine density in visual cortical neurons (Parnavelas et al., 1973), suggesting that morphological changes induced by deafferentation were bidirectional. It was later shown that these effects were not exclusive of

the visual cortex. Surgical lesions of the perforant path – the afferent fibers from the entorhinal cortex into the dentate gyrus – caused an initial loss of dendritic spines of granule cells, which was followed by a recovery to prelesion levels due to subsequent axonal sprouting (Parnavelas et al., 1974). These early observations consolidated the concept that changes in the levels of afferent input evoked an adaptive response in terms of dendritic spine density in several brain regions.

4.34.5.1 Ongoing Synaptic Activity

Because dendritic spines represent the postsynaptic compartment of excitatory synapses, the aforementioned *in vivo* studies were followed by varied manipulations of glutamatergic synaptic transmission in brain slices and cultured neurons. Long-term exposure to gamma-aminobutyric acid$_A$ ($GABA_A$) receptor antagonists, which increases neuronal excitability and thus excitatory synaptic drive, caused a significant reduction in spine density in pyramidal neurons within organotypic cultures of hippocampal slices (Muller et al., 1993; Thompson et al., 1996). Due to the excessive recurrent innervation in long-term hippocampal slice cultures (Gutierrez and Heinemann, 1999), this manipulation may have evoked pathological levels of excitability, leading to spine loss as described in epilepsy patients (Scheibel et al., 1974). On the other hand, exposing cultured hippocampal neurons to $GABA_A$ receptor antagonists for shorter periods led to an increase in dendritic spine density (Papa and Segal, 1996). Additional studies in cultured neurons support the hypothesis that moderate levels of excitatory synaptic transmission promotes spine growth and formation while excessive levels cause spine shrinkage and loss, representing an adaptive response mediated by intracellular Ca^{2+} levels (Korkotian and Segal, 1999a,b; Segal et al., 2000). In fact, dendritic spines are rapidly formed and orient themselves toward a local source of glutamate (Richards et al., 2005). On the other side of the coin, weeklong pharmacological blockade of NMDARs or Na^{+}-dependent action potentials reduced spine density in CA1 pyramidal neurons in slice cultures (Collin et al., 1997). Furthermore, spontaneous quantal excitatory synaptic transmission, i.e., miniature synaptic currents or 'minis,' seemed to be sufficient for the maintenance of spines, as long-term blockade of AMPARs or inhibition of vesicular neurotransmitter release with *Botulinum* neurotoxins led to a decrease in spine density in hippocampal pyramidal neurons maintained in organotypic slice cultures (McKinney et al., 1999). The spine loss in these chronic blockade experiments may result from the prolonged absence of excitatory synaptic input (7 days *in vitro*), because shorter periods of inactivity (48 h) did not cause spine loss but rather a change in the proportion of morphological spine types (Tyler and Pozzo-Miller, 2003). In fact, even shorter inactivity (~8 h) induced filopodia and immature synapse formation in CA1 pyramidal neurons of acute hippocampal slices (Kirov and Harris, 1999; Petrak et al., 2005). Such brief periods of inactivity actually promote the active searching of presynaptic partners by filopodia and immature dendritic spines (Richards et al., 2005) that, if unsuccessful, would lead to the collapse and pruning of spines. It has been proposed that highly motile immature dendritic spines containing only NMDARs (i.e., silent synapses) (Isaac et al., 1995; Liao et al., 1995) may later stabilize by the acquisition of AMPARs (Shi et al., 1999). Taken together, these observations provide a physiological mechanism for deafferentation-induced spine loss and support the early notion that the levels of afferent synaptic input directly influence neuronal morphology, e.g., spine synapse density.

4.34.5.2 Homeostatic Plasticity

Due to the duration (days to weeks) and generalized extent of the experimental manipulations of synaptic activity, the aforementioned experiments may reflect cell-wide global adaptations as thought to occur during homeostatic synaptic plasticity. Homeostatic mechanisms maintain an 'optimal' level of input to a neuron by regulating the strength of afferent excitatory and inhibitory synapses, by modulating intrinsic neuronal excitability, and by altering synapse number (Turrigiano and Nelson, 2004; Davis, 2006). Despite the lack of changes in spine density in cultured developing neurons after activity blockade (Davis and Bezprozvanny, 2001; Burrone and Murthy, 2003), CA1 pyramidal neurons in acute slices exhibit more – and longer – spines after blockade of synaptic transmission (Kirov and Harris, 1999). In addition to the duration of the activity blockade (hours in acute slices vs. days in cultures), the developmental age of the neurons in these studies was different. The absence of synaptic activity during the critical period of synaptogenesis and pruning in the hippocampus (postnatal day 6 through postnatal day 16) did not evoke an homeostatic modulation in spine number (Kirov et al., 2004a), despite the

well-characterized changes in quantal scaling of glutamate receptors and neuronal excitability in developing cultured neurons (Turrigiano, 1999). Instead, homeostatic plasticity of spine density seems to appear once the adult-like dendritic complement of spines is achieved (Kirov et al., 2004a). Complementary studies focused on the morphological consequences of activity-dependent manipulations of glutamatergic synaptic transmission with 'Hebbian' features resembling associative learning (Hebb, 1949), such as LTP and LTD of excitatory synaptic transmission (Malenka and Bear, 2004). Since this topic has been reviewed extensively over the years (e.g., Wallace et al., 1991; Geinisman, 2000; Yuste and Bonhoeffer, 2001; Segal, 2005), we will briefly discuss the main classical findings in addition to the most recent observations employing state-of-the-art imaging technologies.

4.34.5.3 Long-Term Potentiation

Instead of reflecting an adaptive response to prolonged and widespread changes in synaptic input, LTP is induced and expressed by a small number of activated synapses. Due to several of its properties (e.g., input specificity, associativity, cooperativity), LTP has become the most recognized synaptic model of associative learning and memory (Bliss and Collingridge, 1993). As pointed out earlier, the need for a structural change to underlie the longevity of memories has been repeatedly speculated (Cajal, 1893; Tanzi, 1893; Hebb, 1949). In fact, this possibility was explicitly proposed as one of the potential mechanisms for the enhancement of excitatory transmission in the very first description of LTP (Bliss and Lomo, 1973). In this groundbreaking paper, Bliss and Lomo quoted Rall, stating that

> . . . a reduction in the resistance of the narrow stem by which spines are attached to the parent dendrite . . .

may change the relative weight of synapses and thus contribute to the observed potentiation of excitatory transmission (Rall, 1970, as cited in Bliss and Lomo, 1973; Rall and Segev, 1987; Diamond et al., 1970, as cited in Rall and Segev, 1987). Soon thereafter, Van Harreveld and Fifkova tested this hypothesis by using pioneering rapid freezing methods of tissue preparation for EM following *in vivo* stimulation protocols described in the first LTP papers. In these studies, dendritic spines within the outer two thirds of the dentate molecular layer – which received the stimulated perforant path fibers – had larger heads as well as wider and shorter necks, features observed as early as 2 min and lasting as long as 23 h from the stimulation (Van Harreveld and Fifkova, 1975; Fifkova and Van Harreveld, 1977; Fifkova and Anderson, 1981). The layer specificity and time course of these changes strongly suggest that they are directly correlated with the potentiation of synaptic responses, despite the lack of electrophysiological confirmation of LTP induction and the identification of activated synapses.

The pioneering ultrastructural studies by Van Harreveld and Fifkova were followed by a series of EM observations after LTP-inducing afferent stimulation both *in vivo* and in the more amenable *in vitro* brain slice preparation, which sometimes included contradicting observations:

1. Increase in the proportion of shaft synapses, without changes in spine number, size, or form in area CA1 *in vivo* and *in vitro*, although much of these observations might have come from relatively aspiny inhibitory interneurons (Lee et al., 1979, 1980).
2. Layer-specific increases in the proportion of spines with concave heads and larger PSDs, without net changes in the total number density of spine or shaft synapses on dentate gyrus granule cells (Desmond and Levy, 1983, 1986a,b, 1990).
3. Increase in the number of 'sessile' (likely stubby spines) and shaft synapses in area CA1 of *in vitro* hippocampal slices, without detectable changes in the number of spines or in the dimensions of their heads and necks, although typical cup-shaped spines seemed to have been replaced by more flat spine profiles (Chang and Greenough, 1984).
4. Decrease in the number of spine synapses and a parallel increase in shaft synapses on dentate granule cells after high-frequency stimulation of the perforant path in acute hippocampal slices (Gomez et al., 1990). Intriguingly, these changes were correlated with the magnitude and success rate of LTP induction observed in rats with different inborn learning capacity.
5. Increase in the ratio of perforated to nonperforated spines synapses in the dentate molecular layer in chronically implanted rats 1 h after perforant path stimulation, without changes in the total number of spine synapses (Geinisman et al., 1991). These studies were later followed by the observation of an increase in the number of spine synapses with multiple, completely partitioned transmission zones (Geinisman et al., 1993).
6. Increase in spine number in dentate gyrus after *in vivo* stimulation of perforant path fibers, as well as the proportion of 'bifurcated' spines (Trommald

et al., 1996), thought to originate from the splitting of spine synapses bearing perforated PSDs (Peters and Kaiserman-Abramof, 1969).

7. Increase in multiple-bouton spine synapses with perforated PSDs in area CA1 of slice cultures, which were known to have been activated during LTP-inducing afferent stimulation (Buchs and Muller, 1996; Toni et al., 1999, 2001). In these studies, synapses activated during the high-frequency conditioning stimulus – and thus presumably potentiated – were identified at the EM level using a Ca^{2+} precipitate protocol (Buchs et al., 1994).

8. No changes in the absolute number of spine synapses in area CA1 of acute slices (Sorra and Harris, 1998). However, these and other observations in acute hippocampal slices may be confounded by the fact that spine density is increased by temperature changes during slice preparation (Kirov et al., 1999, 2004b; Bourne et al., 2006).

9. Increase in spine synapses with perforated PSDs in dentate granule cells after successfully maintained LTP in aged rats, while spine branching was only observed after high-frequency stimulation without sustained potentiation (Dhanrajan et al., 2004).

10. Increase in the number of spines with perforated PSDs and of multiple-synapse boutons in area CA1 after chemically induced LTP in acute slices (Stewart et al., 2005).

Despite the increasing sophistication of the morphological studies at the EM level listed above, especially with regard to the confirmation of LTP induction and the identification of potentiatied synapses, it was clear from the beginning that time-lapse imaging of live neurons before, during, and after LTP induction would yield more consistent results. To this aim, repeated laser-scanning confocal microscopy was performed in live acute hippocampal slices to follow spine morphology during chemically induced LTP, a procedure that would ensure that most synapses in the slice are potentiatied (Hosokawa et al., 1995). The appearance of new dendritic spines in these DiI-labeled (DiI is a long-chain dialkylcarbocyanines dye) CA1 pyramidal neurons was a rare – and statistically insignificant – event. However, small spines were seen to extend, and others appeared to change their orientation with respect to the parent dendrite. Circumventing the optical limitations of confocal microscopy applied to acute slices, imaging live neurons within organotypic slice cultures (Gahwiler, 1981; Stoppini et al., 1991; Pozzo Miller et al., 1993) by multiphoton excitation microscopy – a.k.a. two-photon microscopy (Denk and Svoboda, 1997) – yielded a much clearer picture. High-frequency afferent stimulation was shown to induce the formation of protrusions that resembled dendritic filopodia in CA1 pyramidal neurons expressing enhanced GFP (eGFP) (Maletic-Savatic et al., 1999). Some of these synaptically induced filopodia persisted for the duration of the imaging session (up to 1 h) and later acquired a bulbous head – suggesting that they developed into spines – and their appearance required NMDAR activity, as LTP induction does. Similar spine formation associated with successful LTP induction was observed in CA1 pyramidal neurons injected with the fluorescent dye calcein (Engert and Bonhoeffer, 1999). These studies further showed that spine growth occurred selectively within a synaptically active region, which was restricted to a ~30-μm area, while some spines were seen to randomly disappear in distant regions where synaptic transmission was blocked, suggesting an homeostatic balance in spine number. Similar filopodia and spine formation was also shown to occur in CA1 pyramidal neurons after the intracellular application of autophosphorylated Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), an enzyme implicated in LTP induction (Jourdain et al., 2003).

In addition to spine number, time-lapse imaging by multiphoton excitation microscopy during and after LTP induction in hippocampal slices also revealed rapid changes in spine morphology, which resembled the spine head swelling initially described by Van Harreveld and Fifkova at the EM level (Van Harreveld and Fifkova, 1975). Induction of LTP by high-frequency stimulation in acute slices from eGFP-expressing mice caused a transient expansion of activated CA1 neuron spines, which were identified by synaptically mediated Ca^{2+} elevations (Lang et al., 2004). The predicted increase in spine volume observed in these studies was detected as early as 30 s after stimulation and lasted for 10–20 min. On the other hand, repetitive (1 min at 1 Hz) miniature excitatory postsynaptic current (mEPSC)-like two-photon uncaging of MNI-glutamate on individual CA1 neuron spines led to equally rapid (1–5 min) and selective enlargement of stimulated spines that was transient in large mushroom spines but persistent in small spines, lasting up to 100 min (Matsuzaki et al., 2004). Furthermore, the enduring spine enlargement was associated with an increase in AMPAR-mediated currents and required NMDAR activity, CaMKII, and actin polymerization. A similar photorelease approach – but using ultraviolet (UV) uncaging and wide-field fluorescence imaging in thinner slice

cultures – failed to reveal any morphological changes in spines during the potentiation of AMPAR-mediated currents (Bagal et al., 2005). However, the potentiation of uncaging currents in the latter studies was induced by a single UV flash paired with postsynaptic depolarization, suggesting that structural remodeling of dendritic spines may require repeated activation of NMDARs, as during LTP of synaptic responses. Further support to this idea comes from spine size changes after a global induction of LTP in slice cultures. CA1 pyramidal neurons were transfected with the fluorescent protein tDimer-dsRed and glutamate receptor subunits tagged with pH-sensitive GFP to monitor morphological changes and receptor trafficking simultaneously (Kopec et al., 2006). Generalized induction of 'chemical' LTP (Otmakhov et al., 2004) caused a significant increase in spine volume that was maximum by 2 min after the application of the chemical LTP-inducing cocktail. Furthermore, the observed spine enlargement preceded the membrane insertion of AMPA receptor subunits, suggesting that different mechanisms mediate structural and functional changes at individual spines after synaptic potentiation.

Regarding the molecular machinery responsible for the structural plasticity of dendritic spines, it was originally proposed that the actin cytoskeleton plays a critical role (Crick, 1982; Fifkova and Delay, 1982). Only recently it was shown that LTP-inducing stimulus enlarged spine heads in parallel with an increase in their content of filamentous actin relative to globular actin as imaged by fluorescence resonance energy transfer (FRET) (Okamoto et al., 2004). Furthermore, the latter studies in CA1 neurons in slice cultures showed that LTD-inducing stimulus led to the opposite results, namely a reduction in F-actin relative to G-actin and a reduction in spine size, suggesting that spine structural plasticity is bidirectional. In addition to multiple signaling pathways, in particular the one mediated by Rho and Rac small GTPases (Nakayama et al., 2000; Tashiro et al., 2000), several scaffold proteins and actin binding proteins converge on the actin cytoskeleton to regulate spine morphology and dynamics, an issue that has been recently reviewed (Tada and Sheng, 2006).

4.34.5.4 Long-Term Depression

Further evidence of the bidirectional nature of spine structural plasticity is beginning to emerge. GFP-expressing CA1 pyramidal neurons in slice cultures of Thy-1-GFP mice rapidly and persistently lost their dendritic spines after their afferent inputs were stimulated at low frequency (900 pulses at 1 Hz) (Nagerl et al., 2004), a protocol well known to induce NMDAR-dependent LTD (Dudek and Bear, 1992; Mulkey and Malenka, 1992). In addition, CA1 neurons individually filled with the fluorescent dye calcein showed spine shrinkage leading to spine retraction in response to low-frequency stimulation (Zhou et al., 2004). Similar to the requirements for LTD induction, spine retraction and pruning were also sensitive to NMDAR antagonists and calcineurin, an inhibitor of protein phosphatases (Nagerl et al., 2004; Zhou et al., 2004), strengthening the view that dendritic spines are formed and eliminated in an activity-dependent manner that correlates with bidirectional changes in synaptic efficacy.

4.34.6 Structural Plasticity of Dendritic Spines Induced by Experience and Behavioral Learning

The seminal ideas proposed by Hebb regarding associative learning sparked experimental research not only at the cellular level, but also on the structural consequences of behavioral training, spatial learning, or differential experience (Bailey and Kandel, 1993). Apparently inspired by Hebb's anecdotal report of the enhanced learning ability of laboratory rats when housed as home pets during their development (Hebb, 1949), Rosenzweig and colleagues showed that raising rodents in 'enriched' environments led to neurochemical and anatomical changes in the cerebral cortex (reviewed in Rosenzweig and Bennett, 1996). Among the anatomical consequences of rearing rodents in enriched environments was an increase in dendritic spine number in the visual cortex (Globus et al., 1973; Greenough and Volkmar, 1973), an observation in keeping with the concept that continuous use is critical for the maintenance and even the formation of new synaptic contacts on dendritic spines (e.g., Globus and Scheibel, 1966; Valverde, 1967).

Because these enriched complex environments were initially considered as visual and motor challenges, the majority of the studies focused in those brain regions thought to be engaged in such behaviors (e.g., somatosensory, motor, and visual cortices). It was later realized that a complex environment also represents a challenge for spatial navigation abilities, likely engaging the hippocampus, a region well known for the functional and structural plasticity of its excitatory spine synapses. Indeed, rats that were repeatedly trained in a complex environment (up to 5

floors connected by ladders, 4 h per day for 14–30 days) exhibit higher spine density in basal dendrites of CA1 pyramidal neurons, an observation that correlated with an improved performance in the Morris water maze compared to untrained littermates (Moser et al., 1994, 1997). Similar increases in spine density were also observed in the CA1 region of mice (Rampon et al., 2000) and marmosets, New World monkeys (Kozorovitskiy et al., 2005). However, an 'enriched' environment consists of a combination of enhanced social interactions, cognitive stimulation, and physical exercise in addition to requiring spatial navigation abilities. Not surprisingly then, environmental enrichment enhances several aspects of hippocampal function, including neurogenesis, LTP, and spatial learning (van Praag et al., 2000). It remains to be established which features of the enriched environment are more important for those lasting functional outcomes.

Learning-specific hippocampal-dependent associative tasks, such as trace eye blink conditioning or odor discrimination, were also correlated with increases in spine density on CA1 neurons, although on their apical dendrites (Leuner et al., 2003; Knafo et al., 2004). In contrast, unbiased stereological analyses failed to detect differences in spine synapse number on CA1 pyramidal neurons after Morris water maze training (Rusakov et al., 1997; Miranda et al., 2006) or trace eye blink conditioning (Geinisman et al., 2000). However, the latter study did reveal an increase in the area of the PSD of nonperforated spine synapses (Geinisman et al., 2000). These discrepancies may originate from 'snapshot' observations of likely transient changes made at different time points through a continuous distribution of morphological states. Indeed, learning-induced increases in spine density of dentate gyrus granule cells were transient, with maximum levels observed 6–9 h after passive avoidance (O'Malley et al., 1998) or spatial water maze training (O'Malley et al., 2000; Eyre et al., 2003), and returning to control levels after 24–72 h. Despite the attractiveness of the possibility that dendritic spines and memory formation mutually interact – i.e., "learning makes more spines" and "more spines promotes learning" – their causal relationship, if any, has eluded more than 100 years of intensive research and continues to raise alternative interpretations (Moser, 1999; Segal, 2005).

Although we have focused on the structural plasticity of dendritic spines in the hippocampus, it should be noted that recent advances in multiphoton excitation microscopy have allowed the study of dendritic spines in cortical regions of the intact brain *in vivo*. Due to the obvious limitation of accessibility, most of the work so far has focused in the cerebral cortex, where long-term imaging has demonstrated that subpopulations of spines appear and disappear while others remain for months (Grutzendler et al., 2002; Trachtenberg et al., 2002; Holtmaat et al., 2005). Furthermore, spines in the somatosensory barrel cortex are modulated by sensory experience (Trachtenberg et al., 2002; Zuo et al., 2005; Holtmaat et al., 2006), and such experience-driven spine formation precedes synapse formation (Knott et al., 2006). However, the only report of dendritic spine imaging in the hippocampus *in vivo* showed a remarkable stability, at least for up to 4 h, even after induction of epileptic seizures with the muscarinic agonist pilocarpine or the $GABA_A$ antagonist bicuculline (Mizrahi et al., 2004). However, the conditions used in this study for imaging spines in the intact hippocampus *in vivo* (e.g., halothane anesthesia and blunt resection of the overlaying cerebral cortex) may have affected their physiological behavior. Thus, it remains to be determined whether dendritic spines in the intact hippocampus *in vivo* display the types of rapid structural rearrangements they are capable of *in vitro* in response to varying levels of afferent synaptic activity. A further challenge will be to relate synaptic activity-dependent rapid spine plasticity with the morphological remodeling described after experience-driven hippocampal-dependent learning.

4.34.7 Structural Plasticity of Dendritic Spines Induced by Neuromodulators: Ovarian Hormones and Neurotrophins

In addition to the ongoing levels of excitatory afferent input, exposure to enriched environments and learning spatial navigation tasks, dendritic spines of hippocampal neurons are also sensitive to slow-acting neuromodulators. Due to their established role during brain development and throughout aging, some of the most attractive molecular candidates for modulating spine structural plasticity include the ovarian steroid hormones (i.e., estradiol and progesterone) and members of the family of neurotrophins, such as brain-derived neurotrophic factor (BDNF). As we shall discuss below, the interplay between estradiol and BDNF actions on hippocampal neurons is

reflected not only in the modulation of dendritic spine density, but also in the modulation of hippocampal synaptic plasticity thought to underlie associative learning and memory.

4.34.7.1 Estradiol

Traditionally, the effects of steroid ovarian hormones have been defined as 'organizational' and 'activational.' Organizational effects are permanent structural changes induced by hormones during a critical period of brain development, while activational effects represent the initiation or termination of previously established neuronal circuits (Arnold and Gorski, 1984). Initial studies of the effects of steroid hormones on neuronal and synaptic structure focused in sexually dimorphic brain regions that control reproductive behaviors, such as the arcuate and ventromedial nuclei of the hypothalamus (Matsumoto and Arai, 1979, 1986; Carrer and Aoki, 1982; Frankfurt et al., 1990; Pozzo Miller and Aoki, 1991, 1992). Relevant to learning and memory, several studies had shown that elevations in the circulating levels of estradiol induced experimentally, or those achieved during the proestrus in rodents, resulted in enhanced neuronal excitability in the hippocampus (Woolley and Timiras, 1962; Terasawa and Timiras, 1968; Kawakami et al., 1970). Inspired by the growing literature reporting that enhanced afferent synaptic activity promoted dendritic spine growth in several brain regions, McEwen and colleagues first demonstrated that ovariectomized female rats had reduced spine density in CA1 pyramidal neurons compared to sham-operated controls (Gould et al., 1990). Later they showed that this effect was reversible upon treatment with estradiol and progesterone (Gould et al., 1990; Woolley and McEwen, 1993), and that in fact spine density fluctuated during the estrous cycle in a manner that is consistent with the effects of estrogen and progesterone (Woolley et al., 1990). Similar effects of estradiol were observed in cultured hippocampal neurons (Murphy and Segal, 1996) and CA1 pyramidal neurons maintained in organotypic slice culture (Pozzo-Miller et al., 1999b). Furthermore, CA1 pyramidal neurons in the hippocampus of young and aged nonhuman primates (African green monkeys and Rhesus monkeys) also lose their spines after ovariectomy, an effect that is reversed by estrogen-replacement therapy (Leranth et al., 2002; Hao et al., 2003). Curiously, hippocampal CA1 neurons in ovariectomized mice of the C57BL/6J strain commonly used for genetic knockout and transgenic approaches did not exhibit changes in spine density after estrogen exposure, although the proportion of their mushroom Type-II spines was increased (Li et al., 2004). Since mushroom spines are thought to represent 'potentiated' spines (Kasai et al., 2003), these observations suggest a role of estrogen in dendritic spine structural plasticity during activity-dependent synaptic potentiation.

The changes in spine density induced experimentally by estradiol or during the estrous cycle were accompanied by increases in the binding sites for NMDARs (Weiland, 1992), enhanced immunofluorescence of the NR1 receptor subunit (Gazzaley et al., 1996), upregulation of NMDAR-mediated synaptic potentials (Woolley et al., 1997; Foy et al., 1999), and enhanced synaptic NMDAR-mediated Ca^{2+} elevations in spines of CA1 pyramidal neurons (Pozzo-Miller et al., 1999b). This enhancement of NMDAR-mediated synaptic transmission has consequences for long-term synaptic plasticity. Indeed, the likelihood for the induction of LTP is elevated at proestrous (when estradiol is highest) (Warren et al., 1995) and by estradiol treatment *in vivo* (Cordoba Montoya and Carrer, 1997), as well as in acute brain slices (Foy et al., 1999). The functional consequences of estradiol-replacement therapy in ovariectomized rats may also involve changes in the ratio of NMDAR-to AMPAR-mediated synaptic responses (Smith and McMahon, 2005). In these experiments, the magnitude of LTP after estradiol treatment was larger only when spine density increased simultaneously with an elevation in NMDAR transmission relative to AMPAR transmission, suggesting that the increase in functional synapse density alone is not sufficient to support heightened plasticity. Rather, estradiol may increase LTP via enhancing NMDAR transmission, likely through receptor insertion into newly formed or preexisting synapses. The mechanisms of estradiol action leading to spine formation resemble other forms of activity-dependent plasticity due to the requirement of NMDARs (Woolley and McEwen, 1994; Murphy and Segal, 1996), as well as activation of cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) and CREB binding protein (CBP) (Murphy and Segal, 1997).

Parallel to these cellular and molecular studies, clinical studies in postmenopausal women receiving estrogen-replacement therapy initially reported improvements in mood and cognitive functions (Sherwin, 1996), although some lingering issues remain unanswered (Sherwin, 2005). The similarities in downstream signaling and cellular effects between ovarian hormones and neurotrophins such as BDNF

(Toran-Allerand, 1996) have suggested a potential neuroprotective role for estrogens in age-related memory loss and Alzheimer's disease dementia (Gibbs and Aggarwal, 1998; Lee and McEwen, 2001). BDNF is a polypeptide related to nerve growth factor (NGF) that has been shown to provide neurotrophic support for cholinergic (Knusel et al., 1992; Widmer et al., 1993), serotonergic (Mamounas et al., 1995), and dopaminergic (Hyman et al., 1994; Yurek et al., 1996) neurons. Thus, BDNF may influence hippocampal function by maintaining the innervation of one or more of these neurotransmitter systems. In addition, the expression of BDNF mRNA in the hippocampus fluctuates during the estrous cycle and is increased after estrogen replacement in ovariectomized rats (Gibbs, 1998). Hence, increases in BDNF expression following estrogen treatment may help to enhance and maintain proper hippocampal function. However, estradiol was shown to downregulate BDNF levels in cultured hippocampal neurons, leading to spine formation by reduced GABAergic inhibition and increased excitation (Murphy et al., 1998). BDNF itself blocked the effects of estradiol on spine formation, and BDNF depletion with selective antisense oligonucleotides or with anti-BDNF antibodies mimicked the effects of estradiol, increasing spine density (Murphy et al., 1998). As we shall see in the next section, the interplay between estrogen and BDNF may be more complex than initially proposed. We next discuss the actions of BDNF on the structure and function of hippocampal neurons, with particular attention to dendritic spines and their role in synaptic plasticity.

4.34.7.2 Brain-Derived Neurotrophic Factor

Neurotrophins are secretory proteins involved in neuronal survival and differentiation (Barde, 1989). Four neurotrophins have been identified in mammals, and all are widely expressed in the CNS: NGF, BDNF, neurotrophin-3 (NT3), and NT4/5 (Lewin and Barde, 1996). They exert their effects by binding to pan-neurotrophin $p75^{NTR}$ receptors and to specific tyrosine kinase receptors, members of the *trk* family of proto-oncogenes related to insulin and epidermal growth factor receptors (Barbacid, 1993). NGF binds selectively to tyrosine kinase A (TrkA), BDNF and NT4/5 to TrkB, and NT3 to TrkC and, with a lower affinity, to TrkB as well (Chao, 1992). Binding of a neurotrophin to a specific Trk receptor stimulates its kinase activity, leading to the activation of phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK, also known as ERK), and phospholipase C-γ (PLC-γ) (Segal and Greenberg, 1996; Huang and Reichardt, 2003). Interaction of neurotrophins with $p75^{NTR}$ results in the activation of different signaling cascades mostly involved in the regulation of cell fate, such as nuclear factor kappa B (NF-κB) or c-*jun* N-terminal kinase (Kaplan and Miller, 2000). In addition to their well-established role in neuronal survival and differentiation during development, neurotrophins are strong candidates for providing the molecular signaling pathways that mediate the complex interactions between internal developmental programs and external environmental factors, ultimately leading to the appropriate development of dendrites and synapses (McAllister et al., 1999). Furthermore, BDNF has recently emerged as a fundamental modulator of hippocampal function, where its levels are amongst the highest in the brain (Murer et al., 2001). Together with the dendritic localization of GFP-tagged BDNF to dendritic process in cultured hippocampal neurons (Hartmann et al., 2001; Kojima et al., 2001; Brigadski et al., 2005), the significant release of native (i.e., endogenous) BDNF in response to stimulus patterns most amenable for LTP induction (Balkowiec and Katz, 2002; Gartner and Staiger, 2002; Aicardi et al., 2004) strongly supports the role of this prominent neurotrophin in activity-dependent functional and structural plasticity of hippocampal synapses. In the remaining paragraphs of this chapter we will focus on the morphological actions of BDNF, as its role in hippocampal synaptic transmission and plasticity in the context of learning and memory has been extensively reviewed (Lo, 1995; Thoenen, 1995, 2000; Black, 1999; Schinder and Poo, 2000; Poo, 2001; Tyler et al., 2002a,b; Vicario-Abejon et al., 2002; Lu, 2003; Bramham and Messaoudi, 2005).

Neurotrophins were shown to increase the length and complexity of dendrites of cortical pyramidal neurons when applied to slice cultures of ferret visual cortex (McAllister et al., 1995). Cortical pyramidal neurons in slice cultures treated with Trk 'receptorbodies' to scavenge endogenous neurotrophins showed limited dendritic growth and even dendritic retraction (McAllister et al., 1997), confirming that endogenous neurotrophins play a critical role in dendritic development. In addition, cortical pyramidal neurons that overexpress BDNF exhibit increased dendritic arborization, as well as enhanced rates of spine retraction and formation (Horch et al., 1999; Horch and Katz, 2002). More relevant to hippocampal function, BDNF increases spine density in CA1

pyramidal neurons, an effect blocked by the scavenger TrkB-immunoglobulin G, the receptor tyrosine kinase inhibitor k-252a, and inhibitors of the MAPK/ERK signaling cascade (Tyler and Pozzo-Miller, 2001; Alonso et al., 2004). Sustained Ca^{2+} elevations set off by BDNF-induced Ca^{2+} mobilization from inositol triphosphate (IP_3)-sensitive intracellular stores followed by transient receptor potential, canonical subfamily (TRPC)-mediated capacitative Ca^{2+} entry (Amaral and Pozzo-Miller, 2005) may play a critical role in BDNF-induced increase in spine density. Indeed, inhibition or siRNA-mediated knockdown of TRPC channels prevented not only the BDNF-induced sustained depolarization and dendritic Ca^{2+} elevation, but also the increase in spine density (Amaral and Pozzo-Miller, 2007). Moreover, the BDNF-induced structural remodeling of spiny dendrites of CA1 pyramidal neurons has consequences for Ca^{2+} signals evoked by coincident pre- and postsynaptic activity. Dendritic Ca^{2+} signals evoked by coincident excitatory postsynaptic potentials (EPSPs) and backpropagating action potentials (bAPs) were always larger than those triggered by bAPs in CA1 neurons exposed to BDNF (Pozzo-Miller, 2006), representing a potential consequence of neurotrophin-mediated dendritic remodeling of hippocampal neurons. BDNF promoted spine formation also in granule cells of the dentate gyrus (Danzer et al., 2002) and in cultured hippocampal neurons, where the effect was 'gated' by cAMP (Ji et al., 2005); but see earlier discussion of the opposite effect of BDNF in the context of estradiol-induced spine formation reported by Murphy et al. (1998).

In addition to increasing spine density *per se*, BDNF also increased the proportion of stubby spines (Type-I) under conditions of *both* action potential-dependent *and* independent synaptic transmission (Tyler and Pozzo-Miller, 2003). Even when SNARE (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor)-dependent vesicular synaptic transmission was abolished with *Botulinum* neurotoxin C, BDNF was still capable of inducing spine formation. Under these conditions, however, BDNF selectively increased the proportion of thin spines (Type-III), while decreasing the proportion of stubby spines (Type-I). Consistent with an activity-dependent process of morphological differentiation of synapses, the effects of BDNF on hippocampal spines suggest that it cooperates with spontaneous miniature synaptic activity to sculpt the fine structure of CA1 pyramidal neurons in the postnatal hippocampus. Since BDNF also increases quantal transmitter release from presynaptic terminals (Tyler and Pozzo-Miller, 2001; Tyler et al., 2002b; Tyler et al., 2006), its role in spine growth and their morphological sculpting spans the synaptic cleft.

The activation of membrane-bound $p75^{NTR}$ and Trk receptors by neurotrophin binding – in addition to physical interactions between them – organizes precise signaling cascades that control their varied actions throughout development, such as cell survival, differentiation, neurite outgrowth, and synaptic function (Patapoutian and Reichardt, 2001; Hempstead, 2002; Roux and Barker, 2002; Chao, 2003; Huang and Reichardt, 2003; Nykjaer et al., 2005). Recent observations regarding the differential role of the two types of membrane receptors have uncovered an intriguing level of complexity in neurotrophin signaling, namely opposing functional actions of $p75^{NTR}$ and Trk receptors (Lu et al., 2005). For example, while Trk receptors are essential for neuronal survival, $p75^{NTR}$ has been implicated in neuronal death. Similarly, $p75^{NTR}$ signaling has been linked to the inhibition of axonal growth, an opposing effect to that of Trk receptors. Lastly, while TrkB receptors have been shown to be critical for the role of BDNF in hippocampal LTP (Minichiello et al., 1999, 2002; Xu et al., 2000), recent reports indicate that $p75^{NTR}$ is necessary for the induction and/or expression of hippocampal LTD (Rosch et al., 2005; Woo et al., 2005). With regard to dendritic organization, $p75^{NTR}$ and Trk receptors also seem to have functional antagonisms. TrkB activity has been shown to modulate dendritic growth (Yacoubian and Lo, 2000), while a recent report using $p75^{NTR}$ knockout and $p75^{NTR}$ overexpressing mice indicates that these receptors negatively modulate dendritic morphology in hippocampal pyramidal neurons (Zagrebelsky et al., 2005), further strengthening the 'yin and yang' model of functional antagonism between Trk and $p75^{NTR}$ signaling (Lu et al., 2005).

What is the contribution of these receptors to BDNF-induced changes in spine morphology and density? It is tempting to extend the 'yin and yang' model of functional antagonism also to the modulation of spine number and form. Indeed, dendritic spine density in hippocampal pyramidal neurons was higher in $p75^{NTR}$ knockout mice, which also showed a reduction in the proportion of stubby (Type-I) spines (Zagrebelsky et al., 2005). On the other hand, mice with a conditional deletion of the TrkB receptor have reduced spine density and a higher proportion of long spines (Luikart et al., 2005; von Bohlen und Halbach et al., 2006). In addition, the proportion of

stubby (Type-I) spines was reduced in CA1 pyramidal neurons of adult transgenic mice expressing a dominant-negative TrkB (Chakravarthy et al., 2006). Intriguingly, brief exposures to k-252a, an inhibitor of receptor tyrosine kinases such as TrkS, caused a significant increase in spine density in CA1 pyramidal neurons expressing enhanced yellow fluorescent protein (eYFP) (Chapleau and Pozzo-Miller, unpublished data). However, most of these spines are of the long and thin Type-III, known to be highly motile and unstable structures (Dailey and Smith, 1996; Dunaevsky et al., 1999) and characteristic of immature synapses (Sorra and Harris, 2000). The fact that longer exposures to k-252a by itself caused spine loss (Tyler and Pozzo-Miller, 2001; Alonso et al., 2004) suggests that an initial increase in long and thin spines may precede spine regression leading to spine pruning (Segal et al., 2000). Alternatively, these observations may reflect the functional antagonism between $p75^{NTR}$ and Trk receptor signaling for dendritic spine formation and maintenance (Lu et al., 2005). In this interpretation, preferential activation of $p75^{NTR}$ by spontaneously released BDNF under conditions of Trk receptor inhibition (i.e., in the presence of k252a) leads to an initial and transient increase in thin and unstable spines, followed by a persistent spine loss. Further complexity was uncovered by the outgrowth of dendritic filopodia through the interaction of the truncated TrkB receptor (TrkB.T1) with $p75^{NTR}$, intriguingly enough, in the absence of neurotrophin binding (Hartmann et al., 2004). The combination of all these structural and physiological effects in the hippocampus may underlie the role of BDNF in the consolidation of synaptic plasticity and hippocampal-dependent learning and memory (Tyler et al., 2002a; Bramham and Messaoudi, 2005).

4.34.8 BDNF, MeCP2, and Dendritic Spine Pathologies in Rett Syndrome

Neurological disorders associated with mental retardation (MR) are characterized by a prevalent deficit in cognitive function and adaptive behavior that range in phenotype severity and are often accompanied by specific symptoms. MR-associated disorders that have environmental, neurodegenerative, or genetic origins have long been associated with structural anomalies of dendritic spines that are a common feature of various neurological disorders (Fiala et al., 2002). The pioneering studies by Huttenlocher (1970, 1974), Marin-Padilla (1972b, 1976), and Purpura (1974, 1975a) described strikingly similar abnormalities in the dendritic organization of cortical neurons from human patients with unclassified MR-associated disorders. Pyramidal neurons of the cerebral cortex showed a reduction in the number and length of dendritic branches, a significant loss of dendritic spines, and a predominance of long 'tortuous' spines. Interestingly, no other neuropathologies were found in these patients. Since then, similar features of dendritic branching, as well as spine density and morphology, have been described in other MR-associated genetic developmental disorders, ranging from autosomal genetic forms of MR (e.g., Down syndrome, Angelman syndrome) to X chromosome–linked forms of MR (e.g., Rett syndrome, fragile X syndrome) (Kaufmann and Moser, 2000; Fiala et al., 2002; Newey et al., 2005). Thus, it seems that dendritic and spine anomalies are the major brain pathologies underlying the cognitive impairments observed in humans with MR, because such dendritic and spine changes will reduce postsynaptic surface area and the density of mature excitatory synapses.

One MR-associated disorder with an intriguing link to BDNF signaling is Rett syndrome (RTT) (*See also* Chapter 4.43). RTT is an X-linked neurodevelopmental disorder and the leading cause of severe mental retardation in females, affecting 1:10,000–20,000 births worldwide without predisposition to a particular racial or ethnic group (Percy, 2002; Neul and Zoghbi, 2004). Patients with RTT are born healthy and achieve standard developmental milestones until 6–18 months of age, when they begin a regression period associated with loss of acquired cognitive, social, and motor skills (Armstrong, 1997). Later symptoms and deficits include irregularities in motor activity, characterized by a stereotypic hand movement or useless hand movements, altered breathing patterns, gait and motor imbalance, and continued cognitive decline. As the period of regression concludes, the individuals are often left in a severely impaired condition, with a majority of children developing seizure activity, although seizure frequency diminishes with age. As the children get older, a period of stabilization occurs where they develop greater communication abilities with their eyes, yet motor function continues to regress gradually (Percy and Lane, 2005). Loss-of-function mutations in the gene encoding methyl-CpG-binding protein 2 (MeCP2) have been recently identified in RTT patients (Amir et al., 1999). MeCP2 binds specifically to CpG-methylated DNA and is thought to inhibit gene transcription by recruiting corepressor

and histone deacetylase complexes and altering the structure of genomic DNA (Nan et al., 1998). Because other neurodevelopmental disorders with autistic features have also been associated with MeCP2 dysfunction, the role of MeCP2 in neuronal function may extend beyond RTT (Percy, 2002). Intriguingly, one of the targets of MeCP2-mediated transcriptional repression is the gene encoding BDNF, whereby MeCP2 binds to and represses the transcription of the promoter region of the *BDNF* gene (Chen et al., 2003; Martinowich et al., 2003).

The neuropathology of RTT reveals several areas of abnormal brain development (Armstrong et al., 1995; Bauman et al., 1995a,b). Several studies reported abnormal dendritic structure in the frontal cortex (Jellinger et al., 1988), and pyramidal neuron dendritic area and growth are significantly decreased in the frontal and motor cortices, as well as in the *subiculum*, while dendritic length was not affected in the hippocampus (Armstrong et al., 1995). Another group reported a similar dendritic appearance; in addition, they observed a thickening of dendrites (Cornford et al., 1994). Finally, a reduction in the levels of microtubule-associated protein-2 (MAP-2), a dendritic protein involved in microtubule stabilization, was found throughout the neocortex of RTT brains (Kaufmann et al., 1995, 2000). Relevant to the role of neurotrophins in dendritic spine formation and maturation, RTT brains have reduced spine density in dendrites of the frontal cortex (Belichenko et al., 1994). In addition, the levels of cyclooxygenase-2, a protein enriched in dendritic spines, are reduced in the frontal cortex in RTT (Kaufmann et al., 1997). With regard to synapse density, reduced levels of the synaptic vesicle protein synaptophysin were detected in the motor, frontal, and temporal cortices by immunofluorescence (Belichenko et al., 1997), although another study reported unaltered synaptophysin levels in frontal cortex and cerebellum (Cornford et al., 1994).

To further the understanding of RTT, a number of different mouse models have been generated, either carrying a null deletion of *MECP2* (Chen et al., 2001; Guy et al., 2001) or expressing a truncated, nonfunctional form of the wild-type MeCP2 protein (*MECP2*^{308}) (Shahbazian et al., 2002). These mice have common phenotypes, including delayed onset of symptoms (at approximately 5 weeks of age) and motor impairment and abnormal gait, whereby the null *MECP2* mice have hindlimb irregularities, while the *MECP2*^{308} mice have forelimb impairment (Shahbazian and Zoghbi, 2002; Armstrong, 2005). In addition, null *MECP2* mice have altered synaptic transmission and plasticity, including impaired hippocampal LTP (Asaka et al., 2006), and reduced excitatory transmission in the hippocampus (Nelson et al., 2006) and neocortex (Dani et al., 2005). Deficits in hippocampal LTP and hippocampal-dependent learning and memory were also observed in the *MECP2*^{308} mice expressing the truncated protein (Moretti et al., 2006). On the other hand, transgenic mice that mildly overexpress wild-type MeCP2 (~2 times wild-type levels) display a higher rate of hippocampal-dependent learning and enhanced LTP, though they developed a neurological phenotype that included seizures after 20 weeks of age (Collins et al., 2004).

Intriguingly, observations regarding neuronal and synaptic morphology in these mouse models of RTT have produced varying results, sometimes inconsistent with the neuropathology found in RTT patients (Armstrong et al., 1995; Bauman et al., 1995a,b). Pyramidal neurons of layer II/III of the neocortex of null *MECP2* hemizygous mice (MECP2$^{-/y}$) are smaller and have reduced dendritic branching as observed in RTT brains, although no differences were found in dendritic spine density (Kishi and Macklis, 2004), a distinctive feature observed in one study of the cortex of RTT patients (Belichenko et al., 1994). Another study of the somatosensory cortex of null *MECP2* hemizygous mice (MECP2$^{-/y}$) reported that dendrites of layer II/III neurons were thinner and had fewer spines than those of wild-type littermates (Fukuda et al., 2005). On the other hand, transient overexpression of wildtype MeCP2 in cultured cortical neurons led to a significant increase in dendritic and axonal length and arborization, while neurons overexpressing a nonfunctional, truncated form of MeCP2 (*MECP2*^{293}) only showed increases in dendritic and axonal branching (Jugloff et al., 2005). Quantitative analyses of dendritic morphology in neurons from layers III and V of the frontal cortex of *MECP2*^{308} mice, which express a truncated *MECP2* allele, reported no major abnormalities in dendritic branching (Moretti et al., 2006). Further analyses at the EM level in area CA1 of the hippocampus yielded no differences in the density of asymmetric synapses and in the number of synaptic vesicles, either docked at the active zone or in the main vesicle cluster within the terminals. However, the mean length of the PSD was smaller in *MECP2*^{308} mice, driven by a concomitant increase in the smaller PSDs and a decrease in the larger ones (>150 nm) (Moretti

et al., 2006). Since the size of the active zone is tightly correlated with the size of the PSD across the synaptic cleft (Harris and Sultan, 1995; Boyer et al., 1998; Schikorski and Stevens, 1999), the observation of smaller PSDs and similar numbers of docked vesicles in the *MECP2*^{308} mice suggests that the density of docked vesicles is increased in the mutant active zone. These apparent discrepancies clearly warrant further investigations of the role of wild-type and mutant MeCP2 on dendritic and synaptic morphology in specific brain regions as a potential underlying mechanism for the functional impairments observed in animal models and RTT patients.

The molecular pathways contributing to the pathogenesis of Rett syndrome remain unclear. Considering that the best-characterized function of MeCP2 is as transcriptional repressor, numerous gene expression profiles have been conducted on RTT patients and in the available mouse models of RTT (Colantuoni et al., 2001; Tudor et al., 2002; Ballestar et al., 2005; Nuber et al., 2005; Delgado et al., 2006). However, these studies identified several genes that are either upregulated or downregulated, but their contribution to the disease remains unknown. A more targeted approach, such as chromatin immunoprecipitation, has identified the promoter region of the *BDNF* gene as a target of MeCP2 transcriptional control (Chen et al., 2003; Martinowich et al., 2003). These studies demonstrated that MeCP2 binds to and represses the transcription of mouse *BDNF* promoter IV, equivalent to the rat *BDNF* III promoter, which is well known to be activated by neuronal activity and the ensuing voltage-gated Ca^{2+} influx (Tao et al., 1998). As expected, the levels of *BDNF* exon IV transcript were low in cultured cortical neurons from wildtype mice in the absence of neuronal activity (i.e., in the presence of tetrodotoxin, TTX). On the other hand, neurons from *MECP2* null mice showed a twofold increase in their basal levels of *BDNF* exon IV transcript, as predicted from its transcriptional repressor function. Furthermore, *BDNF* exon IV transcript levels induced by a strong depolarizing stimulus (i.e., KCl) were similar between control and mutant cells, due to the large increase observed in wild-type neurons and the already elevated levels in *MECP2* null neurons, which were unresponsive to KCl depolarization (Chen et al., 2003). Considering that BDNF is a positive modulator of excitatory synaptic function (Thoenen, 2000; Poo, 2001; Tyler et al., 2002a,b; Lu, 2003; Bramham and Messaoudi, 2005), the observation of elevated BDNF levels (at least in terms of its activity-dependent mRNA transcripts) was unexpected and somewhat puzzling. However, BDNF protein levels measured by enzyme-linked immunosorbent assay (ELISA) were found to be lower in brain samples of *MECP2* null mice at 6–8 weeks of age compared to wild-type controls, a difference not observed at 2 weeks (Chang et al., 2006). In addition, conditional deletion of the *BDNF* gene in *MECP2* null mice exacerbated the onset of the RTT-associated phenotypes of the *MECP2* null animals, which included hypoactivity in the running wheel and reduced action potential frequency in pyramidal neurons of acute cortical slices *in vitro*. Surprisingly, overexpression of *BDNF* slowed down the disease progression in *MECP2* null mice, with increased wheel running behavior and augmented action potential firing in cortical neurons (Chang et al., 2006). Since BDNF mRNA and protein levels are tightly regulated by neuronal activity, the reduced firing frequency of neurons from *MECP2* null mice (Dani et al., 2005) may cause the reduced BDNF protein levels measured by ELISA (Chang et al., 2006). The discrepancy between the elevated basal levels of *BDNF* exon IV transcripts in cultured *MECP2* neurons (Chen et al., 2003) and the reduced BDNF protein levels in *MECP2* brain tissue (Chang et al., 2006) likely originates from different basal conditions (TTX in culture vs. naive fresh brain samples), developmental age (embryonic vs. postnatal), or the modulation of mRNA translation into protein. Whether direct or indirect, the relationship between MeCP2 function and BDNF-mediated signaling seems potentially relevant to the speculated impairments in Rett syndrome, especially with regard to synaptic function (Dani et al., 2005; Asaka et al., 2006; Moretti et al., 2006; Nelson et al., 2006).

Unfortunately, the few available studies of neurotrophin levels in RTT patients have not yielded results consistent with the 'BDNF hypothesis of Rett,' at least at a first approximation. First, reduced NGF levels were observed in cerebrospinal fluid (CSF) of RTT patients, a difference not found in blood serum levels, while BDNF was unaffected in CSF or blood serum RTT samples (Lappalainen et al., 1996; Vanhala et al., 1998; Riikonen and Vanhala, 1999; Riikonen, 2003). Second, the expression levels of NGF and its receptor TrkA were reduced in postmortem RTT brains, as assessed by immunohistochemistry (Lipani et al., 2000), while the number of basal forebrain neurons expressing $p75^{NTR}$ was unaffected in RTT brains (Wenk and Hauss-Wegrzyniak, 1999). Third, NGF serum levels

tend to decrease with age in RTT patients, opposite to the characteristic developmental increase in healthy individuals (Calamandrei et al., 2001). Taken together, these few reports reveal our incomplete understanding of the potential and intriguing role of neurotrophin signaling in the pathogenesis of Rett syndrome. Furthermore, they underscore the need for further cellular and molecular studies, perhaps at the single-cell level, of the consequences of mutant MeCP2 expression on neurotrophin expression, targeting, and/or release, as well as signaling through its $p75^{NTR}$ and Trk receptors.

4.34.9 Final Considerations

The observations reviewed in this chapter provide strong evidence that dendritic spines are highly specialized neuronal compartments that are exquisitely tuned to sense ongoing and fluctuating levels of afferent synaptic activity, which likely play a fundamental role in synaptic integration and plasticity. However, most of the experimental evidence is so far correlative: for example, while LTP or behavioral learning cause structural changes, those changes may not contribute to the synaptic potentiation or the formation of the engram. On the other hand, the new spines formed after LTP induction may not initially participate in synaptic transmission, but rather may be the sites of future synaptic plasticity (i.e., NMDA-only silent synapses). In any case, dendritic spines are prime examples that function affects structure and vice versa in a global and ongoing homeostatic balance (i.e., negative feedback), which can be locally modified by rapid 'Hebbian' positive-feedback changes. After more than a century of their discovery and the speculation that 'mental exercise' may promote their formation and growth, dendritic spines still represent a truly fascinating 'thorny' issue in our quest for the neurobiological basis of learning and memory.

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4.35 Integrins and Cadherins – Extracellular Matrix in Memory Formation

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4.35.1 Introduction

Certain types of molecules and biochemical processes have surfaced recurrently as important for learning and memory. For instance, one theme that frequently emerges is that signal transduction through the second messenger cyclic AMP is particularly important for many forms of learning. The MAP kinase pathway is also frequently implicated along with a host of other cellular molecules to include other protein kinases, transcription factors, metabolic enzymes for neurotransmitter synthesis and degradation, phosphatases, and so on. The widespread involvement of many cellular signaling pathways and many different classes of molecules is due to the fact that learning occurs through broad changes in the overall physiology of the relevant neurons.

One area of the cell that must play a major role in the modulation of cell–cell communication underlying learning is the surface – the interface for junctional communication. It is thus reasonable to believe that major classes of cell surface proteins, including the cell adhesion receptors, might be critical for learning to occur. Cell adhesion receptors serve two broad roles. First, they provide for the adhesive interactions necessary to maintain stable contacts between a neuron and the extracellular matrix or with other cells through ligands expressed on these adjacent cells. Second, they mediate cellular signaling processes and in some cases do this bidirectionally – from inside to out and from outside to in. Furthermore, the major classes of cell adhesion receptors reviewed here, the integrins and the cadherins, signal to the actin cytoskeleton to mediate changes in neuron and synapse structure. Since neuron and synapse structure are intimately related to function, these important attributes of the integrin and cadherin class of cell adhesion receptors position these molecules to be of central importance to various forms of synaptic and behavioral plasticity.

4.35.2 Structure of Integrins

Integrins are glycosylated transmembrane heterodimers composed of noncovalently bound α and β subunits. To date, 18 α and 8 β subunits have been characterized in mammals that together form at least 24 integrin heterodimers. Both subunits have (1) a large extracellular domain (ectodomain) from 778 to 1104 amino acids in length that together associate to form a globular, ligand-binding site, (2) a single-pass transmembrane (TM) domain, and (3) a short cytoplasmic domain from 30 to 70 amino acids in length, with the exception of the β4 subunit, which has a cytoplasmic domain of 1088 amino acids. The

cytoplasmic domain lacks enzymatic activity and interacts with a large number of molecules involved in signal transduction and cytoskeletal association. About half of the α subunits contain within the N terminus an additional I-domain in which a metal ion is coordinated at the ligand-binding interface through a conserved five–amino acid motif, the metal ion-dependent adhesion site (MIDAS). Variant sequences in the extracellular and/or cytoplasmic domain for several α and β subunits produced by alternative splicing have been reported (reviewed in Sastry and Horwitz, 1993; van der Flier and Sonnenberg, 2001), thus further expanding the structural and functional complexity of the family.

Several major advances have been made over the last few years in understanding the structural changes that take place during integrin activation (also referred as priming, i.e., the changes that promote ligand binding), with $\alpha_{IIb}\beta_3$ and $\alpha V\beta_3$ being used as prototypical molecules for these studies (**Figure 1(a)**). Although some differences exist in the details, a general conclusion is that global conformational changes underlie integrin activation. Integrin activation via inside-out signaling is triggered by the binding of the cytoskeletal protein talin to the cytoplasmic tail. This binding appears to be a final common step of intracellular signaling cascades (Tadokoro et al., 2003; Calderwood et al., 2004). Recent evidence has suggested that the cytoplasmic domains of the α and β subunits are clasped close to each other through a weak interaction at the membrane-proximal region in the resting state (Hughes et al., 1996; Vinogradova et al., 2002; Kim et al., 2003). Binding of talin to the β subunit (Vinogradova et al., 2002; Garcia-Alvarez et al., 2003; Ulmer et al, 2003) may disrupt this interaction between the two cytoplasmic tails, leading to a spatial separation of the subunit tails and an overall conformational change in the heterodimer.

The inside-out activation signal generated through the conformational change in the cytoplasmic tails must propagate to the ectodomain via the TM domain. It is not surprising that the TM domain has also been found to be critical in the conformational change that converts an integrin heterodimer from a low to a high ligand binding affinity state. Using disulfide bond scanning of the exofacial portions of α_{IIb} and β_3 TM domains, Luo et al. (2004) showed that restraining the two TM domains in the specific heterodimeric helical interface locks integrins in a low-affinity state. Conversely, mutations that disrupt interactions in the helical interface lead to the disassociation of the interface, most likely through a partial lateral separation of the two subunits, and an increase in ligand binding affinity (Takagi et al., 2002a; Li et al., 2005; Luo et al., 2005; Partridge et al., 2005).

The three-dimensional structure of the integrin ectodomain in both the resting and active states was recently clarified through X-ray crystallography, nuclear magnetic resonance, and electron microscopy. The first breakthrough came in 2001 when M. Amin Arnaout and colleagues successfully determined the crystal structure of the extracellular segment of $\alpha V\beta 3$ (Xiong et al., 2001). The studies revealed a structure with 12 domains assembled into an oval head connected to two legs that fold severely backward at an approximately 135° angle. A seven-bladed β-propeller domain from αV and a MIDAS-containing domain (βA) from $\beta 3$ formed the putative ligand-binding head. Subsequent studies from these and other investigators showed that binding of the cyclic peptide containing an RGD sequence, a motif found in many extracellular matrix (ECM) proteins such as fibronectin, laminin, vitronectin, and tenascin, indeed occurred in the groove between the β-propeller and the βA domain, accompanied with changes in both the tertiary and quaternary structure of the integrin subunits (Takagi et al., 2002; Xiong et al., 2002). However, it remains unclear whether activation and ligand binding are accompanied with the extension of the bent conformation, as evidence that supports both an extended (Nermut et al., 1988; Beglova et al., 2002; Takagi et al., 2002) and nonextended (Calzada et al., 2002; Butta et al., 2003; Adair et al., 2005) ligand-bounded ectodomain exists.

4.35.3 Expression of Integrins in the Adult Brain

The diversity of the integrin family makes it necessary to examine the regional, cellular, and subcellular expression patterns of individual members in order to understand how integrins are involved in various processes in the adult central nervous system. Using RNA *in situ* hybridization to adult rat brain sections, Pinkstaff et al. (1998, 1999) provided a comprehensive description of the regional and cellular localization of gene expression for nine α ($\alpha 1$–$\alpha 8$, αV) and five β ($\beta 1$–$\beta 5$) integrin subunits. A substantial amount of valuable information on the RNA expression in the brain can also be found from a number of online resources

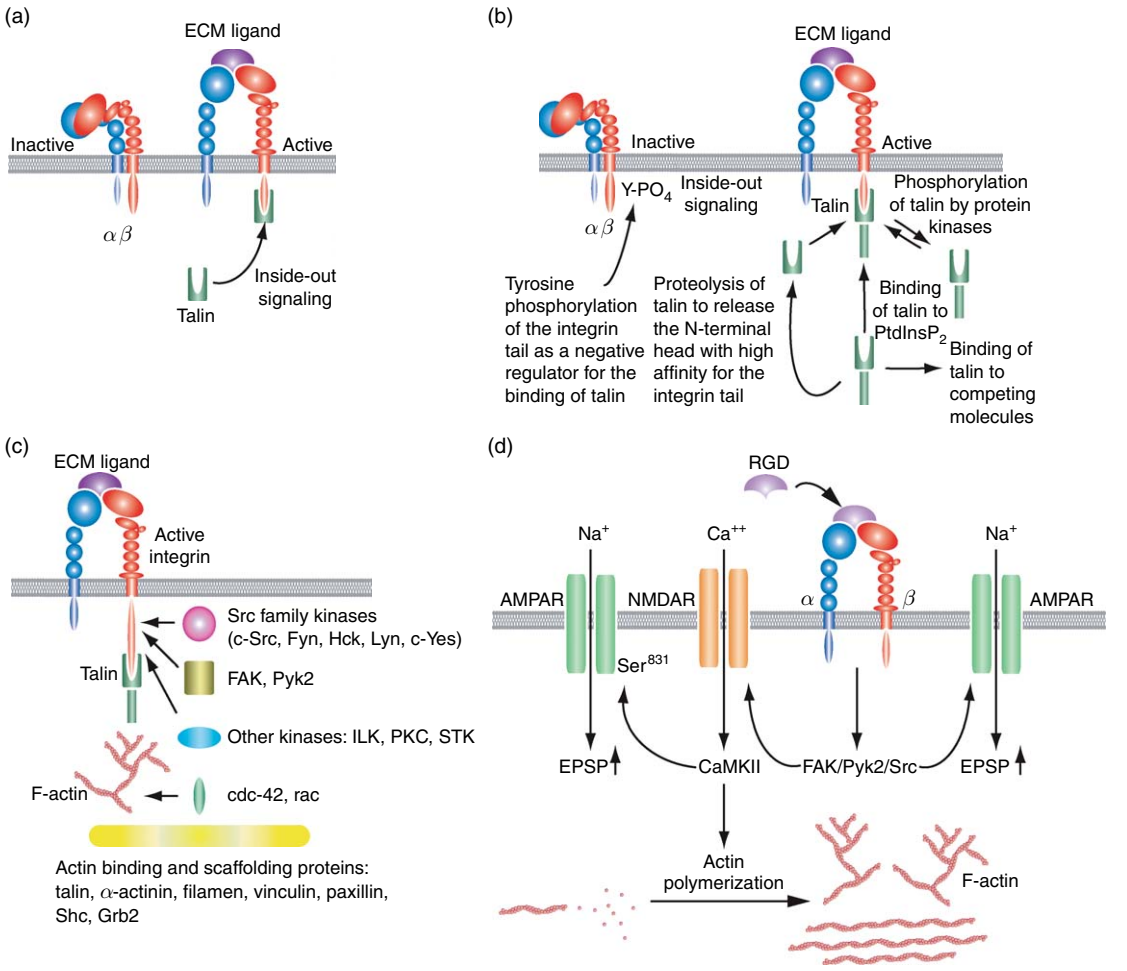


Figure 1 Integrin activation and signaling. (a) Inside-out signaling. In the resting state, the cytoplasmic domains and the transmembrane domains of the α and β subunits remain in close proximity to each other through weak interactions. Binding of talin to the cytoplasmic tail of the β subunit triggers separation of the two subunits, which leads to conformational changes in the extracellular domain and subsequently a higher affinity for the ligand. (b) Schematic diagram of some of the endpoints of cellular signaling that could lead to integrin activation through inside-out mechanisms. Tyrosine phosphorylation of integrin subunits alters the capacity to bind talin. Processing of talin by proteolysis creates a head domain of talin that has higher affinity to the β-integrin tail. Binding of $PtdInP_2$ by talin increases its affinity for β-integrin tails. Integrins compete for talin binding with other cellular molecules that have affinity for talin. Phosphorylation of talin may influence its ability to bind integrin tails. (c) Biochemical signaling pathways that are activated after ligand binding to integrins. Various members of the Src family of protein kinases are activated by integrin signaling. Members of the focal adhesion family of protein kinases and other types of protein kinases are activated after ligand binding to integrins. Small GTPases cdc42 and rac influence the actin cytoskeleton and are activated via integrin signaling. Other actin binding and scaffolding proteins are attached to focal complexes and participate in integrin signaling. (d) Schematic diagram of possible signaling cascades in integrin-induced potentiation of synaptic currents. Binding of RGD-containing peptides results in the activation of the Src and FAK/Pyk2 kinase pathways, which in turn promotes the phosphorylation of *N*-methyl-D-aspartate (NMDA) receptor subunits and the concomitant increase in the Ca^{++} influx. Activation of CaMKII caused by the increase in Ca^{++} leads to the phosphorylation of AMPA receptor subunits and the subsequent increase in synaptic currents. Activation of Src family kinases by integrins also leads to the increase in synaptic currents through a mechanism that is independent of CaMKII Thr^{286} and GluR1 Ser^{831} phosphorylation. Activation of CaMKII also induces structural reorganization at the activated synapses through actin polymerization and the formation of F-actin.

(St. Jude's Children's Research Hospital, 2004; Allen Institute for Brain Science, 2007; Max-Planck-Institute for Biophysical Chemistry, 2007). With the exception of $\alpha 2$, $\beta 2$, and $\beta 3$, RNA transcripts for all the surveyed integrin subunits are expressed in multiple brain regions. Extensive

overlapping patterns of expression were observed for a number of subunits. For example, $\alpha 3$, $\alpha 5$, $\alpha 7$, αV, and $\beta 1$ were detected in all of the neocortical layers, and $\alpha 1$, $\alpha 3$, $\alpha 5$, $\alpha 8$, and $\beta 1$ were detected in both the hippocampal CA1 and CA3 subfields. Since different integrin receptors can also overlap for their ligands and intracellular signaling pathways, neurons that express multiple integrins can potentially be stimulated by combinations of ligands and respond with the mobilization of multiple signaling pathways. The overlapping expression patterns, on the other hand, can provide a compensatory mechanism when one of the integrin receptors is missing. This potential redundancy needs to be accounted for when considering phenotypes from the integrin mutants.

Immunohistochemical studies, although available for only a limited number of integrin subunits, provide valuable additional information on the cellular localization of the receptors. At the level of light microscopy, immunoreactivity for $\alpha 1$, $\alpha 3$, $\alpha 5$, $\alpha 8$, $\beta 1$, and $\beta 8$ is generally observed broadly across the adult mammalian brains (Grooms et al., 1993; Einheber et al., 1996; Murase and Hayashi, 1998; Nishimura et al., 1998; Rodriguez et al., 2000; Bi et al., 2001). However, there are discrepancies between the reported mRNA and protein expression patterns from different studies for certain integrin subunits, probably due to technical limitations in the sensitivity of the probes used for RNA *in situ* hybridization experiments or the specificity of antibodies used for immunochemistry. For instance, although mRNA was undetectable in the cortical layers for $\alpha 1$ (except layer 5) and $\alpha 8$ (except layer 6) (Pinkstaff et al., 1999), both $\alpha 1$ and $\alpha 8$ immunoreactivity were observed in neurons throughout cortical layers 2–6 (Einheber et al., 1996; Murase and Hayashi, 1998). Some studies have also noted cellular compartmentalization of integrin immunoreactivity. Strong $\alpha 5$ immunoreactivity was observed within the primary apical dendrites of hippocampal CA3 pyramidal cells, with no detectable immunoreactivity in the basal dendrites (Bi et al., 2001), and $\alpha 8$ immunoreactivity has been reported as intensely punctate in the proximal portions of the apical dendrites (Einheber et al., 1996). Such compartmentalization and nonuniform distribution of immunoreactivity suggests a role of integrin receptors in the regional and/or synaptic specificity of neuronal properties such as synaptic plasticity.

In support of the possibility that integrins are involved in synaptic functions, biochemical and ultrastructural studies have demonstrated the presence of integrin receptors at the synaptic contacts. Integrins subunits $\alpha 3$, $\alpha 5$, αV, $\beta 1$, and $\beta 8$ have been localized in the synaptosomes isolated from hippocampus and cortex (Nishimura et al., 1998; Kramar et al., 2002; Chan et al., 2003). From immunoelectron microscopy experiments, strong immunoreactivity for $\alpha 3$ (Rodriguez et al., 2000), $\alpha 8$ (Einheber et al., 1996), $\beta 1$ (Schuster et al., 2001), and $\beta 8$ (Nishimura et al., 1998) has been detected in subsets of dendritic spines, most often close to or coincident with the postsynaptic density. In contrast, the examined presynaptic terminals were rarely labeled, and when they were, the immunoreactivity was weak. Interestingly, both immunopositive and -negative synapses can be detected from the same dendrite, and they can sometimes be found adjacent to each other, even making contacts with the same axonal terminal (Einheber et al., 1996; Schuster et al., 2001). The mechanism by which integrin receptors are precisely targeted to the specific synapses is unknown. Nor is it known whether multiple integrin receptors colocalize at the same synapse. Understanding these issues is critical for providing insights about the functional roles of integrins in the adult central nervous system.

4.35.4 Biochemical Signaling through Integrins

The accumulated evidence consistent with the idea that integrin activation is mediated through the binding of talin to the cytoplasmic tail of β-integrin has been a major step forward in conceptualizing the integrin-proximal steps involved in integrin signaling (Campbell and Ginsberg, 2004; Ginsberg et al., 2005). Talin is an integrin and actin-binding protein that colocalizes with integrins at sites of cell-substratum contact. Overexpression of the N terminus of talin activates integrins (Calderwood et al., 1999), and knockdown of talin expression blocks integrin activation (Tadokoro et al., 2003). Thus, the critical processes that lead to the activation of integrins via intracellular signaling potentially include the abundance of talin, its subcellular distribution, and the molecular modifications that might enhance or weaken its affinity for β-integrin tails.

The signaling pathways in neurons for which talin is a downstream target are largely unknown. However, there are several potential mechanisms (Cambell and

Ginsberg, 2004) that neurons could employ to alter talin binding to integrins (**Figure 1(b)**):

1. Modification of integrin tails by tyrosine phosphorylation inhibits the binding of talin in some cell types, providing a negative regulator for integrin activation.
2. The head domain of talin, which includes the integrin binding site, has a higher affinity for integrins than the complete molecule. The proteolytic cleavage of talin therefore offers a regulatory mechanism for integrin activation through talin binding.
3. The binding of phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$) to talin induces a conformational change that enhances talin's affinity for integrins. Cellular signaling processes that lead to an accumulation of $PtdInsP_2$ could therefore cause integrin activation.
4. Talin is known to bind other molecules besides integrins in the cell, such as the PtdInsP2 biosynthetic enzyme, phosphatidyl-(4)-phosphate 5-kinase type Iγ. Negative regulation of integrin activation could be effected by promoting talin's interaction with the cellular competitors for integrin binding (Calderwood et al., 2004).
5. Talin is the target for multiple protein kinases, likely including protein kinase C, protein kinase G, Akt, and the MAP Kinases. These phosphorylation events may have some role in regulating the binding of talin to integrins.

The activation of integrins is not a slow process but occurs within seconds and is transient, at least as revealed by cell-based adhesion assays using cells other than neurons (Constantin et al., 2000; Arnaout et al., 2005). The rapidity of these responses suggests that the adhesive-deadhesive responses of cellular subdomains, such as synapses, could occur within time frames of less than 1 s. Similarly, the formation of focal adhesion complexes containing integrins at the leading edge of motile cells grows to a size that can be detected by immunofluorescence in less than 1 min (Zaidel-Bar et al., 2004). Thus, many integrin-based signaling processes must be engaged by the time that these assemblies are visibly detectable.

These observations indicate that integrins can hypothetically be involved in processes that mediate short-term (seconds to minutes) neuronal processes as well as medium-term (minutes to hours) and long-term (hours to days) processes that may ensue.

The conformational change in integrin structure induced by the binding of talin alters the affinity of integrins for extracellular ligands. The ligands that interact with integrins are numerous and diverse (Plow et al., 2000). In general, ligands that participate in normal physiological responses fall into two classes: (1) ligands in the extracellular matrix that provide a basis for the integrins in adhesive functions to extracellular support scaffolds and (2) counter-receptors that are displayed for cell–cell interactions. Some of the better-characterized integrin ligands include collagen, fibrinogen, matrix metalloprotease-2, laminin, vitronectin, and thrombospondin (Plow et al., 2000; van der Flier and Sonnenberg, 2001). Each individual integrin heterodimer has the potential to interact with numerous ligands, and each ligand can interact with several different integrin heterodimers.

All of the integrin ligands listed above characterized from studies using tissues other than from the central nervous system have been reported to be expressed in neurons and/or glia. However, their specific central nervous system functions generally remain unexplored. Some surprising molecules that function as integrin ligands have been recently reported to be expressed in the central nervous system. Pasterkamp et al. (2003) reported that the molecule semaphorin 7A, a member of the semaphorin family of axon-repulsive guidance molecules, enhances axon outgrowth, and that this activity is dependent on β1-integrin receptors. Similarly, Dulabon et al. (2000) demonstrated that reelin, a large glycoprotein secreted principally by Cajal-Retzius cells in the marginal zone of the developing cerebral cortex, interacts with $\alpha 3\beta 1$ integrins to inhibit cortical neuron migration in the process of forming a normally layered cerebral cortex. Thus, semaphorin 7A and reelin, which have been shown to be expressed in the adult brain, must be added to the list of putative integrin receptors in processes underlying normal memory formation.

The binding of talin activates integrins, and activated integrins then bind to their endogenous ligands. Ligand binding to integrins then triggers the formation of different types of integrin signaling assemblies and diverse cell signaling events (Zaidel-Bar et al., 2004). These assemblies are likely to include dozens and perhaps hundreds of integrin and associated signaling molecules (Liu et al., 2000; Ginsberg et al., 2005) by the time that they can be visualized by immunofluorescence. This complexity has kept the details of such signaling complexes a secret. However, a few generalities have emerged regarding the major intermediates of integrin

signaling. Some of these are potentially relevant to memory formation.

One major theme that has emerged is that many different protein kinases are activated by binding directly or indirectly to integrins, providing a plausible mechanism for initiating intracellular signaling processes (**Figure 1(c)**). For instance, the Src family of protein kinases is frequently implicated in integrin signaling and are known to be required for integrin-mediated cellular responses (Ginsberg et al., 2005). The enzymes c-Src and Fyn bind directly to the tail of β3-integrin through a domain that is separate from the talin-binding region (Arias-Salgado et al., 2005), and other Src kinases including Hck, Lyn, and c-Yes bind to the tails of β1- and β2-integrins (Arias-Salgado et al., 2003). Thus, the activation of Src kinases by binding to the cytoplasmic domains of β-integrins appears to be an early and proximal event for integrin signaling.

Focal adhesion kinase (FAK) and proline-rich tyrosine kinase 2 (Pyk2) are the sole members of the FAK family of nonreceptor protein tyrosine kinases (Parsons, 2003). FAK colocalizes with integrins at cell-substratum contact sites and is thought to bind directly to β-integrin tails through sequences located at the N terminus of the kinase, although sequences at the C terminus known as the focal adhesion targeting (FAT) domain are necessary and sufficient for targeting the molecule to focal adhesions. Pyk2 is also a downstream effector of integrins with similar association and targeting properties (Litvak et al., 2000; Butler and Blystone, 2005).

The phosphorylation state and tyrosine kinase activity of FAK are regulated by integrin–ligand association, with the phosphorylation at Tyr-397 correlating with increased catalytic activity of FAK. This phosphorylation creates a high-affinity binding site for the SH2 domain of the Src family kinases and other enzymes such as phospholipase C-γ (PLC-γ) and phosphoinositide 3-kinase (Parsons, 2003).

Other kinases that potentially bind directly to the cytoplasmic tails of β-integrins include integrin-linked kinase (ILK), protein kinase C, and spleen tyrosine kinase (Syk) (Liu et al., 2000; Tadokoro et al., 2003; Ginsberg et al., 2005). In addition, integrins bind to numerous other proteins, some of which provide a platform for other signaling molecules. Four actin-binding proteins are among the best-characterized proteins that bind to β-integrin tails: talin, α-actinin, vinculin, and filamen (Liu et al., 2000; Hynes, 2002; Critchley, 2004). Some of the molecules that participate in integrin signaling complexes are illustrated in **Figure 1(c)**, although the specifics of many of the various molecular interactions are not yet known. However, some studies have revealed that antagonistic interactions occur: the binding site on the cytoplasmic tail of β-integrin overlaps with the binding site for talin, such that these proteins compete for binding to integrin tails (Kiema et al., 2006). Other interactions are likely to be cooperative. More complete lists of the molecules engaged in integrin signaling complexes can be found in many reviews on integrin signaling (Liu et al., 2000; van der Flier and Sonnenberg, 2001; Hynes, 2002)

The actin cytoskeleton is known to be one of the most important cellular targets of integrin signaling (**Figure 1(c)**; Wiesner et al., 2005). But how do the ligand-bound integrins signal for cytoskeletal rearrangements? As already mentioned, talin contains binding sites for the cytoplasmic tail of β-integrin as well as actin binding sites. Thus, the mere binding of talin and associated conformation changes is sufficient for the establishment of connections to and/or reorganization of the actin scaffold. Subsequent binding of talin to another actin-binding protein, vinculin, could establish secondary crosslinks to the actin cytoskeleton. In addition, some small GTPase molecules, such as rac and cdc-42, are activated by tyrosine phosphorylation by the src-family of protein tyrosine kinases. These GTPases are linked to the early phases of cell protrusion during cell migration and the remodeling of the actin cytoskeleton through actin-nucleating factors like Arp2/3 (Wiesner et al., 2006). Thus, these same molecules may therefore be involved in filopodia outgrowth and perhaps rapid modifications of synapse structure (DeMali et al., 2003).

4.35.5 Integrin-Dependent Synaptic Transmission and Plasticity

The possibility that integrins are involved in synaptic physiology was first reported 15 years ago when Xiao et al. (1991) observed that incubating hippocampal slices with RGD-containing peptides produced a reversible, dose-dependent decay of long-term potentiation (LTP) after stimulation of the Shaffer collaterals in the CA1 area over a period of 40 min but had no effect 1–2 min after the LTP-inducing high-frequency stimulation. This suggested that integrins are required for the stabilization rather than the induction of LTP. This idea was further substantiated in later studies showing that application of the RGD-containing peptides 10 min before to

10 min after LTP induction, but not 25 min or more after, caused a steady decay of the potentiation, suggesting that integrin activation and signaling occurring over several minutes after LTP induction was necessary for the stabilization (Stäubli et al., 1998). In addition to RGD peptides, function blocking antibodies against $\alpha 3$ and $\alpha 5$, but not αV and $\alpha 2$, as well as disintegrins, which are small, high-binding-affinity, RGD-containing integrin inhibitors found in various snake venoms, were also shown to block the stabilization of LTP in hippocampal slices (Chun et al., 2001; Kramár et al., 2002). More recently, Chan et al. (2003) have used a genetic approach as a more direct way of identifying and examining the functional roles of integrin subunits in synaptic plasticity. Consistent with the previous studies, animals heterozygous for an $\alpha 3$ null mutation exhibited a more rapidly decaying LTP compared to their wild-type littermates. The magnitude of LTP induced in the heterozygotes was not significantly different from control animals immediately after the tetanus, but it decayed rapidly so that a significant difference in magnitude was detected at 40 min. Surprisingly, when combined with the heterozygous $\alpha 8$ mutation, the double heterozygote $\alpha 3/+$; $\alpha 8/+$ produced a more severe deficiency immediately after LTP induction that has not been detected in previous studies using integrin antagonists. When a heterozygous $\alpha 5$ mutation was added to the double mutant, the triple heterozygotes showed a further reduction in the magnitude of potentiation. Thus, results obtained with mutants confirm the primary role of $\alpha 3$ in promoting the stability of LTP. They further suggest some redundancy and a role for $\alpha 8$ for the induction or stability of LTP within seconds or minutes after tetanus when $\alpha 3$ also is limiting, and for $\alpha 5$ on the magnitude of LTP that can be generated when both $\alpha 3$ and $\alpha 8$ are limiting. Furthermore, these results also suggest a different temporal requirement for integrins at different stages of LTP, with $\alpha 8$ more likely acting during and minutes after LTP induction and $\alpha 5$ during stabilization of LTP. A similar effect on LTP like that produced by the triple heterozygote $\alpha 3/+$; $\alpha 5/+$; $\alpha 8/+$ was obtained using conditional forebrain-specific knockouts of $\beta 1$-integrin, further confirming the adult physiological role of integrins in modulating LTP (Chan et al., 2006). In addition to LTP in the hippocampus, evidence indicating a function for integrins in other forms of synaptic plasticity has been obtained from studies focusing on different brain regions and from different animal models. In the cerebellum, a distinct type of long-lasting potentiation, named rebound potentiation (RP), is induced by postsynaptic depolarization at the GABAergic synapses between inhibitory interneurons and a Purkinje neuron (Kano et al., 1992). It was shown that activation of integrins by extracellular Mn^{2+} suppressed RP induction, and such suppression could be blocked by function blocking antibodies against the $\alpha 3$ or $\beta 1$ subunit. Overexpression of an $\alpha 3$ subunit in the Purkinje neurons also impaired RP induction (Kawaguchi and Hirano, 2006). These results thus suggest a critical role of $\alpha 3\beta 1$ integrin in negatively regulating the long-term plasticity at inhibitory synapses on cerebellar Purkinje neurons. In *Drosophila*, mutations in *volado*, the gene encoding the αPS3-integrin, result in significant defects in two forms of Ca^{2+}-dependent short-term facilitation, paired-pulse facilitation and frequency-dependent short-term facilitation, as well as in impaired posttetanic potentiation at the neuromuscular junction (Rohrbough et al., 2000).

How do integrins modulate synaptic plasticity? Given the properties of integrins and their ability to interact with a large number of molecules on both sides of the synaptic membrane, it is conceivable that they could modulate the adhesiveness of the cell membrane to the membrane of the synaptic partner, thus altering the physical structure of the synapse. An alternative is that they may modulate the properties of ion channels through intracellular signaling. Evidence supporting the latter possibility has been reported recently in which infusions of the RGD-containing peptides to hippocampal slices caused an approximately twofold increase in the amplitude and duration of *N*-methyl-D-aspartate (NMDA) receptor-mediated synaptic currents that was blocked by Src kinase inhibitor PP2 or function blocking anti-$\beta 1$ integrin antibodies (Lin et al., 2003; Bernard-Trifilo et al., 2005). Remarkably, a corresponding increase in the tyrosine phosphorylation of NMDA receptor subunits NR2A and NR2B was also observed in the treated hippocampal slices after the physiological testing (Bernard-Trifilo et al., 2005). When kinase activity was examined in the treated synaptoneurosomes isolated from adult rat forebrain, it was found that both the RGD-containing peptide and fibronectin induced a $\beta 1$ integrin-dependent, rapid increase in tyrosine phosphorylation of FAK, Pyk2, and other Src family kinases (Bernard-Trifilo et al., 2005). In addition to its influence in NMDA receptor-mediated currents, the RGD-containing peptide was also found to enhance the slope and amplitude of the excitatory postsynaptic responses through changes in

alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type glutamate receptors, and such enhancement was found to be blocked by a mixture of function-blocking antibodies against integrin $\alpha 3$, $\alpha 5$, and αV subunits; the NMDA receptor antagonist aminophosphonovaleric (APV); the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) inhibitor KN-93 and, similar to that of NMDA receptor–mediated currents, by Src tyrosine kinase inhibitor PP2 (Kramár et al., 2003). Interestingly, the RGD-containing peptide also induced an increase in phosphorylation at CaMKII autophosphorylation site Thr^{286} and at GluR1 Ser^{831}, and both of these increases were markedly attenuated by APV but not by PP2, suggesting that the effect on RGD-induced AMPA-type currents by NMDA receptor is probably through these two phosphorylation events, whereas the effect by Src family kinases is through either other activation sites on these two molecules or other pathways. Chan et al. (2006) have also recently demonstrated that forebrain conditional $\beta 1$-integrin knockouts have impaired basal excitatory synaptic transmission through AMPA receptors at the same Shaffer collateral pathway. Taken together, a model emerges from these studies in which binding of some $\beta 1$ integrins results in the activation of the Src/FAK/Pyk2 kinase pathway, which in turn promotes the phosphorylation of NMDA receptor subunits and the concomitant increase in the Ca^{2+} influx (**Figure 1(d)**). Activation of CaMKII caused by the increase in Ca^{2+} concentration leads to the phosphorylation of AMPA receptor subunits and the subsequent increase in synaptic currents. Activation of Src family kinases by integrins also leads to the increase in synaptic currents through a mechanism that is independent of CaMKII Thr^{286} and GluR1 Ser^{831} phosphorylation. At the *Drosophila* larval neuromuscular junction (NMJ), integrins have also been shown to be involved in synaptic transmission. At reduced external Ca^{2+} concentration, Volado mutants showed a highly significant elevation in excitatory junctional currents (EJC) amplitude over the control animals (Rohrbough et al., 2000). Interestingly, since the mutant NJMs exhibit no changes in the postsynaptic miniature EJC amplitude or frequency sufficient to account for the large difference in the evoked EJC amplitude under the same recording conditions, it was concluded that Volado was involved specifically in the regulation of Ca^{2+}-dependent, evoked neurotransmitter release processes.

It has been well established that one of the key targets of the integrin-mediated signaling pathways is the actin cytoskeleton (Matus, 2000). This is important because it is widely believed that structural changes in synaptic morphology are necessary for memory (Lamprecht and LeDoux, 2004; Maviel et al., 2004) and synaptic plasticity. In neurons, actin filaments are highly concentrated in dendritic spines. Besides providing structural integrity, recent studies have shown that actin cytoskeleton is highly dynamic and its reorganization is involved in regulating the synaptic plasticity of the cells. For instance, it has been shown that stimuli that induce LTP spur actin polymerization resulting in a rapid and persistent increase in F-actin content within the dendritic spines (Fukazawa et al., 2003; Lin et al., 2005; Kramár et al., 2006), whereas manipulations that block the induction of LTP or reverse LTP within minutes after it is induced disrupt the increase (Lin et al., 2005; Kramár et al., 2006). Furthermore, inhibition of actin filament assembly in hippocampus by various agents such as latrunculin and cytochalasin impairs both HFS- and TBS-induced LTP, especially during consolidation at the late phase (Kim and Lisman, 1999; Krucker et al., 2000; Fukazawa et al., 2003). These effects on LTP produced by blocking actin cytoskeleton assembly are reminiscent of the effects produced by inhibiting integrin function, and together with the fact that there is a close interaction between integrins and the actin cytoskeleton, provide another possible mechanism by which integrins modulate LTP. Two recent studies support this hypothesis. Shi and Ethell (2006) have shown that treatment of cultured hippocampal neurons with RGD-containing peptides induced the elongation of existing dendritic spines and promoted the formation of new filopodia. In addition, immunofluorescent labeling of F-actin showed that these effects were accompanied by actin reorganization and synapse remodeling and were mediated by integrins, since they were partially blocked by function-blocking antibodies against $\beta 1$ and $\beta 3$ integrins. Furthermore, this RGD-induced actin reassembly was suppressed by the NMDA receptor antagonist MK801 and CaMKII inhibitor KN93 as in the integrin-mediated enhancement of AMPA-type currents described above, thus suggesting that integrins confer structural plasticity of spines through NMDA receptor- and CaMKII-dependent actin reorganization. Kramár et al. (2006) have shown that the actin reassembly caused by the LTP-inducing stimulation is blocked by function-blocking antibodies against $\beta 1$

integrins when they were applied shortly after the stimulation. Therefore, both the reorganization of the actin cytoskeleton and the expression of LTP require the functions of β1 (and possibly β3) integrins. It is unknown but particularly interesting how actin reassembly is mediated through NMDA receptor/CaMKII signaling, given that integrins are already known to physically link to the actin cytoskeleton through direct and indirect interactions with a large number of proteins that bind to the actin cytoskeleton.

However, there is no reason to exclude the possibility that shorter-lasting forms of memory may also employ actin-based structural plasticity of the synapse as a mechanism, albeit with changes that may be more modest than those observed after long-term memory. Indeed, changes in dendritic spine morphology have been observed in electron micrographs as early as 2 min after the induction of LTP (Lamprecht and LeDoux, 2004). Recent imaging studies of spine volume after repetitive or LTP-inducing stimulation revealed both rapid (Lang et al., 2004; Matsuzaki et al., 2004) and long-lasting spine enlargement (Matsuzaki et al., 2004), with increases in spine volume being detectable in less than 1 min after stimulation. Increases in spine volume were noticeable in both small and large spines, but persistent long-lasting changes in volume were detectable primarily in spines that were small prior to stimulation. Taken together, these studies indicate that integrin receptors can modulate neuronal synaptic plasticity through changes in the spine actin cytoskeleton and may provide a functional link between short- and long-term synaptic structural plasticity.

4.35.6 Integrins and Learning and Memory

The relatively large body of evidence showing that integrins are involved in the modulation of synaptic physiology and plasticity strongly suggests that they may function in memory formation and behavior. Indeed, the first direct evidence of integrin function in memory formation was obtained from the study of *Volado* mutants in *Drosophila* in which the mutants displayed impaired olfactory memories within 3 min of training, which could be rescued by conditional expression of a *Volado* transgene during adulthood, thus indicating that the integrin is required for the physiological processes underlying short-term memory formation (Grotewiel et al., 1998). The behavioral effect on short-term memory, although observed and reproduced in another laboratory (Tamura et al., 2003), has been difficult to observe routinely and reproducibly compared to other authentic learning mutants, suggesting that there are unknown and uncontrolled environmental factors that influence behavioral performance of the mutants. In vertebrates, mice with reduced expression of α3, α5, and α8 integrin subunits are defective in the hippocampus-dependent spatial reference memory in the Morris water maze but have normal fear conditioning (Chan et al., 2003). Furthermore, the forebrain and excitatory neuron-specific knockout of β1 integrin are impaired in a hippocampal-dependent, nonmatching-to-place T-maze working memory task (Chan et al., 2006). Additional support for integrin function in memory formation is provided indirectly from studies on integrin-associated protein (IAP). IAP is a member of the immunoglobulin (Ig) superfamily that has been shown to be physically and functionally associated with the several integrins, though they are also known to bind ligands other than integrins (Brown and Frazier, 2001). A reduction or elimination of IAP activity by injection of antisense oligonucleotides complementary to the IAP transcript, by gene-targeting or by function-blocking antibodies against IAP, significantly impaired memory retention in a one-way inhibitory avoidance paradigm using rats and also reduced the amplitude and slope of excitatory postsynaptic potential (EPSP) in hippocampal LTP (Huang et al., 1998; Chang et al., 1999, 2001).

4.35.7 Structure of Cadherins

A second major family of cell adhesion receptors that has been shown to be essential for synaptic function is the cadherins. Cadherins comprise a large superfamily of single-pass transmembrane glycoproteins that mediate the Ca^{2+}-dependent, adherin-based cell–cell interactions. In mammals, at least 80 members, approximately 20 of which belong to the classic cadherin subfamily and the remainder collectively belong to the protocadherin family (protocadherins and cadherins-related neuronal receptors), have been found in the nervous system with different adhesive and signaling properties, making them one of the most molecularly diversified groups of receptors found in the CNS (reviewed in Redies, 2000; Yagi and Takeichi, 2000). Owing to the complexity of the family, we focus our discussions on the classic

cadherins since they have been most extensively studied and should provide a general model of how cadherins function in synaptic physiology and plasticity, memory formation, and behavioral plasticity.

The structure of classic cadherins consists of an extracellular region with the five characteristic and tandemly arranged so-called EC domains (EC1–EC5) of approximately 110 amino acids, each with internal sequence homology, a transmembrane domain, and a highly conserved cytoplasmic domain that interacts with the actin cytoskeleton and mediates signaling through binding of catenins. Details of the three-dimensional structure of cadherins and their interactions have been elucidated through crystallographic and biochemical studies (Shapiro et al., 1995; Nagar et al., 1996; Tamura et al., 1998; Pertz et al., 1999; Tanaka et al., 2000; Boggon et al., 2002). In brief, the current view is that binding of Ca^{2+} to the binding pockets located between the EC domains stabilizes and rigidifies the elongated rod-like structure (**Figure 2(a)**). Monomeric cadherin molecules within the same membrane form *cis*-interaction pairs (strand-dimer) via lateral dimerization and clustering. Strong intercellular adhesion is achieved when the membrane-distal EC1 domain from the strand-dimers of one cell interact with the EC1 domain from the strand-dimers emanating from the opposite cell in the *trans* configuration, forming a zipper-like structure. However, in contrast to the integrins, it is unknown whether intracellular signaling can affect the adhesive properties of the molecules and the conformational coupling among the cytoplasmic, transmembrane, and extracellular domains.

Protocadherins were originally identified by PCR using degenerate primers for EC domains of classic cadherins to identify additional members of the cadherins family (Sano et al., 1993). Many more members of the protocadherin family have since been identified based on their similarity in the extracellular and transmembrane domains (Kohmura et al., 1998; Wu and Maniatis, 1999). One of the remarkable features of this family is its molecular diversity. In contrast to classic cadherins, protocadherins contain varying numbers of EC domains in their extracellular domain and vastly divergent cytoplasmic domains that suggest different intracellular interacting partners and functions, whereas CNR cadherins have six EC domains and a distinct cytoplasmic domain that interacts with Fyn. As with the classic cadherins, the EC domains in protocadherins have also been shown or implicated to be able to undergo homophilic interactions (Sano et al., 1993; Yamagata et al., 1999).

4.35.8 Expression of Cadherins in the Adult Brain

Owing to the diversity of the family, it is not surprising that the expression patterns in the adult brain for most of the cadherin family members have not been characterized. However, based on the more than 30 cadherins that have been studied in detail, it has become clear that most cadherins are expressed in a restricted and differential manner.

More importantly, a number of cadherins and their catenin-binding partners have been found by various approaches to be localized at the synaptic junctions as an integral part of the synaptic complex. For example, immunoprecipitation studies have identified N-cadherin as a major component of the rat forebrain postsynaptic density (PSD) preparations (Beesley et al., 1995). EM and confocal studies have revealed the synaptic presence of N-cadherin in the chick optic tectum (Yamagata et al., 1995; Uchida et al., 1996) and the hippocampus and cerebellum (Fannon and Colman, 1996; Benson and Tanaka, 1998; Bozdagi et al., 2000), cadherin-11 in the hippocampus (Manabe et al., 2000); CNR1 protein in the neocortex (Kohmura et al., 1998), and R-cadherin and T-cadherin in the developing chick retina (Miskevich et al., 1998). Several remarkable features, which may be generally true for other neural classic cadherins, have emerged from these studies:

1. The cadherin/catenin complex is localized at both the pre- and postsynaptic membranes of the same synaptic clefts, which is consistent with and reflective of their homophilic binding properties.
2. The cadherin/catenin complex is found adjacent to and often partially surrounding the active zones on the presynaptic membrane. The functional significance of this spatial localization appears to be related to synaptic vesicle trafficking and localization. By deleting β-catenin in hippocampal pyramidal neurons, Bamji et al. (2003) have shown that the β-catenin mutant brains have a selective reduction of undocked vesicles by 40% per synapse, with no detectable change for docked vesicles, suggesting that β-catenin is important for localizing the reserved pool of synaptic vesicles. Bamji et al. (2006) have further shown that

dissociation of the cadherin-β-catenin adhesion complex is required for the BDNF-mediated mobilization of synaptic vesicles at the synapses.

3. Different cadherins are localized at functionally distinct types of synapses. For example, Benson and Tanaka (1998) have demonstrated that N-cadherin is concentrated only at the PSD-95-positive excitatory synaptic sites in mature hippocampal neurons in culture or *in vivo* and is absent from glutamic acid decarboxylase (GAD)-positive inhibitory synaptic sites.

4. Each synapse appears to express only one member of the cadherin family, although multiple members can exist in the same neurons. For example, although the expression of both N-cadherin and E-cadherin are widespread and closely coincident, their expression patterns do not overlap but instead are distinct and mutually exclusive between synapses of the same cell (Fannon and Colman, 1996).

5. The expression domains of α- and β-catenin are broader than that of individual cadherins. For example, while N-cadherin is present only at the excitatory synapses in the mature hippocampal neurons, β-catenin is localized to both the excitatory and inhibitory synapses, suggesting that other cadherin proteins are expressed in the inhibitory synaptic sites (Benson and Tanaka, 1998).

Together, these characteristic features of cadherins support the general hypothesis that the cadherin/catenin complex could be involved not only in providing synaptic adhesion but also in mediating synapse target specificity, formation and/or stabilization of synapses during early brain development, and conceivably, some of the synaptic changes that occur in the adult due to activity.

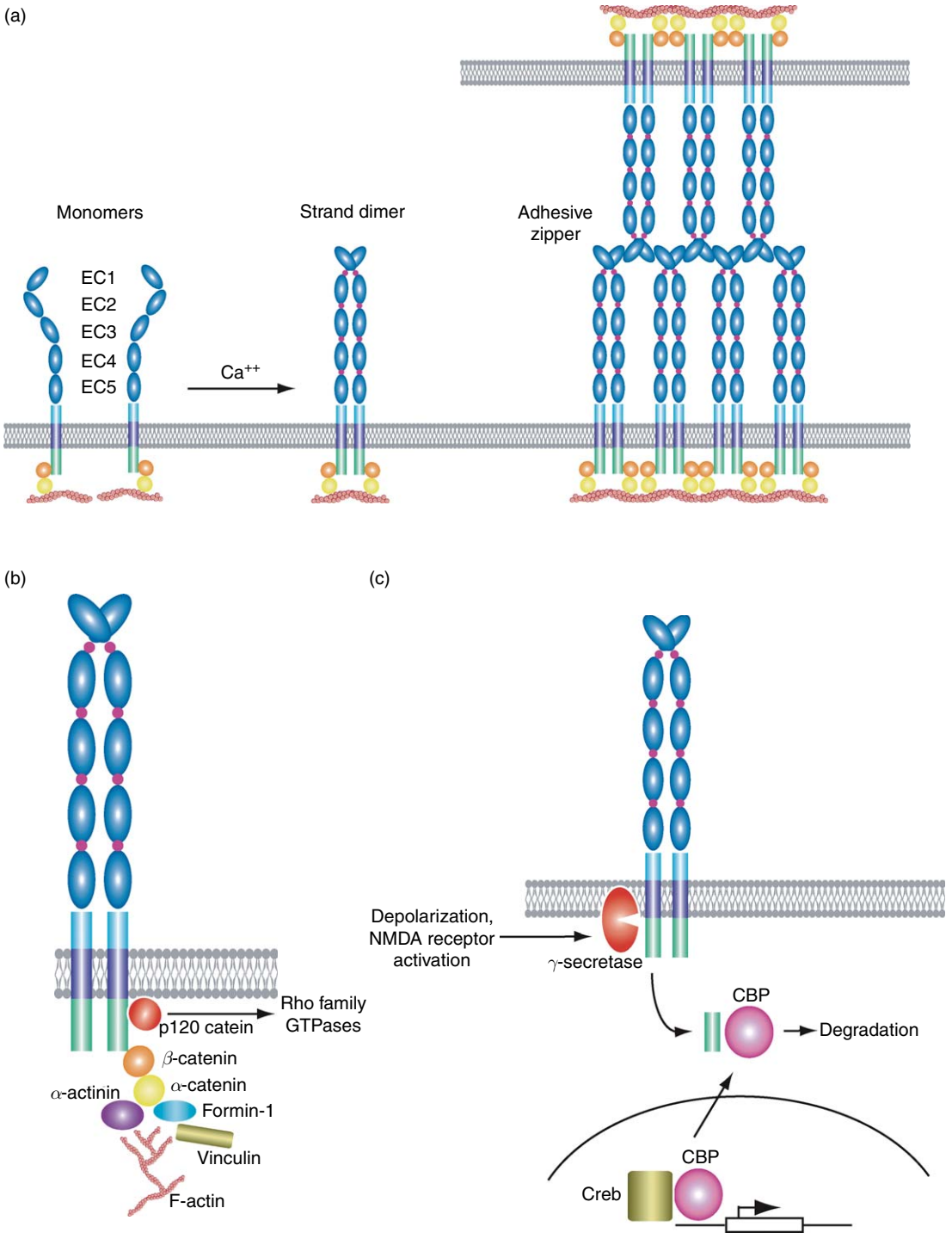
4.35.9 Biochemical Signaling through Cadherins

The accumulated data on cadherin function in the nervous system indicate that they are critical for axon targeting and synapse formation, maturation, stability, and plasticity through their roles as homophilic cell adhesion and signaling molecules (Junghans et al., 2005; Salinas and Price, 2005; Takeichi, 2007). Although cadherins may signal bidirectionally like the integrins, using both outside-inside and inside-outside signaling mechanisms, the evidence for inside-outside signaling is currently scant (Gumbiner, 2005). Furthermore, some of the mechanisms for outside-inside cellular signaling are only superficially understood. The complexity of the cadherin family of cell adhesion molecules suggests that their signaling mechanisms will prove to be diverse and perhaps cell-type specific. Therefore, the summary of cadherin signaling mechanisms provided below is limited to a few specific examples, which may or may not prove to be generally employed by different members of the cadherin family or even the same cadherin expressed in different cell types.

Like the integrins, neural activity is known to induce signaling through the cadherin pathway. Depolarization induces the mobilization of the cytoplasmic protein β-catenin from dendritic shafts into spines and leads to an increased association of β-catenin with cadherins and subsequent morphological and functional changes in synapses (Murase et al., 2002). This process is regulated by tyrosine phosphorylation of β-catenin. Dephosphorylation of tyrosine-654 of β-catenin is essential for the movement of β-catenin into spines.

Like the integrins, a major target for cadherin signaling is the actin cytoskeleton. Cadherins are thought to be able to induce actin filament formation in several different ways (**Figure 2(b)**). First, cadherin/catenin complexes recruit and activate proteins involved in actin polymerization, such as Arp2/3 and formin, thus establishing sites for the assembly of actin filaments (Bamji et al., 2005; Gumbiner, 2005). Second, after β-catenin binds to cadherins, it further associates with α-catenin, which either serves as a physical link between the cadherin/β-catenin complex or is involved in regulating the link of cadherins to the actin cytoskeleton through other molecular bridges (Bamji, 2005; Gates and Peifer, 2005). Third, cadherins interact with p120 cadherin via a segment close to the plasma membrane and can regulate the activity of the Rho family of GTPases (Bamji, 2005; Salinas and Price, 2005). This family of GTPases, as described earlier, are involved in regulating the state of the actin cytoskeleton. This signaling pathway may be involved in stabilizing the adhesive contacts established by the cadherins (Goodwin et al., 2003).

The cadherins may also be involved in modulating signaling through the transcription factor Creb (**Figure 2(c)**; Marambaud et al., 2003; Junghans et al., 2005; Salinase and Price, 2005). Cadherins are known to associate with presenilin 1, a γ-secretase also involved in the processing of amyloid precursor protein, resulting in the production of Aβ peptide. The stimulation of the γ-secretase activity of presenilin 1 by membrane depolarization or activation of the


(a)
Monomers
Strand dimer
Adhesive zipper
EC1
EC2
EC3
EC4
EC5
Ca++
(b)
Rho family GTPases
p120 catein
β-catenin
α-catenin
α-actinin
Formin-1
Vinculin
F-actin
(c)
Depolarization, NMDA receptor activation
γ-secretase
CBP
Degradation
CBP
Creb

NMDA receptor releases an intracellular peptide from cadherins. This peptide decreases cAMP response element binding protein (CREB)-mediated transcriptional activity by increasing the degradation of the CREB-binding protein, CBP. This is important because CREB activity has been widely implicated in the processes of long-term memory, suggesting that regulatory processes that influence the proximity of cadherins to γ-secretase and/or the activity of γ-secretase activity on cadherins are directly involved in long-term memory processes.

Other evidence has begun to emerge (see following) indicating that cadherins also participate in signaling through other molecules besides those mentioned above. It seems likely that future research will uncover many other new pathways for cadherin-based signaling that may underlie various forms of memory.

4.35.10 Cadherin-Dependent Synaptic Transmission and Plasticity

Accumulating evidence has made it clear that the functional roles for cadherins at synaptic sites provide more than just a static adhesiveness between the apposed pre- and postsynaptic membranes. For example, Tang et al. (1998) have shown that pretreatment of hippocampal slices with function-blocking antibodies raised against the extracellular domain of N- or E-cadherin significantly attenuated LTP at the Schaffer collateral-CA1 synapses. Infusion of peptides containing the His-Ala-Val (HAV) consensus sequence, which is known to be antagonistic for the formation of strand-dimers, also reduced LTP significantly. Notably, the inhibitory effect of the antibodies and the peptides is specific for the expression of early-phase LTP (E-LTP) since other physiological properties such as basal synaptic transmission, paired-pulse facilitation, posttetanic potentiation, and postsynaptic NMDA receptor–mediated currents were indistinguishable from the control slices. Similarly, Bozdagi et al. (2000) showed that LTP in area CA1 was disrupted by blocking N-cadherin adhesion functions with function-blocking antibodies. However, in contrast to the previous results, only late-phase LTP (L-LTP) was found to be defective. The reported difference in the time period in which N-cadherin functions are required during LTP between the two studies could be due to the differences in the protocol used for LTP induction, since a more intense stimulation protocol, such as the one used in the study by Tang et al., may have caused a more severe reduction in the extracellular Ca^{2+} and resulted in less stable N-cadherin complexes. An example for the importance of extracellular Ca^{2+} in affecting cadherin-dependent LTP induction was provided by a study on Arcadlin, a protocadherin-8-like molecule whose mRNA is dramatically induced in brain neurons after strong stimulation. It was shown that in the presence of Ca^{2+} in the medium, blocking antibodies against Arcadlin had no effect on LTP induction. However, by incubating the slices briefly with the antibodies in a Ca^{2+}-free medium before stimulation, the antibodies were able to block LTP even if Ca^{2+} was added back to the medium during the recordings (Yamagata et al., 1999). It is believed that, as in classic cadherins, Ca^{2+} is required for Arcadlin to exist at the synapses in a homophilic bound state that is resistant to the blocking antibodies. Depleting extracellular Ca^{2+}

Figure 2 Cadherin structure, adhesion, and signaling. (a) Model of structural rearrangements and adhesion of classic cadherins. Binding of Ca^{++} to the binding pockets located between the EC domains stabilizes and rigidifies the elongated rod-like structure. Monomeric cadherin molecules within the same membrane form a strand-dimer via lateral association and clustering. Strong intercellular adhesion is achieved when the membrane-distal EC1 domain from the strand-dimers of one cell interact with the EC1 domain from the strand-dimers emanating from the opposite cell in the *trans* configuration, forming an adhesive zipper. (b) Cytoplasmic signaling of cadherins. Cadherins signal to the Rho family of small GTPases through the association of p120 catenin. They also signal more directly through the actin cytoskeleton to effect changes in cell shape by binding β-catenin. β-catenin associates with α-catenin, which in turn binds to the actin cytoskeleton and/or regulates the association of cadherins to other molecular bridges, such as formin-1, α-actinin, or vinculin. (c) Nuclear signaling of cadherins. Cadherins signal to the nucleus to alter Creb signaling via the release of an intracellular fragment of cadherins. Depolarization or the activation of the NMDA receptor activates a γ-secretase, which cleaves and releases a peptide from the cytoplasmic tail of cadherins. This fragment is thought to sequester the co-activator protein, CBP, and target it to degradative pathways, thus inhibiting the signaling through CREB/CREB-binding protein (CBP) complexes. NMDA, *N*-methyl-D-aspartate.

destabilizes the intermolecular bond and renders the antibodies accessible for their function-blocking effect.

Molecular genetic approaches have also demonstrated the roles for cadherins/catenin complexes in synaptic plasticity. In the hippocampus, cadherin-11 is among one of the classic cadherins with strong expression (Suzuki et al., 1997; Manabe et al., 2000). Surprisingly, it was shown that LTP in the CA1 area was enhanced in the cadherin-11-deficient mice, in contrast to the strong reduction observed by blocking the functions of N-cadherin described above (Manabe et al., 2000). The mechanism underlying the observed LTP enhancement in these mutant animals remains unclear. Another mutant that was studied in detail physiologically was δ-catenin (δ-cat), a neuron-specific catenin that has been shown to colocalize with N-cadherin, PSD-95, and presenilin-1 (PS1) (Israely et al., 2004). The δ-cat$^{-/-}$ animals exhibit a significant deficit in PPF at multiple interpulse intervals. When tested for LTP, these mutants showed an abnormal enhancement of potentiation when a high-frequency stimulation was used for the induction, but a reduction when a low-frequency stimulation was used instead. Therefore, these results indicate that δ-cat is critically involved in both short-term and long-term synaptic plasticity. Interestingly, a significant reduction of N-cadherin and PSD-95 protein level was detected in the brain lysates from the δ-cat$^{-/-}$ animals when compared to the wild-type controls, while the level of PS1, E-cadherin, and synaptophysin remained unaffected. Since both N-cadherin and PSD-95 have been shown to be involved in LTP (Migaud et al., 1998; Tang et al., 1998; Ehrlich and Malinow, 2004), and PSD-95 is also linked to signaling cascades involved in the induction of LTP, the impaired synaptic plasticity and the nonlinear effect on LTP changes in the δ-cat$^{-/-}$ mice may be due to secondary effects caused by disrupting δ-catenin expression.

More recently, Jüngling et al. (2006) have generated N-cadherin knockout neurons in culture by *in vitro* differentiation of N-cadherin-deficient embryonic stem cells, thereby circumventing the early embryonic lethality of the N-cadherin null mutant animals. Physiological analysis on these N-cadherin knockout synapses revealed that during high activity there was a deficit in the readily releasable vesicle pool available for exocytosis, and consequently an impaired short-term plasticity, as evident by the increased paired-pulse depression. Interestingly, the same impaired short-term plasticity was still observed in synapses formed between wild-type neocortical neurons and ES-cell-derived N-cadherin-absent neurons in a heterogeneous culture with selective postsynaptic absence of N-cadherin. This result suggests the postsynaptic N-cadherin can regulate the release properties of the presynaptic sites in a retrograde manner, presumably through homophilic interaction with the apposed presynaptic partner. Taken together, the results from these different experimental approaches have demonstrated that different functional cadherin/catenin complexes are required for multiple forms of synaptic plasticity in the adult brain.

How do cadherins modulate synaptic plasticity? Since different mechanisms are known to underlie different forms of synaptic plasticity, it is likely that cadherins impart their effects by interacting, directly or indirectly, with different molecules and signaling cascades at specific synaptic sites. Both paired-pulse facilitation and paired-pulse depression are traditionally attributed to increased or decreased, respectively, neurotransmitter release from the presynaptic terminal (Zucker, 1999; O'Donovan and Rinzel, 1997). The impairment in these two forms of short-term plasticity observed in the δ-cat$^{-/-}$ animals and in the N-cadherin$^{-/-}$ cultured neurons is likely a consequence of the deficit in the transmitter vesicle pool as mentioned above (Bamji et al., 2003; Jüngling et al; 2006), although this deficit has not been described in the δ-cat$^{-/-}$ animals. The mechanism by which cadherin/catenin complexes regulate the vesicle pool at the active zone is unknown. However, by deleting specific domains of the β-catenin protein, Bamji et al. (2003) provided an additional important finding that the primary mechanism by which β-catenin regulates the reserve pool of synaptic vesicles is through recruitment of PDZ proteins to the cadherins, and not through the cadherin-actin cytoskeleton association. Consistent with this idea, β-catenin has been shown to bind directly through its carboxyl terminus to a PDZ domain of MALS/Velis, a component of the MALS/CASK/Mint-1 complex that regulates neurotransmitter release (Perego et al., 2000; Olsen et al., 2006).

Recent studies have also shed light on how cadherin/catenin complexes are involved in LTP. It has been long established that the NMDA receptor is one of the key factors in the induction of LTP. Remarkably, proteomic characterization of the NMDA receptor multiprotein complex has identified N-cadherin as being physically linked to the receptor

(Husi et al., 2000). The functional significance for such a close association between the two molecules might be tied by the studies by Tanaka et al. (2000), which showed that, in response to synaptic stimulation, N-cadherin dimerizes and becomes markedly resistant to degradation by proteases, indices that have been used to measure enhanced, strong intercellular adhesion. Importantly, the activity-induced dimerization and increase in protease resistance was attenuated by APV and was therefore required for NMDA receptor function. This activity-induced, NMDA receptor-dependent increase of adhesive interactions at the synapses might contribute to the potentiated response in LTP, although a direct demonstration of whether or how changes in synaptic adhesion affect synaptic plasticity is lacking. Nevertheless, L-LTP is accompanied by an increase in N-cadherin synthesis and dimer formation, implicating enhanced adhesion between the pre- and postsynaptic membranes in the process (Bozdagi et al., 2000). In addition to the enhanced adhesion at the synaptic junctions, LTP is also associated with the delivery of alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate receptors (AMPARs) to synapses (Heynen et al., 2000). Remarkably, recent evidence has shown that AMPARs associate with N-cadherin and β-catenin *in vivo* (Nuriya and Huganir, 2006). In primary cultures of hippocampal neurons, both GluR1 and GluR4 showed colocalization with N-cadherin clusters at synaptic sites by immunocytochemistry. When GluR1 was immunoprecipitated from the rat cortical membrane fraction, both N-cadherin and β-catenin were specifically coimmunoprecipitated. In addition, it was found that lowering the extracellular Ca^{2+} concentration increased the interaction between AMPAR receptors and N-cadherin, suggesting that extracellular Ca^{2+} negatively regulates this association. Interestingly, when N-cadherin was overexpressed in either heterologous cells or cortical neurons, the surface expression of GluR1 was specifically increased significantly. Conversely, overexpression of GluR1 also increased the surface expression of N-cadherin. Therefore, the association between the AMPA receptors and N-cadherin appears to regulate their surface expression in a mutual manner and provides an attractive mechanism for the potentiation of synaptic transmission that contributes to LTP.

LTP is also associated with structural remodeling and synapse formation, especially during the stabilization and maintenance phase (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Toni et al., 1999). Evidence from several studies indicates that cadherins are involved in activity-dependent changes in synaptic structure and function. Murase et al. (2002) showed that neural activity induced a persistent, NMDA receptor-dependent redistribution of β-catenin and an increase in its association with synaptic cadherins. In addition, a point mutation in β-catenin that increased its localization in dendritic spines resulted in expanded areas of postsynaptic PSD-95 protein clusters within the spines, as well as increased size and intensity of associated presynaptic synapsin-1 clusters, suggesting an overall increase in the synaptic contact area. Furthermore, these morphological changes were accompanied by a higher miniature excitatory postsynaptic current, indicating an increase in synaptic strength. In line with these results, αN-catenin, one of the linker molecules between cadherins and actin cytoskeleton through binding with β-catenin, was shown to be essential for dendritic spine stability in cultured hippocampal neurons (Abe et al., 2004). In αN-catenin-deficient cells, many spines showed unusually dynamic deformation of their heads, with abnormal, rapid protrusion and retraction of filopodia from most of these spines, indicating that the synaptic contacts in these αN-catenin deficient cells are unstable. Conversely, overexpression of αN-catenin led to an enhancement of spine stability, and consequently an increase in density as well. Notably, the expression level αN-catenin at the synapses is activity dependent, since its synaptic accumulation was suppressed by tetrodotoxin (TTX), a neural activity blocker, but was enhanced by bicuculline, a gamma-aminobutyric acid (GABA) antagonist. The synaptic accumulation of αN-catenin is functionally significant, since it was shown that excess αN-catenin could render spines resistant to the TTX treatment. Together, these studies suggest that the cadherin/catenin complexes contribute to enhanced synaptic size and density by conferring both strength and stability to synapses in an activity-dependent manner.

4.35.11 Cadherins and Learning and Memory

Compared to the extensive studies of the function of cadherin/catenin complexes at the molecular and cellular level, relatively little is known regarding their contributions to normal behavior. This is primarily due to the fact that most of the members in the cadherin and catenin families are essential during early development, and mutations are therefore

embryonic lethal, and the generation of conditional mutants has been lacking. Nevertheless, a few studies have been reported that did indicate that cadherin/catenin complexes are indeed involved in certain behavioral or learning and memory paradigms. For example, animals deficient in cadherin-11, which is not an essential cadherin, showed reduced fear- or anxiety-related responses as assayed by the elevated plus-maze behavioral test (Manabe et al., 2000). Deletion of δ-cat resulted in impairment in Pavlovian fear conditioning and in motor coordination, although their exploratory behavior and spatial learning remained intact (Israely et al., 2004). Surprisingly, mice expressing the dominant negative cadherin for both N- and E-cadherin in the adult forebrain showed only abnormal rearing behavior in the open field test, with no detectable defect in spatial learning and anxiety-related behaviors (Edsbagge et al., 2004). It may be that functional redundancy occurs in these animals that masks the *in vivo* functions, since multiple cadherins are expressed in the same neurons. Certainly, additional conditional knockouts, and perhaps in genetic combinations, will be needed to uncover the roles of cadherin/catenin complexes in learning and memory.

4.35.12 Cross-Talk among Cell Adhesion Receptors for Potential Synaptic and Behavioral Functions

It is clear from the above discussion that integrins and cadherins, the two major families of cell adhesion receptors, are critical for the regulation of synaptic functions. Both families provide a physical link between the extracellular environment and the actin cytoskeleton and the reorganization of the cytoskeleton in response to cell stimulation. Despite this commonality, studies of their signaling mechanisms have been performed separately. However, there now exists clear evidence for crosstalk between distinct members of these two families. In colon cancer cells, elevated Src activity disrupts the membrane localization of E-cadherin. This Src-induced redistribution of E-cadherin is dependent on functional αV- and β1-integrin subunits, along with FAK with intact Src-dependent phosphorylation acceptor sites (Avizienyte et al., 2002). Yano et al. (2004) have shown that siRNA-induced reduction in FAK and paxillin protein levels in HeLa cells inhibits the formation of N-cadherin-based cell–cell adhesions. In addition, signals involving FAK are implicated in downregulating the activity of Rac1, a Rho family GTPase stimulated by integrins (DeMali et al., 2003), at sites thought to be important for the formation of N-cadherin-based adhesions in motile cells. Therefore, FAK signaling pathways are linked to mechanisms that downregulate Rac1 activity, which is important for efficient formation and maintenance of cadherin-based cell–cell adhesions in motile cells. Rap 1, which is another small GTPase regulator of the inside-out signaling of integrins (Bos et al., 2003; Caron, 2003), has been shown to play a critical role in the integrin-cadherin crosstalk. In cultured epithelial cells, disassembly of adheren junctions triggered by internalization of E-cadherin led to Src-dependent Rap1 activation and the concomitant formation and localization of Rap1/E–cadherin–catenin complex at the recycling endosome compartment (Balzac et al., 2005). Remarkably, this E-cadherin endocytosis-dependent activation of Rap1 was associated with and was required for the assembly of integrin-dependent cell-matrix contacts, therefore suggesting a major role of Rap1 in relaying information from E-cadherin-based to integrin-based adhesive structures. Thus, an emerging theme is that the Src/FAK protein kinases and the ras/rho GTPases signaling pathways are pivotal links for the integrin–cadherin crosstalk, probably as effectors that directly influence actin polymerization. It will be interesting to determine whether similar crosstalk between these two protein families and the underlying mechanisms exist in the nervous system, and whether such crosstalk is essential for the regulation of synaptic plasticity and memory formation.

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4.36 Presynaptic Mechanisms in Plasticity and Memory

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4.36.1 Introduction

Presynaptic proteins and presynaptic plasticity have been implicated in learning and memory through decades of study in highly tractable, 'simpler' model systems, such as the marine mollusk *Aplysia* (Frost et al., 1988; Kandel, 2001; Roberts and Glanzman, 2003). In mammalian synaptic plasticity and learning and memory, however, the role of presynaptically mediated short- and long-term plasticity, presynaptic release machinery, and presynaptic signal transduction has received much less attention. This is likely due to a focus on proteins involved in long-term potentiation (LTP) in area CA1 of the hippocampus (*See* Chapters 4.16, 4.20, 4.29, 4.30, 4.32, and 4.39), which is expressed in part through postsynaptic molecular changes (Chen and Tonegawa, 1997; Silva et al., 1997; Martin et al., 2000). Given the overlap in molecular mechanisms between invertebrate and mammalian learning and memory, it is likely that presynaptic mechanisms will also play a prominent role in memory formation in the mammalian brain (Abbott and Regehr, 2004).

Presynaptic plasticity commonly refers to functional changes in synaptic strength that are due to alterations in the amount of transmitter release. Activity-dependent changes in transmitter release can be transient (up to a few minutes) or long-lasting (typically more than an hour). This chapter mainly discusses our current understanding of the molecular basis of presynaptic long-term plasticity and the potential role of this form of plasticity in learning and memory. It begins with an overview of molecular mechanisms of neurotransmitter release, followed by discussion of what we consider best examples of long-term, activity-dependent presynaptic plasticity that may be relevant to learning and memory in the mammalian brain. Finally, a review of recent studies using mammalian genetic models to link presynaptic proteins and presynaptic plasticity to learning and memory is presented. Portions of this chapter are based on or excerpts from previous reviews by the authors (Chevaleyre et al., 2006; Powell, 2006).

4.36.2 Neurotransmitter Release

Release of neurotransmitter from presynaptic terminals requires the highly regulated fusion of synaptic vesicle membrane with the plasma membrane in a specialized, electron-dense region known as the active zone. The details of this process and its regulation have been thoroughly reviewed (Sudhof, 2004), and a brief overview is provided here to orient the reader. Synaptic vesicles first dock at the active zone, and then an ATP-dependent priming reaction makes them competent for exocytosis when an action potential triggers Ca^{2+} influx into the presynaptic terminal via voltage-gated Ca^{2+} channels. Multiple studies have identified several important components of the molecular machinery responsible for synaptic vesicle fusion, its regulation by Ca^{2+}, and modulation of neurotransmitter release (**Figure 1**).

At the core of neurotransmitter release are the soluble N-ethylmaleimide-sensitive-factor attachment protein receptor (SNARE) proteins synaptobrevin/vesicle-associated membrane protein (VAMP), syntaxin, and SNAP-25. Synaptobrevin is a synaptic vesicle protein, whereas syntaxin and SNAP-25 are associated with the active zone. When docked vesicles are primed for release, SNARE complexes are formed that bring the synaptic vesicle into tight proximity to the active zone membrane (Sudhof, 2004).

The Ca^{2+} sensor for fast neurotransmitter release is synaptotagmin (Littleton et al., 1993; Geppert et al., 1994; Fernandez-Chacon et al., 2001). Synaptotagmin associates constitutively with SNARE complexes and, on binding Ca^{2+}, results in vesicle fusion and release of neurotransmitter (Sudhof, 2004).

As alluded to later, synaptic vesicle, cytoplasmic, plasma membrane, and active zone-associated proteins can regulate this process. These proteins can affect vesicle availability, docking, priming, Ca^{2+} triggering, and even Ca^{2+} entry, all of which can affect both the probability of neurotransmitter release and how neurotransmitter release changes as a function of patterns of presynaptic action potential firing.

Neurotransmitter release is a highly regulated process. Presynaptic ionotropic and metabotropic receptors can be activated in a homosynaptic (i.e., same presynaptic terminal) or heterosynaptic

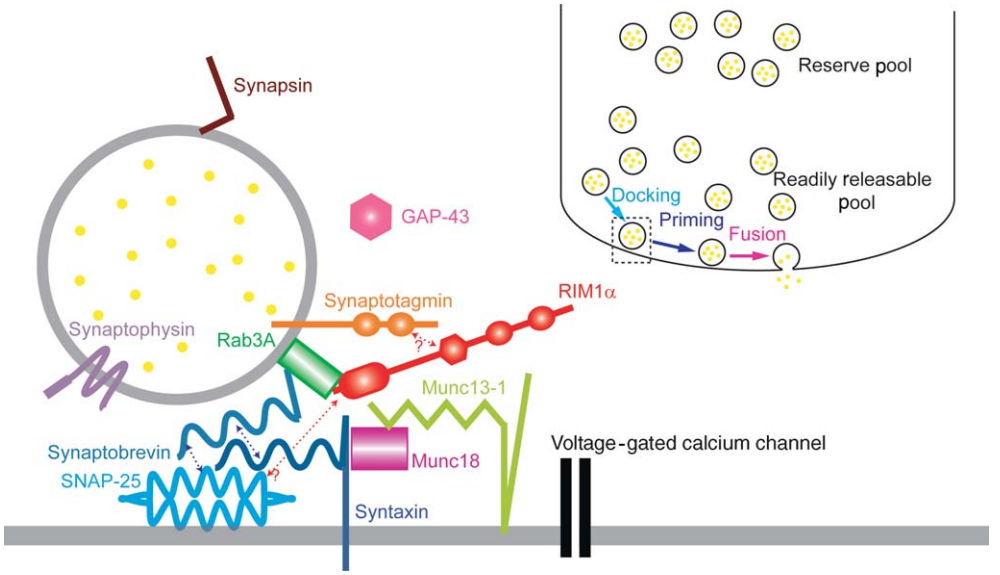


Figure 1 Schematic diagram of a presynaptic terminal and presynaptic proteins (Sudhof, 2004). Inset depicts a presynaptic terminal with reserve pool and readily releasable pool of synaptic vesicles. Also depicted are three final steps in neurotransmitter release: docking, priming, and fusion. The dashed box represents the region magnified in the main figure to the left. Main figure to the left depicts a single synaptic vesicle, synaptic vesicle–associated proteins, active zone proteins, and other proteins. The SNARE complex proteins, which interact to mediate fusion of the synaptic vesicle (synaptobrevin, SNAP-25, and syntaxin), are shown in shades of blue. Known interactions between proteins are depicted as bidirectional arrows. A question mark indicates that the interaction has not been completely validated. Additional possible interactions between the proteins depicted are described in the text. GAP-43, growth-associated protein-43. Figure modified from Powell CM (2006) Gene targeting of presynaptic proteins in synaptic plasticity and memory: Across the great divide. *Neurobiol. Learn. Mem.* 85: 2–15, with permission from Elsevier.

(i.e., neighboring presynaptic terminals) manner to modulate neurotransmitter release (Miller, 1998; MacDermott et al., 1999; Meir et al., 1999; Schoepp, 2001; Engelman and MacDermott, 2004). In addition, presynaptic receptors can be activated by retrograde signals generated from postsynaptic cells (Alger, 2002). The effects mediated by presynaptic receptors are typically short lasting. However, there is growing evidence demonstrating that transient activation of these receptors (i.e., presynaptic metabotropic receptors) can induce long-term changes in transmitter release.

4.36.3 Presynaptic Short-Term Plasticity

The probability of neurotransmitter release in response to a given action potential is robustly modulated by the pattern of presynaptic activation. In effect, the presynaptic terminal acts as a computational unit that can vary its output depending on the recent pattern of input. This type of presynaptic regulation of neurotransmitter release is generally referred to as short-term plasticity. For the purposes of this discussion, short-term plasticity refers to alterations in presynaptic release lasting for at most a few minutes. A brief overview of short-term plasticity is provided here, while more detailed accounts are available elsewhere (Fisher et al., 1997; Zucker and Regehr, 2002; Sudhof, 2004).

4.36.3.1 Facilitation/Depression

Paired-pulse facilitation (PPF) is a form of short-term plasticity lasting on the order of hundreds of milliseconds (**Figure 2(a)**). When two presynaptic action potentials occur within 25–400 ms, the postsynaptic response to the second action potential is increased dramatically relative to the first. This is widely held to be due to residual Ca^{2+} in the presynaptic terminal from the first action potential adding to the Ca^{2+} influx from the second pulse (Katz and Miledi, 1968; Zucker and Regehr, 2002). The larger presynaptic Ca^{2+} leads to facilitated or increased neurotransmitter release on the second 'pulse' or action potential. PPF is not limited to only the second of two presynaptic activations. In fact, responses to high-frequency stimulation beyond the second pulse can be facilitated even further; hence the more general term 'synaptic facilitation.' The magnitude of PPF varies depending on the initial probability of neurotransmitter release (Pr). At low baseline Pr, PPF is large (i.e., the response to the second 'pulse' is proportionately much larger than the first). At higher initial Pr, PPF can be smaller or can even reverse to become paired-pulse depression (PPD, with the response to the second pulse being smaller than the first). As a result, changes in PPF following an

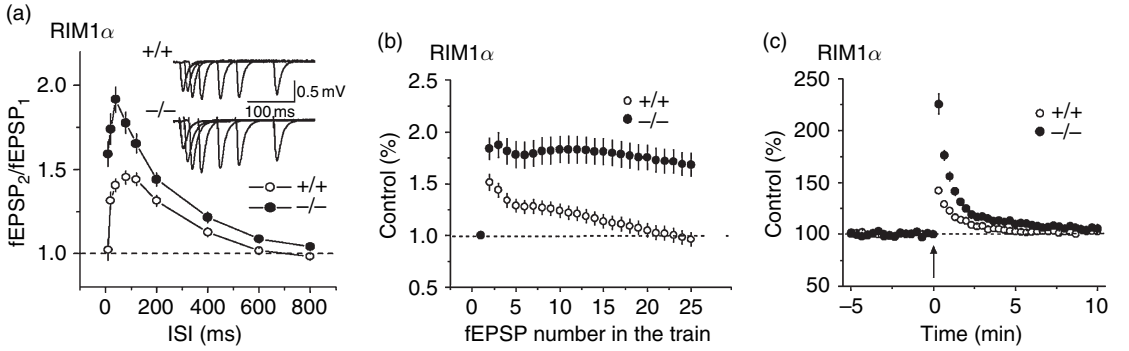


Figure 2 Examples of short and long-term presynaptic plasticity. (a) Example of paired-pulse facilitation (PPF) in wild-type (open circles, +/+) and Rab3A interacting molecule (RIM1α) knockout (filled circles, –/–) mice. The ratio of the first extracellularly recorded field excitatory postsynaptic potential (fEPSP) to that of the second fEPSP elicited at different interstimulus intervals (ISI) is plotted. RIM1α knockout mice show increased PPF compared to wild-type mice. Inset shows examples of fEPSPs elicited at various ISIs after the initial fEPSP. (b) Example of synaptic depression. In wild-type mice, stimulation at 14 Hz elicits an initial facilitation followed by synaptic depression. Depression in RIM1α–/– mice is markedly decreased. (c) Example of post-tetanic potentiation (PTP). In the presence of the *N*-methyl-D-aspartate inhibitor 4-aminophosphonovaleric acid to block long-term potentiation in area CA1 of the hippocampus, a high-frequency, tetanic stimulation (arrow) elicits immediate potentiation of the synaptic response lasting 2–3 min. PTP in RIM1α–/– mice is significantly enhanced. From Schoch S, Castillo PE, Jo T, et al. (2002) RIM1alpha forms a protein scaffold for regulating neurotransmitter release at the active zone. *Nature* 415: 321–326; used with permission from Nature Publishing Group.

experimental manipulation are often taken as an indication of changes in Pr, though alternative explanations can be offered, making PPF a useful tool in the study of synaptic plasticity mechanisms. The role of this robust integration of presynaptic activity in information processing remains a mystery, though initial studies of how PPF might play a role in behavior are detailed in the section titled 'Links between short-term presynaptic plasticity and learning and memory.'

Prolonged high-frequency presynaptic activation eventually leads to a decrease in neurotransmitter release known as synaptic depression (**Figure 2(b)**). This results in a lower steady-state level of neurotransmitter release that can take several minutes to recover. Synaptic depression is commonly taken to be the result of a decrease in the readily releasable pool (RRP) of synaptic vesicles, though the precise molecular mechanisms are poorly understood (Zucker and Regehr, 2002). The maximum number of synaptic vesicles released by a very strong, brief stimulus defines the RRP. Experimentally, the RRP can be defined by examining neurotransmitter release rates during sustained high-frequency stimulation, among other paradigms (Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996; Goda and Stevens, 1998). Application of hypertonic sucrose is commonly used to measure the RRP. Hypertonic sucrose-induced release correlates well with that induced by high-frequency stimulation and with the number of vesicles visualized as 'docked' by electron microscopy (Zucker and Regehr, 2002). As with PPF, RRP measurements can be used to reveal alterations in presynaptic function following a given experimental manipulation.

4.36.3.2 Post-tetanic Potentiation

Post-tetanic potentiation (PTP) refers to an increase in neurotransmitter release after a brief, high-frequency train of action potentials (**Figure 2(c)**). This large enhancement may last on the order of several minutes and is measured after high-frequency stimulation. Although the precise mechanism of PTP remains to be determined, genetic manipulation of certain presynaptic proteins can clearly lead to altered PTP with or without changes in other forms of presynaptic plasticity (Zucker and Regehr, 2002). The time-course of PTP suggests that the relatively large presynaptic Ca^{2+} accumulation during high-frequency stimulation activates a Ca^{2+}-dependent biochemical process that leads to modification of presynaptic proteins. This modification may be slowly reversed over minutes following return of presynaptic Ca^{2+} signal to baseline. Although the exact purpose of PTP in the central nervous system is unclear, initial studies on the behavioral implications of altering PTP are discussed in the section 'Links between short-term presynaptic plasticity and learning and memory.'

4.36.4 Presynaptically Mediated Forms of Long-Term Plasticity

4.36.4.1 CA1 LTP: Evidence for Presynaptic Locus of Expression

The most-studied form of long-term synaptic plasticity is LTP at the Schaffer collateral-to-CA1 pyramidal neuron synapse in the hippocampus (Malenka and Bear, 2004). This form of LTP is induced postsynaptically via *N*-methyl-D-aspartate (NMDA) receptor activation and expressed, at least in part, postsynaptically via increased postsynaptic responsiveness to neurotransmitter release. The postsynaptic induction mechanisms for LTP, including Ca^{2+} influx through NMDA receptors and activation of postsynaptic Ca^{2+}-activated protein kinases, makes postsynaptic expression of CA1 LTP a parsimonious conclusion. Indeed, the belief that NMDA receptor-dependent LTP in area CA1 is expressed via postsynaptic mechanisms is so ingrained in current thought that several chapters in this work are focused largely on postsynaptic expression mechanisms (Chapters 4.16, 4.20, 4.21, 4.22, 4.23, 4.25, 4.27, 4.30). As discussed elsewhere, it seems likely that a portion of CA1 LTP expression is mediated by postsynaptic mechanisms.

A great deal of literature also provides evidence for a presynaptic component of CA1 LTP expression. Initial studies were based on metabolic labeling of glutamate and measuring release of labeled glutamate *in vivo* (Dolphin et al., 1982). These were followed by a flurry of studies applying quantal analysis to hippocampal synapses suggesting an increase in neurotransmitter release (Malinow and Tsien, 1990; Bekkers and Stevens, 1990; Malinow, 1991; Nicoll and Malenka, 1999). These studies led to important work identifying candidate retrograde messengers, molecules that could be generated postsynaptically, diffuse across post- and presynaptic membranes, and induce changes in the presynaptic neuron. Additional studies using sophisticated imaging of presynaptic vesicles suggest altered presynaptic function during CA1 LTP expression (Malgaroli et al., 1995; Zakharenko et al., 2001; Emptage et al., 2003). A more recent study finds

that the presynaptic proteins RIM1α and Rab3A may be required for successful expression of a protein kinase A (PKA)-dependent, late phase of LTP, implying that CA1 LTP lasting beyond 2–3 h may also be expressed in part via presynaptic mechanisms (Huang et al., 2005).

4.36.4.2 Mossy Fiber Long-Term Potentiation/Depression

Following the initial discovery of LTP at the perforant path to dentate granule neuron synapse by Bliss and Lomo (1973), it soon became apparent that LTP could also occur in area CA1 and at the dentate granule neuron-to-CA3 pyramidal neuron synapse (Alger and Teyler, 1976). The discovery of the role of NMDA receptors in CA1 LTP led to a focus on CA1 LTP due to the mechanistic implications of NMDA receptor involvement, though NMDA receptor-independent forms of LTP have also been reported at this synapse (Grover and Teyler, 1990; Powell et al., 1994).

Work by several laboratories has revealed a clearly different form of LTP in area CA3 of the hippocampus that is induced and expressed by mechanisms distinct from those of LTP in area CA1. Unlike CA1 LTP, this form of LTP does not require NMDA receptor activation (Harris and Cotman, 1986) and has been most studied at the mossy fiber-to-area CA3 pyramidal neuron synapse in the hippocampal formation (mossy fiber LTP or mfLTP) (Johnston et al., 1992; Nicoll and Schmitz, 2005). All available evidence suggests that mfLTP is expressed via an increase in presynaptic neurotransmitter release (Nicoll and Malenka, 1995; Weisskopf and Nicoll, 1995; Tong et al., 1996). Induction of mfLTP appears to require activation of PKA, whereas early phases of CA1 LTP do not (Hopkins and Johnston, 1988; Weisskopf et al., 1994; Huang and Kandel, 1994; Huang et al., 1995; Nguyen and Kandel, 1997; Villacres et al., 1998). The major events and molecules thought to be involved in mfLTP are depicted schematically in **Figure 3**.

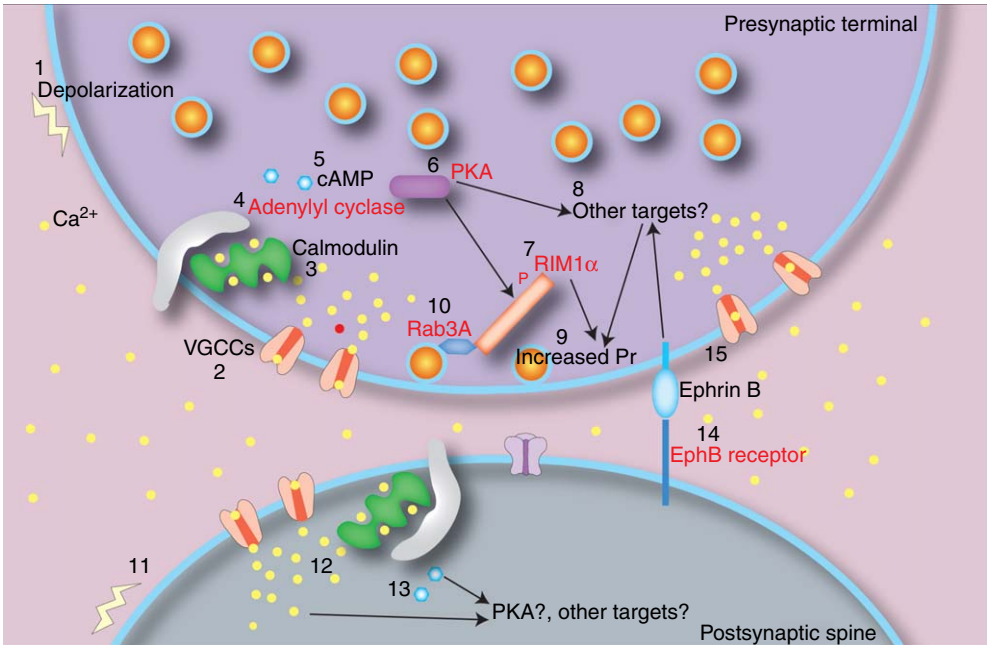


Figure 3 Schematic diagram of major processes thought to be involved in mossy fiber long-term potentiation (mfLTP). In one scenario, presynaptic depolarization (1) activates voltage-gated Ca^{2+} channels (VGCCs), (2) allowing significant Ca^{2+} entry. Ca^{2+} binds calmodulin (3), which activates Ca^{2+}/calmodulin-sensitive adenylyl cyclases (4) leading to increased cyclic adenosine monophosphate (cAMP) (5) and activation of protein kinase A (PKA) (6), which then phosphorylates RIM1α (7) or other targets (8), which leads directly or indirectly to increased probability of neurotransmitter release (Pr) (9). Rab3A (10), which interacts with RIM1α, is also required for induction/maintenance of mfLTP. Other reported contributors are postsynaptic depolarization (11), Ca^{2+} influx (12), and cAMP (13), as well as the postsynaptic signaling molecule EphB (14) and its presynaptic ligand Ephrin B (15). Genetic deletion of the molecules labeled in red is known to lead to decreased mfLTP.

Beyond the lack of NMDA receptor activation and a requirement for PKA activation, studies regarding induction mechanisms for mfLTP have produced different results. The mechanism and location of mfLTP induction are thus still unresolved. One series of studies suggests that that mfLTP can be induced purely by presynaptic activation (Castillo et al., 1994; Weisskopf et al., 1994; Nicoll and Malenka, 1995; Weisskopf and Nicoll, 1995; Tong et al., 1996; Mellor and Nicoll, 2001), requiring presynaptic Ca^{2+} influx via R-type voltage-dependent Ca^{2+} channels (Breustedt et al., 2003; Dietrich et al., 2003) and cAMP/PKA signaling (Weisskopf et al., 1994; Huang et al., 1995; Villacres et al., 1998; Wang et al., 2003). Another series of experiments indicates that mfLTP induction requires postsynaptic Ca^{2+} influx, depolarization, and cAMP (Williams and Johnston, 1989; Jaffe and Johnston, 1990, Urban et al., 1996; Yeckel et al., 1999; Alle et al., 2001; Contractor et al., 2002; Wang et al., 2004), necessitating the involvement of a retrograde messenger for presynaptic expression. Indeed, postsynaptic induction of mfLTP does not preclude presynaptic expression. In fact, transsynaptic interactions between postsynaptic EphB tyrosine kinase receptors and presynaptic B-ephrins have been implicated (Contractor et al., 2002; Armstrong et al., 2006). However, it is unclear how exactly mossy fiber repetitive stimulation triggers retrograde signaling or how presynaptic ephrins enhance transmitter release. Several pharmacological and mouse knockout studies (Contractor et al., 2001; Lauri et al., 2001; Bortolotto et al., 2003; Schmitz et al., 2003; Breustedt and Schmitz, 2004) support the notion that presynaptic kainate receptors are not essential for mfLTP but play a modulatory role in triggering this form of plasticity. Thus, activation of kainate autoreceptors by endogenous glutamate would increase presynaptic calcium entry during tetanization, thereby facilitating the induction of mfLTP (reviewed in Bortolotto et al., 2003; Nicoll and Schmitz, 2005). Long-term depression (LTD) at this synapse has been shown to be induced postsynaptically (Lei et al., 2003). It is worth noting that regardless of the induction mechanism, several studies have implicated increased presynaptic neurotransmitter release as the final common pathway for mfLTP expression (Nicoll and Malenka, 1995; Weisskopf and Nicoll, 1995; Tong et al., 1996). Thus, any remaining uncertainty is limited to the pre- versus postsynaptic requirement for mfLTP induction.

The molecular players involved in presynaptic mfLTP induction/expression are slowly being revealed. Presynaptic Ca^{2+}/calmodulin-sensitive adenylyl cyclases and presynaptic activation of PKA are critical for induction of mfLTP (Hopkins and Johnston, 1988; Huang et al., 1995; Villacres et al., 1998; Wang et al., 2003). In addition, the synaptic vesicle-associated small GTPase (GTP: guanosine triphosphate) Rab3A is required for mfLTP induction/expression (Castillo et al., 1997). Rab3A, however, is not a substrate for PKA *in vitro*, prompting a search for Rab3A interacting molecules with PKA phosphorylation sites. Rabphilin and the Rab3A interacting molecule (RIM1α) were then identified as PKA substrates that interacted with Rab3A. The PKA substrate RIM1α was found to be required not only for mfLTP but also for cerebellar parallel fiber to Purkinje cell LTP (pfLTP), highlighting that LTP expression at the hippocampal mossy fibers and cerebellar parallel fibers is mechanistically similar. However, at least for mfLTP, rabphilin was not required (Schluter et al., 1999). The discovery that the PKA substrate and active zone protein RIM1α and its vesicle-associated binding partner Rab3A were required for mfLTP induction/expression led to a specific, testable hypothesis that phosphorylation of RIM1α by PKA was required for mfLTP. The PKA site in RIM1α was subsequently identified, and RIM1α constructs with a serine to alanine substitution at this site were made (Lonart et al., 2003). Using a cerebellar culture system, this PKA/RIM hypothesis was tested at the parallel fiber-to-Purkinje neuron synapse. In RIM1α knockout mice, presynaptic cerebellar LTP was absent and could be rescued by wild-type RIM1α. This form of cerebellar LTP was not rescued by RIM1α containing a single serine-to-alanine point mutation at the PKA site, indicating that PKA phosphorylation of RIM1α may be necessary for induction/expression of cerebellar presynaptic LTP (Lonart et al., 2003). This negative finding has not yet been replicated, and a similar experiment has not been performed in mfLTP. Thus, although the presence of RIM1α is important for mfLTP induction/expression, it is not clear whether PKA phosphorylation of RIM1α is required for mfLTP. Even if PKA phosphorylation of RIM1α were required for mfLTP or cerebellar LTP, it would still not be clear whether PKA phosphorylation of RIM1α is the direct cause of increased neurotransmitter release or simply necessary for some other parallel mechanism to lead to increased neurotransmitter release. Interestingly, even in

RIM1α knockout mice, the adenylyl cyclase activator forskolin can still increase neurotransmitter release, suggesting that cyclic adenosine monophosphate (cAMP) or PKA activation can increase neurotransmitter release via mechanisms independent of RIM1α (Castillo et al., 2002; Schoch et al., 2002). This finding does not preclude RIM1α's involvement in mfLTP, but does complicate the interpretation that presynaptic cAMP activates PKA, leading to phosphorylation of RIM1α and the subsequent increase in neurotransmitter release.

The idea that mfLTP is due to a modification of the release machinery has been challenged by a study showing that hyperpolarization-activated cation channels (Ih) are necessary for mfLTP (Mellor et al., 2002). Activation of presynaptic Ih channels, by depolarizing mossy fibers and presumably increasing presynaptic Ca^{2+} influx, would facilitate transmitter release. Against this notion, another study showed normal mfLTP after pharmacological blockade of Ih channels (Chevaleyre and Castillo, 2002). Two independent groups showed that presynaptic Ca^{2+} entry remains unchanged during mfLTP (Regehr and Tank, 1991; Kamiya et al., 2002), supporting a model for mfLTP expression that involves persistent modification of presynaptic molecular targets residing downstream of Ca^{2+} entry. At the moment, the existence of Ih channels on mossy fiber terminals awaits discovery, and a role of Ih in mfLTP remains unproven.

Mossy fiber synapses also express presynaptic forms of LTD. At mossy fiber-to-CA3 pyramidal cell synapses, LTD is typically induced by moderate rates of stimulation ($\sim$1 Hz) for several minutes (Kobayashi et al., 1996). This form of LTD is induced by the activation of presynaptic metabotropic glutamate receptor 2 (mGluR2) (Yokoi et al., 1996) and is expressed as a reduction in glutamate release (Kobayashi et al., 1996). Tzounopoulos et al. (1998) reported that mGluR2 activation leads to a reduction in adenylyl cyclase activity followed by a decrease in PKA activity (Tzounopoulos et al., 1998). In addition, this study showed that mossy fiber LTD (mfLTD) requires Rab3A. Based on these observations, the authors suggested that mfLTD involves reversal of the presynaptic process involved in mfLTP. However, this model has been disputed by a more recent report showing that RIM1α, which is required for mfLTP, is not required for mfLTD (Castillo et al., 2002). Mossy fiber-to-interneuron synapses also express a presynaptic form of LTD (Lei and McBain, 2004). Interestingly, this form of plasticity is induced by the activation of postsynaptic Ca^{2+}-permeable alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs) but it is expressed presynaptically as a reduction in transmitter release, suggesting that some retrograde signal is required. How a postsynaptic rise in Ca^{2+} triggers presynaptic LTD remains unclear, but a recent study suggested that activation of presynaptic mGluR7 is necessary (Pelkey et al., 2005). Further studies will have to identify the molecular targets downstream from mGluRs mediating these forms of mfLTD.

4.36.4.3 Endocannabinoid Long-Term Depression

Endogenous cannabinoids (endocannabinoids, or eCBs) are signaling molecules that have emerged as key mediators of presynaptic plasticity in several brain areas, including those critically involved in learning and memory, such as the hippocampus, amygdala, striatum, and cerebellum (for a review see Chevaleyre et al., 2006) (*See* Chapter 4.37). In eCB-mediated plasticity, postsynaptic activation induces production of a lipid eCB messenger that generally moves in retrograde fashion across the synapse, binds to presynaptic cannabinoid receptors, and suppresses transmitter release either transiently or in a consolidated, long-term form.

Several endogenous ligands for the type 1 cannabinoid receptor (CB1R), the principal cannabinoid receptor in the brain, have been identified. Two of the best-characterized eCBs are 2-arachidonoylglycerol (2-AG) and anandamide (AEA). These eCBs are synthesized from membrane-derived lipid precursors. Because eCBs are lipid molecules that can pass through biological membranes, they cannot be stored in vesicles but are instead synthesized on demand by neuronal enzymatic activity (Piomelli, 2003). Notably, the CB1R is among the most highly expressed G protein–coupled receptors in the central nervous system (Herkenham et al., 1990), and it is responsible for most of the behavioral effects of Δ^9-tetrahydrocannabinol (Δ^9-THC), the principal psychoactive component of marijuana (for a more extensive discussion on endocannabinoid signaling, see the excellent recent reviews: Howlett et al., 2002; Freund et al., 2003; Piomelli, 2003; De Petrocellis et al., 2004; Howlett et al., 2004). There are many known types of eCB-mediated plasticity, each acting only at certain synapses and with a specific set of requirements for induction. In this section, we will focus on eCB-mediated long-term depression of transmitter release

(eCB-LTD), which probably constitutes the best-documented and most widely distributed form of activity-dependent long-term presynaptic plasticity in the brain (Gerdeman and Lovinger, 2003; Chevaleyre et al., 2006). Transient forms of eCB-mediated plasticity (Alger, 2002; Kreitzer et al., 2002; Wilson and Nicoll, 2002; Freund et al., 2003; Diana and Marty, 2004) will be briefly mentioned here to facilitate comparison with eCB-LTD.

Critical to our understanding of the induction of eCB-LTD are the mechanisms of eCB release and the signaling events downstream of CB1R activation. eCB production is a complex and tightly regulated, activity-dependent process that can occur as a result of two main mechanisms. The first eCB production mechanism is triggered by an influx of Ca^{2+} into the postsynaptic cell consequent to postsynaptic step depolarization or action potentials. This mechanism is typified by the transient (typically <1 min), eCB-mediated suppression of neurotransmitter release known as *D*epolarization-induced *S*uppression of *I*nhibition or *E*xcitation (DSI or DSE) (Alger, 2002; Kreitzer et al., 2002; Wilson and Nicoll, 2002; Diana and Marty, 2004), but it may also be involved in some forms of eCB-LTD (see later discussion). Postsynaptic Ca^{2+} rises may then be amplified by recruitment of Ca^{2+} release from intracellular stores. How Ca^{2+} promotes eCB release remains unknown. A second eCB production mechanism is triggered by repetitive activity of glutamatergic afferents and activation of group I mGluR, which couples to phospholipase C (PLC). This mechanism underlies most forms of eCB-LTD (Chevaleyre et al., 2006), as well as those short-term eCB-mediated forms of plasticity induced by synaptic activity (Maejima et al., 2001; Brown et al., 2003; Melis et al., 2004). The eCB 2-AG is likely involved because diacylglycerol lipase (DGL), the enzyme that synthesizes 2-AG, is required downstream of mGluR activation (Jung et al., 2005; Chevaleyre et al., 2006). Activation of AMPA receptors may also contribute to eCB-LTD mechanisms (Sjostrom et al., 2003; Soler-Llavina and Sabatini, 2006). Although each eCB production mechanism can be triggered independently of the other, some degree of cooperativity may exist; for example, increased intracellular Ca^{2+} greatly enhances mGluR-induced PLC activity (Hashimotodani et al., 2005; Maejima et al., 2005).

Unlike several common forms of long-term synaptic plasticity that critically depend on NMDA receptor–mediated Ca^{2+} influx for induction (Malenka and Bear, 2004), all known forms of eCB-LTD are independent of postsynaptic NMDA receptors. The newly synthesized eCB traverses the synaptic cleft and binds to presynaptic CB1Rs. The activation of CB1Rs coupled to $G_{i/o}$ proteins can affect many processes potentially involved in synaptic transmission, such as Ca^{2+} channel inhibition, a direct effect on the vesicle release machinery, and/or K^+ channel activation (Ameri, 1999; Mu et al., 2000; Schlicker and Kathmann, 2001; Howlett et al., 2002; McAllister and Glass, 2002; Mukhopadhyay et al., 2002). Although the transient reduction of transmitter release that occurs during DSI/DSE is most likely due to CB1R-dependent modulation of presynaptic voltage-dependent Ca^{2+} channels (Wilson et al., 2001; Varma et al., 2002), the mechanism by which CB1R activation leads to LTD of transmitter release has not yet been identified.

eCB-LTD has been reported in a number of brain structures including the dorsal striatum (Gerdeman et al., 2002; Kreitzer and Malenka, 2005), nucleus accumbens (Robbe et al., 2002), amygdala (Marsicano et al., 2002; Azad et al., 2004), hippocampus (Chevaleyre and Castillo, 2003), neocortex (Sjostrom et al., 2003; Bender et al., 2006), and cerebellum (Soler-Llavina and Sabatini, 2006). This prevalence suggests that eCB-LTD may be a fundamental mechanism for making long-term modifications to neural circuits and behavior. eCB-LTD is defined by certain common features. Induction of eCB-LTD requires the activation of presynaptic CB1Rs, and it is expressed presynaptically as a long-lasting reduction of transmitter release. The requirement for CB1Rs derives from results showing that eCB-LTD induction is blocked by CB1R antagonists and is abolished in CB1R knockout mice. eCB-LTD is likely to be presynaptically expressed because it is associated with enhanced paired-pulse ratio and failure rate. Importantly, once established, maintenance of eCB-LTD does not require continued CB1R activation (see later discussion). It is worth noting that the induction of cerebellar LTD has been recently reported to require eCB signaling (Safo and Regehr, 2005); but because cerebellar LTD is postsynaptically expressed, it constitutes a different class of eCB-mediated plasticity. Where eCB production is triggered by activation of glutamatergic synaptic inputs, eCBs can homosynaptically suppress these same glutamatergic inputs, forming an autoregulatory loop, or heterosynaptically suppress nearby GABAergic (GABA: gamma-aminobutyric acid) inputs (**Figure 4**).

Examples of homosynaptic eCB-LTD can be found at excitatory inputs to medium spiny neurons in the dorsal striatum (Gerdeman et al., 2002) and nucleus accumbens (Robbe et al., 2002) and also at the parallel fiber-to-stellate cell synapse in the

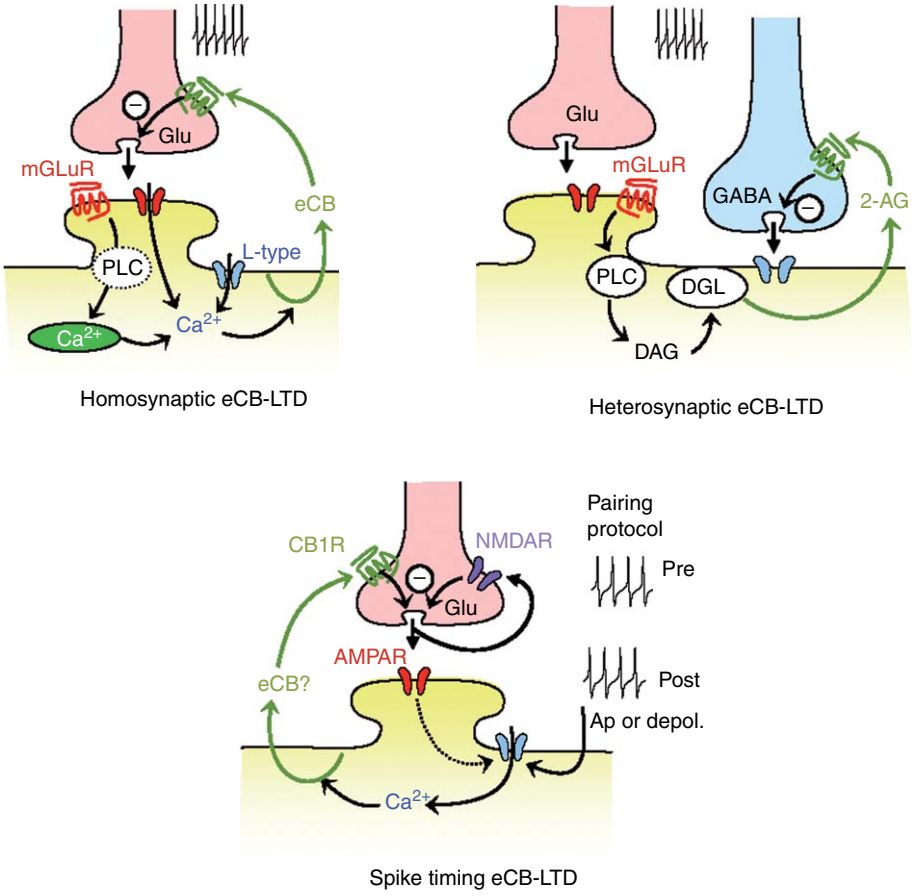


Figure 4 Schematic diagram of endocannabinoid-mediated LTD (eCB-LTD). *Homosynaptic eCB-LTD* has been reported at dorsal striatum and nucleus accumbens glutamatergic synapses. This form of plasticity is induced by repetitive activation of glutamatergic inputs and requires the activation of postsynaptic group I mGluRs (mGluR-I). Although postsynaptic [Ca^{2+}] increase is also required in both cases, the source may vary (intracellular stores in nucleus accumbens and L-type Ca^{2+} channels in the dorsal striatum). Heterosynaptic eCB-LTD occurs at GABAergic (GABA: gamma-aminobutyric acid) synapses in the hippocampus and basolateral amygdala. In both structures, LTD is initiated by glutamate release and group mGluR-I activation but results in a heterosynaptic decrease in GABA release. Unlike homosynaptic eCB-LTD at excitatory synapses, LTD induction at these inhibitory synapses does not require increased postsynaptic [Ca^{2+}]. The two structures differ in their eCB production pathways (see text); for clarity, only the hippocampal signaling pathway is shown. Timing-dependent eCB-LTD has been reported in the neocortex. In this case, LTD is triggered by pairing presynaptic stimulations with postsynaptic depolarizations or action potentials. Increased postsynaptic [Ca^{2+}] is required for eCB release. Interestingly, the induction of timing-dependent eCB-LTD requires activation of both presynaptic Type 1 cannabinoid receptors (CB1R) and presynaptic *N*-methyl-D-aspartate receptors (NMDARs). Glu, glutamate; mGluR, metabotropic glutamate receptor; PLC, phospholipase C; DGL, diacylglycerol lipase; DAG, diacylglycerol; 2-AG, 2-arachidonoylglycerol AMPAR, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor.

cerebellum (Soler-Llavina and Sabatini, 2006). Homosynaptic eCB-LTD is triggered by repetitive stimulation (13 Hz) of prelimbic cortical glutamatergic inputs in the nucleus accumbens (Robbe et al., 2002), high-frequency stimulation (100 Hz) (Gerdeman et al., 2002), and moderate-frequency stimulation of excitatory inputs in dorsal striatum (10–25 Hz) (Ronesi et al., 2004; Kreitzer and Malenka, 2005), and bouts of stimulation (30 Hz) of parallel fibers in cerebellum (Soler-Llavina and Sabatini, 2006). In all cases, the induction of homosynaptic eCB-LTD requires group I mGluR activation and increased postsynaptic Ca^{2+}. Implicated in this [Ca^{2+}] increase are intracellular stores in nucleus accumbens (Robbe et al., 2002), L-type Ca^{2+} channels in the dorsal striatum (Calabresi et al., 1994; Choi and Lovinger, 1997), and postsynaptic calcium accumulation via Ca^{2+}-permeable AMPARs in the cerebellum (Soler-Llavina and

Sabatini, 2006). In the dorsal striatum, D2 receptor activation is also required for induction (Calabresi et al., 1997; Tang et al., 2001; Kreitzer and Malenka, 2005; Ronesi and Lovinger, 2005), and eCB release requires an uncharacterized transporter (Ronesi et al., 2004).

Heterosynaptic eCB-LTD has been reported at inhibitory synapses in the basolateral amygdala (BLA) (Marsicano et al., 2002) and the CA1 area of the hippocampus (Chevaleyre and Castillo, 2003). In both areas, repetitive activity of excitatory synaptic inputs onto principal neurons results in heterosynaptic LTD of GABA release from nearby inhibitory synapses (also known as I-LTD). This heterosynaptic LTD first requires the activation of postsynaptic group I mGluR on principal cells and then presynaptic CB1R on GABAergic terminals. In the hippocampus, eCB-LTD is triggered by stimulation protocols that commonly induce LTP at the Schaffer collateral to CA1 pyramidal cell synapses (i.e., high-frequency stimulation and theta-burst stimulation) and also by moderate-frequency stimulation of Schaffer collaterals (10 Hz) (Chevaleyre and Castillo, 2004). In contrast, BLA eCB-LTD is triggered by low-frequency stimulation (1 Hz) of excitatory inputs (Azad et al., 2004; Marsicano et al., 2002). The hippocampus and amygdala also differ in the nature of the eCB mediating LTD and their eCB production pathways. In the hippocampus, 2-AG release results from PLC-DGL activation (Chevaleyre and Castillo, 2003), whereas in the amygdala, AEA release requires the cAMP-protein kinase A (cAMP-PKA) pathway (Azad et al., 2004). It is unclear how PKA contributes to eCB release. Unlike eCB-LTD at excitatory synapses, LTD induction at inhibitory synapses does not require increased postsynaptic Ca^{2+}.

In addition to homosynaptic and heterosynaptic eCB-LTD, retrograde eCB signaling mediates a form of spike-timing-dependent synaptic plasticity (STDP), which is also known as timing LTD or tLTD. This form of plasticity was initially described at excitatory synapses between pairs of layer-5 pyramidal neurons in the visual cortex (Sjostrom et al., 2003) and more recently at excitatory inputs to layer 2/3 neurons in layer 4 of the somatosensory cortex (Bender et al., 2006). STDP refers to long-term changes in synaptic efficacy triggered by paired action potential firing in pre- and postsynaptic neurons (Abbott and Nelson, 2000; Bi and Poo, 2001; Dan and Poo, 2004). tLTD is triggered by pairing presynaptic stimulations with postsynaptic depolarizations or action potentials. Increased postsynaptic $[Ca^{2+}]$ is required for eCB release. Interestingly, LTD induction requires coactivation of both presynaptic CB1Rs and presynaptic NMDA receptors (see the section 'Presynaptic NMDA receptor-dependent forms of plasticity').

Activation of CB1Rs for several minutes is required for induction of eCB-LTD. In support of this idea, blockade of CB1Rs right after induction protocol (~1 min) abolished the induction of eCB-LTD in the hippocampus (Chevaleyre and Castillo, 2003) and in dorsal striatum (Ronesi et al., 2004). Although eCB-LTD is expressed as a reduction in evoked transmitter release, it does not reflect continued CB1R activation. Once established, eCB-LTD could not be reversed when CB1R antagonists were applied after a postinduction delay of 10 min in hippocampus (Chevaleyre and Castillo, 2003) and dorsal striatum (Ronesi et al., 2004), 15 min in visual cortex (Sjostrom et al., 2003), or 60 min in the nucleus accumbens (Robbe et al., 2002). Together, these observations suggest that eCB-LTD induction requires an additional molecular change at the presynaptic terminal that transduces the transient depression mediated by CB1R activation into a persistent depression that is CB1R independent. An important challenge is to understand what molecular processes occur in the presynaptic terminal during this time window that ultimately lead to a long-lasting change in transmitter release.

Spread of eCB signaling may affect the input specificity of eCB-LTD. Diffusion of eCBs could take place within the cytoplasm or plasma membrane or through the extracellular space, and its influence would be limited not only by dilution but also by transporter uptake and degradation (reviewed in Freund, 2003; Piomelli, 2003). Evidence that eCBs can diffuse between neighboring cells was reported in the hippocampus (Wilson and Nicoll, 2001) and cerebellum (Vincent and Marty, 1993; Kreitzer et al., 2002). In addition, spread of eCB signaling may also occur within or along a single cell. The dependence of eCB-LTD on retrograde signaling by diffusible messengers raises questions about the synapse specificity of eCB-LTD. However, studies in the cerebellum (Brown et al., 2003) and hippocampus (Chevaleyre and Castillo, 2004) suggest that synaptically driven release of eCBs may exert a subtle point-to-point regulation of synaptic inputs. Interestingly, in most synapses expressing eCB-LTD, depression of synaptic transmission induced by a CB1R agonist is fully reversed by CB1R antagonists, clearly suggesting that activation of CB1Rs is necessary but not

sufficient to trigger eCB-LTD. For example, coactivation of presynaptic NMDA receptor activation is also required for the induction of neocortical tLTD (Sjostrom et al., 2003; Bender et al., 2006). Although most forms of eCB-LTD are NMDA receptor independent, it is likely that along with CB1R activation, the induction of eCB-LTD also requires increased presynaptic Ca^{2+} (Chevaleyre et al., 2006), as occurs in the presynaptic terminal during repetitive activity. Thus, integration of these two signals by the presynaptic terminal may confer synapse specificity to this diffusible retrograde messenger–induced process.

What is the role of eCB-LTD in learning and memory? The functional relevance of this form of presynaptic plasticity obviously depends on the synapse type (i.e., excitatory or inhibitory) and brain structure where it is expressed. For example, by removing inhibition, eCBs may exert important effects in regulating the efficacy of excitatory inputs and in controlling the excitability of principal cells. Indeed, eCB-LTD at GABAergic inputs may increase postsynaptic excitability both in the hippocampus (Chevaleyre and Castillo, 2003; Chevaleyre and Castillo, 2004) and amygdala (Azad et al., 2004), two brain areas critically involved in the formation of new memories (LeDoux, 2000; Martin et al., 2000; Eichenbaum, 2004; Squire, 2004). In addition, disinhibition associated with heterosynaptic eCB-LTD facilitates the induction of LTP at hippocampal excitatory synapses (Chevaleyre and Castillo, 2004), a form of plasticity involved in spatial learning (Martin et al., 2000). This facilitation is a clear example of metaplasticity, a long-lasting change in the induction threshold for plasticity (Abraham and Bear, 1996). Thus, by regulating excitability and modifiability of LTP induction at excitatory synapses, eCB-LTD may contribute to experience-dependent changes of the neural circuits involved in memory formation.

In the amygdala, eCBs may play an important role in the extinction of learned fear (Marsicano et al., 2002). In fear conditioning learning, animals associate two stimuli (i.e., a tone and a foot shock) such that the repeated pairing of a tone with a foot shock leads to a freezing response when the tone is presented alone. Over the course of several repeated presentations of the tone without foot shock, animals learn to override this association (see reviews by Myers and Davis, 2002; Maren and Quirk, 2004; Kim and Jung, 2005) and stop freezing, an active process called extinction. Normal extinction of the conditioned fear response, not its acquisition or consolidation, requires intact eCB signaling (Marsicano et al., 2002). For example, in CB1R knockout mice, acquisition and consolidation of the conditioned response were normal, but the rate of extinction was much slower. In addition, when a CB1R antagonist was administered systemically to a trained mouse shortly before the first presentation of an unpaired tone, the degree of extinction that resulted was greatly reduced, an observation that has recently been confirmed by others in both mice (Suzuki et al., 2004) and rats (Chhatwal et al., 2005). eCB-LTD at GABAergic synapses may contribute to extinction of learned fear (Marsicano et al., 2002). However, the precise mechanism by which eCB-mediated disinhibition may lead to extinction learning is unclear. Interestingly, eliminating CB1R activity affects extinction memory in the Morris water maze, which suggests that the eCB system may play a role in facilitating extinction processes in the hippocampus as well (Varvel and Lichtman, 2002; Varvel et al., 2005).

It is commonly believed that long-term synaptic plasticity contributes to the enduring changes in brain reward circuits that follow exposure to drugs of abuse (Berke and Hyman, 2000; Hyman and Malenka, 2001; Nestler, 2001; Thomas and Malenka, 2003; Kauer, 2004; Jones and Bonci, 2005). The widespread nature of eCB-LTD suggests it may be involved in this process. Indeed, experimental evidence suggests that eCB signaling may influence the modifications of the brain reward circuits believed to underlie the rewarding properties of drugs of abuse (for reviews, see De Petrocellis et al., 2004; Lupica et al., 2004; Gardner, 2005). *In vivo* exposure to THC, presumably by inducing functional tolerance of the CB1R, abolishes eCB-LTD in nucleus accumbens (NAc) (Hoffman et al., 2003; Mato et al., 2004). This THC-induced modification of eCB-LTD could affect the development of drug addiction. Although eCB-LTD may contribute to reward-motivated, learned behaviors, direct evidence for this possibility still awaits discovery.

4.36.4.4 Presynaptic NMDA Receptor-Dependent Forms of Plasticity

Most forms of long-term plasticity commonly depend on postsynaptic NMDA receptors. For example, in the 'classic' form of synaptic plasticity at the Schaffer collateral-to-CA1 pyramidal cell synapse, depolarization produced by convergent synaptic inputs or postsynaptic firing removes the Mg^{2+} block of postsynaptic NMDA receptors, allowing

Ca^{2+} entry into target neurons. The NMDA receptor-mediated rise in postsynaptic Ca^{2+} activates a network of kinases and phosphatases that promote enduring changes in synaptic strength (Scannevin and Huganir, 2000; Winder and Sweatt, 2001). Growing evidence indicates that not all NMDA-dependent synaptic plasticity is induced postsynaptically. As discussed later, several studies have revealed presynaptic forms of plasticity that rely on the activation of presynaptic NMDA receptors and may play important roles in learning and memory.

The idea of presynaptic NMDA receptor-dependent forms of plasticity is supported by numerous studies showing a presynaptic location and function of these receptors. First, presynaptic NMDA receptors have been observed on several presynaptic fibers including primary afferent terminals in the spinal cord and brainstem (Liu et al., 1994; Lu et al., 2003); on GABAergic axon terminals in the basal forebrain, thalamus, hypothalamus, and some brainstem nuclei (Paquet and Smith, 2000); as well as on excitatory terminals in the neocortex (Aoki et al., 1994), hippocampus (Siegel et al., 1994), cerebellum (Petralia et al., 1994), and amygdala (Farb et al., 1995). Second, presynaptic NMDA receptors have been reported to enhance spontaneous transmitter release in the entorhinal cortex (Berretta and Jones, 1996; Woodhall et al., 2001), visual cortex (Sjostrom et al., 2003), and cerebellum (Glitsch and Marty, 1999) and to depress evoked transmitter release in the dorsal horn (Bardoni et al., 2004) and cerebellum (Glitsch and Marty, 1999). Together, these studies build a strong case for functional presynaptic NMDA receptors at both excitatory and inhibitory terminals. Furthermore, presynaptic NMDA receptors reportedly mediate enduring forms of transmitter release modulations (see later discussion). Thus, although most studies have examined postsynaptic roles for NMDA receptors, it has become clear that presynaptic NMDA receptors can transiently regulate transmitter release and also induce long-term changes in synaptic efficacy.

In the classic model of NMDA receptor operation, activation of these receptors requires simultaneous glutamate binding and sufficient depolarization to relieve the voltage-dependent Mg^{2+} block of the channel. Given a glutamatergic synapse, how then can presynaptic NMDA receptors be activated, if presynaptic depolarization is generally over by the time glutamate binds to these receptors? This question is particularly relevant when inducing protocols contain moderate (usually physiological) frequencies of presynaptic activity (<30 Hz). Two 'solutions' to this problem have been identified (**Figure 5**). First, glutamate may not arise from the presynaptic terminal but from neighboring afferents (e.g., heterosynaptic plasticity) or from the postsynaptic neuron. In these cases, presynaptic NMDA receptors operate as coincidence detectors of presynaptic depolarization and glutamate release (Duguid and Sjostrom, 2006), thereby conferring the properties of associativity and input specificity (i.e., only active synapses undergo plasticity) to this form of plasticity. Second, glutamate binding alone may be sufficient for activation of presynaptic NMDA receptors. It has been

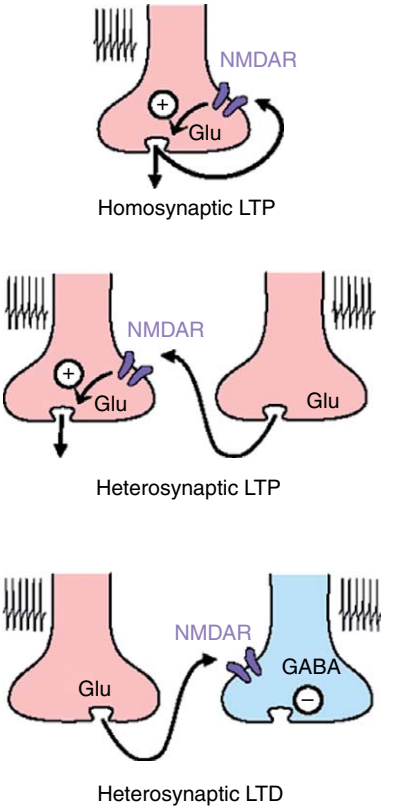


Figure 5 Schematic diagram of presynaptic *N*-methyl-D-aspartate receptor (NMDAR)-dependent forms of plasticity. Homosynaptic long-term potentiation (LTP), a nonassociative form of plasticity, has been observed at thalamic afferents in the central amygdala. Heterosynaptic LTP has been reported at excitatory cortical inputs to principal cells in the lateral amygdala. Glutamate (Glu) released from thalamic terminals following repetitive stimulations activates presynaptic NMDA receptors on cortical afferents, causing a long-lasting increase in transmitter release. Heterosynaptic long-term depression (LTP) occurs in the developing *Xenopus* retinotectal system, where repetitive activity of glutamatergic inputs (and presumably inhibitory inputs) reportedly induces long-term reduction of gamma-amino butyric acid (GABA) release from neighboring inhibitory terminals.

recently established that the subunit composition of NMDA receptors has important implications for the biophysical properties of the receptor. Indeed, NMDA receptors containing the NR2C and NR2D subunits have reduced sensitivity to Mg^{2+} (Kutsuwada et al., 1992; Monyer et al., 1992; Ishii et al., 1993; Monyer et al., 1994; Kuner and Schoepfer, 1996). A recent report provided functional evidence that these receptors may localize to presynaptic fibers (Mameli et al., 2005). As discussed later, there are good examples for both forms of presynaptic NMDA receptor-dependent plasticity. Although the precise mechanism by which a transient activation of presynaptic NMDA receptors leads to long-term changes in transmitter release remains to be identified, these forms of plasticity have already been shown to play an important role in memory formation and in sensory-dependent refinement of neuronal connections in the developing nervous system.

Good examples of presynaptic NMDA receptor-dependent plasticity can be found in the amygdala, a brain structure critically involved in the acquisition and possibly the storage of fear memories (LeDoux, 2000; Davis and Whalen, 2001; Maren, 2001; Maren and Quirk, 2004; Rodrigues et al., 2004). Importantly, some of the best evidence for the involvement of long-term synaptic plasticity in learning comes from fear conditioning (McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997; Rumpel et al., 2005). A key issue is what synaptic inputs are relevant in conditioning-related synaptic plasticity, and whether this plasticity is induced and expressed pre- or postsynaptically (for recent reviews, see Rodrigues et al., 2004; Maren, 2005). Several reports have demonstrated presynaptic forms of plasticity in the amygdala (McKernan and Shinnick-Gallagher, 1997; Huang and Kandel, 1998; Bauer et al., 2002; Tsvetkov et al., 2002; Humeau et al., 2003; Samson and Pare, 2005) that may contribute to fear learning (McKernan and Shinnick-Gallagher, 1997; Tsvetkov et al., 2002; Apergis-Schoute et al., 2005). LTP of thalamic or cortical inputs can be induced by pairing presynaptic and postsynaptic activation. This LTP is induced postsynaptically (involving NMDA receptors and L-type voltage-gated Ca^{2+} channels) but is expressed presynaptically as an increased probability of transmitter release (McKernan and Shinnick-Gallagher, 1997; Huang and Kandel, 1998; Bauer et al., 2002; Tsvetkov et al., 2002). The nature of the retrograde signal mediating this long-term increase in release remains to be identified.

Two recent studies in the lateral and central amygdala reported a form of LTP that is induced and expressed presynaptically and is NMDA receptor-dependent. In the lateral amygdala, repetitive activation of thalamic and cortical excitatory inputs to principal neurons produces LTP of cortical but not thalamic inputs (Humeau et al., 2003). Interestingly, activation of either pathway in isolation had no effect (except after pharmacological inhibition of glutamate uptake), indicating that this form of plasticity is associative. Although bath application of an NMDA receptor antagonist abolished LTP in the lateral amygdala, intracellular blockade of postsynaptic NMDA receptors as well as postsynaptic Ca^{2+} chelation or hyperpolarization did not prevent its induction. Importantly, this LTP was associated with a reduction in PPF, but no increase in quantal size, suggesting changes in synaptic release probability. In addition, LTP was occluded when release probability was artificially increased by raising the extracellular Ca^{2+} concentration. Taken together, these observations led the authors to conclude that glutamate released from thalamic terminals following repetitive stimulations activates presynaptic NMDA receptors on cortical afferents, causing a long-lasting increase in the probability of glutamate release. In the central nucleus of the amygdala, the primary output nucleus, high-frequency stimulation (HFS) of thalamic inputs led to LTP of thalamic responses. Induction of this form of LTP also requires presynaptic NMDA receptors (i.e., LTP was markedly reduced by global NMDA receptor antagonism, but blockade of postsynaptic NMDA receptors had no effect) (Samson and Pare, 2005). This form of LTP is also likely due to an increase in glutamate release as indicated by an associated reduction of PPF. Although changes in PPF generally indicate a presynaptic origin, a network effect cannot be completely discarded. Unlike LTP of cortical afferents in the lateral amygdala, LTP of thalamic afferents in central amygdala is homosynaptic and nonassociative. Recent studies have reported postsynaptically expressed forms of plasticity in the amygdala (Humeau et al., 2005; Rumpel et al., 2005), clearly highlighting the diversity of mechanisms underlying plasticity in this brain structure (Maren, 2005). Future studies will have to determine the precise functional role for the different forms of plasticity at thalamic and cortical inputs in the encoding, storage, and retrieval of fear memories.

Inhibitory synapses also exhibit NMDA receptor-dependent forms of presynaptic plasticity. Activation

of putative presynaptic NMDA receptors exerts complex effects at inhibitory synapses; namely, it increases miniature inhibitory postsynaptic current (mIPSC) frequency in Purkinje cells and interneurons (basket and stellate cells), and it decreases evoked IPSCs at interneuron–interneuron synapses (Glitsch and Marty, 1999) More recently, Duguid and Smart (2004) reported a novel form of plasticity at GABAergic inputs onto Purkinje cells that is caused by the activation of presynaptic NMDA receptors. These investigators showed that depolarization of Purkinje cells induces a potentiation of inhibitory synaptic transmission that lasts several minutes, a phenomenon they called 'depolarization-induced potentiation of inhibition' (DPI). This phenomenon is triggered by an increase in postsynaptic Ca^{2+} and can be evoked by repetitive activation of climbing fibers. DPI is blocked by the NMDA receptor antagonist D-APV and can be mimicked by brief applications of NMDA. Importantly, DPI induction affects mIPSC frequency and the paired-pulse ratio and coefficient of variation of evoked IPSCs, indicating that the site of action is presynaptic. Based on these observations, Duguid and Smart (2004) proposed that a rise in postsynaptic Ca^{2+} following depolarization of the Purkinje cell releases glutamate that diffuses to activate presynaptic NMDA receptors. They also presented experimental evidence that Ca^{2+} influx through NMDA receptors causes Ca^{2+} release from intracellular ryanodine-sensitive Ca^{2+} stores, which then causes an increase in GABA release probability. NMDA receptors are commonly blocked by external Mg^{2+} in a voltage-dependent manner. Interestingly, no prior depolarization of the interneuron was needed to induce DPI, suggesting that presynaptic NMDA receptors on GABAergic terminals have a low Mg^{2+} sensitivity. How glutamate is released from Purkinje cells is still unknown. Although a role of DPI in cerebellar-dependent learning awaits discovery, this potentiation of inhibition may provide a way to reduce Purkinje cell excitability following strong activity. It will be interesting to see if DPI is found in other brain structures as well.

A recent study has provided the first evidence of a presynaptic, NMDA receptor-dependent form of long-term plasticity that can occur *in vivo* (Lien et al., 2006). Lien et al. showed that light stimuli or theta-burst stimulation (TBS) of the optic nerve in the developing *Xenopus* retinotectal system induced LTP of glutamatergic synapses but LTD of GABAergic synapses onto the same tectal neuron. This LTD is likely due to a reduction of GABA release as indicated by an increase in both failure rate and coefficient of variation. Intriguingly, PPF was not changed after LTD induction. Other common manipulations known to decrease Pr in most synapses (i.e., by reducing extracellular Ca^{2+} concentration, blocking presynaptic Ca^{2+} influx with Cd^{2+}, or activating $GABA_B$ receptors) did not affect PPF either, suggesting that PPF may not be a sensitive method to assess Pr at these synapses. As for other presynaptic forms of plasticity that rely on the activation of presynaptic NMDA receptors, the induction of this LTD was blocked by D-APV application but was independent of postsynaptic activity in the tectal neuron, postsynaptic NMDA receptor activation, and postsynaptic Ca^{2+} influx. Although exogenous application of NMDA depressed the amplitude of IPSCs that were evoked at a low frequency (0.1 Hz), this effect was reversed on washout, suggesting that NMDA receptor activation is not sufficient to induce LTD. Interestingly, transient depression induced by exogenous NMDA became long lasting when NMDA application was paired with a weak TBS that by itself was insufficient to induce LTD. These observations strongly suggest that in addition to high-frequency bursts of excitatory fibers, which presumably release enough glutamate to activate presynaptic NMDA receptors on inhibitory fibers, the induction of LTD requires coincident high-level GABAergic activity. In this regard, the heterosynaptic LTD in the developing *Xenopus* retinotectal system constitutes a clear example of presynaptic NMDA receptors functioning as coincidence detectors for glutamatergic and GABAergic activities. Thus, coordinated glutamatergic and GABAergic presynaptic activity can lead to long-term synaptic modifications that may be important not only to experience-dependent changes of neural circuits during development but also to memory formation.

It is worth noting that the LTD reported by Lien et al. in the *Xenopus* retinotectal system resembles the LTD previously described at GABAergic synapses in the hippocampus (Chevaleyre and Castillo, 2003) and the amygdala (Azad et al., 2004). For example, these are all heterosynaptic forms of plasticity requiring repetitive activation of glutamatergic fibers resulting in a long-lasting reduction of GABA release. However, unlike LTD in the hippocampus and amygdala, tectal LTD is NMDA receptor-dependent but cannabinoid-receptor independent, clearly highlighting the variety of mechanisms underlying presynaptic plasticity at GABAergic synapses. Tectal LTD also resembles spike-timing dependent LTD in neocortex (Sjostrom

et al., 2003). In both cases, presynaptic NMDA receptors are necessary but not sufficient to induce LTD. Whereas simultaneous coactivation of presynaptic CB1 receptors is required for the induction of LTD in the visual cortex, GABAergic afferent activity seems to be the additional signal necessary for the induction of tectal LTD. Also, in both forms of plasticity, NMDA receptors appear to operate as coincidence detectors, presumably in a slightly different way than the more classic coincidence detection mediated by these receptors on the postsynaptic side (Duguid and Sjostrom, 2006). Future studies will determine how the presynaptic terminal integrates the diverse signals required for the induction of LTD and also which downstream signaling pathways and presynaptic targets mediate long-term reductions in transmitter release.

4.36.5 Links between Short-Term Presynaptic Plasticity and Learning and Memory

Short-term plasticity includes synaptic plasticity lasting on the order of a few minutes or less. The most commonly studied forms of short-term plasticity include PPF, PPD, synaptic depression, and PTP and augmentation. The physiologic characteristics and mechanisms of these forms of plasticity have recently been extensively reviewed (Zucker and Regehr, 2002). Very few studies have specifically addressed the question of the role of short-term plasticity in learning and memory (see **Table 1**).

Although progress has been made in understanding which presynaptic proteins might be involved in short-term forms of plasticity such as PPF and PTP (Castillo et al., 2002; Schoch et al., 2002; Zucker and Regehr, 2002; Sudhof, 2004), the role of short-term plasticity in information processing and storage remains to be determined (Abbott and Regehr, 2004). Is there a role for short-term plasticity in learning and memory? How could synaptic alterations lasting milliseconds to minutes play such a role? Does short-term plasticity play a role in rapid information processing such as sensorimotor gating or selective attention during learning? A complete picture of how the brain processes and stores information will depend on a fundamental understanding of these questions.

Initial studies suggested that a decrease in PPF may lead to impairments in hippocampus-dependent learning, whereas increased PPF has no effect. Silva and colleagues began with a systematic study of the role of PPF and PTP in learning and memory using mice that were heterozygous for Ca^{2+}/calmodulin-dependent protein kinase II (αCaMKII$^{+/-}$). αCaMKII-deficient mice exhibit increased PTP and decreased PPF in area CA1 of the hippocampus with normal CA1 LTP (Chapman et al., 1995) and were impaired in both fear conditioning and Morris water maze learning (Silva et al., 1996). Thus, increased PTP or decreased PPF was implicated in the learning deficits. In a study by Matilla et al., mice lacking ataxin-1 were found to have decreased PPF with normal PTP and LTP in area CA1 (Matilla et al., 1998). These mice with an apparently isolated decrease in PPF exhibited severe spatial learning deficits in the Morris water maze (Matilla et al., 1998), though baseline Pr was not measured in these mice. These studies suggest that decreased PPF can lead to impairments in hippocampus-dependent learning, whereas increasing PPF has no effect.

In addition to decreased PPF, decreased PTP alone appears sufficient to cause learning and memory deficits. In the same study by Silva et al., mice deficient in synapsin II or synapsins I and II showed decreased PTP, with normal PPF and LTP in CA1, and were impaired in fear conditioning (Silva et al., 1996).

Although decreases in PPF and PTP may lead to learning and memory deficits, it appears that increased PPF alone may not be sufficient to perturb learning and memory. A recent study by Powell et al. (2004) examined two presynaptic protein knockout mice that had previously been extensively characterized electrophysiologically and exhibited increased PPF in area CA1 of the hippocampus. Rab3A$^{-/-}$ mice had increased CA1 PPF with no change in baseline Pr (Castillo et al., 2002; Schoch et al., 2002) and exhibited completely normal water maze and fear conditioning learning (Powell et al., 2004). Two other studies confirmed this lack of learning and memory deficits in Rab3A$^{-/-}$ mice (Hensbroek et al., 2003; D'Adamo et al., 2004). One of these studies of Rab3A$^{-/-}$ mice demonstrated more subtle deficits in working memory and episodic-like memory, though these abnormalities were attributed to altered long-term plasticity at mossy fiber synapses in area CA3 of the hippocampus (D'Adamo et al., 2004). Similarly, synaptotagmin 1 point mutant knockin mice (Syt1R233Q$^{+/+}$) exhibited increased PPF along with an approximately 50% decrease in Pr in area CA1 (Fernandez-Chacon et al., 2001).

Table 1 Summary of short-term plasticity mutants and behavioral results

	PPF	*PTP*	*LTP*	*Fear conditioning*	*Water maze*	*Other*	*Reference(s)*
αCaMKII +/−	↓	↑	Normal	↓	↓		Silva et al., 1996
Ataxin I −/−	↓	Normal	Normal	–	↓		Matilla et al., 1998
Synapsin II −/−	Normal	↓	Normal	↓	–		Silva et al., 1996
Synapsin I/II −/−	Normal	↓	Normal	↓	–		Silva et al., 1996
Synapsin I −/−	↑	Normal	Normal	Normal	Normal		Silva et al., 1996
Rab3A −/−	↑	Normal	Normal	Normal	Normal	↓ mfLTP	Castillo et al., 2002; Powell et al., 2004; Schoch et al., 2002
				Normal	Normal	↓ mfLTP	Hensbroek et al., 2003
				Normal	↓ reversal learning		D'Adamo et al., 2004
				↓ Cue fear conditioning	Normal		Yang et al., 2006
Synaptotagmin 1 R233Q +/+	↑	–	–	Normal	Normal	↓ PrCA1	Powell et al., 2004
Synaptotagmin IV −/−	↑	↑	↑	↓ Passive avoidance (Normal object recognition)	–		Ferguson et al., 2000

PPF, paired-pulse facilitation; PTP, post-tetanic potentiation; Pr, probability of evoked neurotransmitter release; mfLTP, mossy fiber long-term potentiation in area CA3; —, not tested. All electrophysiological data are from area CA1 of the hippocampus unless otherwise specified.

Surprisingly, Syt1R233Q$^{+/+}$ mice showed normal water maze and fear conditioning learning (Powell et al., 2004) despite the significant alteration in Pr in CA1 and likely at other central synapses. Similarly, synapsin I deficient mice showed increased PPF but no deficits in fear conditioning or water maze learning (Silva et al., 1996). In synaptotagmin IV knockout mice, both PPF and PTP were increased, and the mice showed normal novel object recognition but impaired long-term passive avoidance memory (Ferguson et al., 2004). This difference could be due to the different behavioral paradigms compared to the other studies or due to an interaction between increased PPF and increased PTP. Thus, increased PPF alone did not appear to adversely affect learning and memory in the Morris water maze or fear conditioning paradigms, nor did it augment learning.

Although these initial studies are beginning to converge on the idea that PPF and perhaps PTP are necessary for or involved in normal learning and memory, additional confirmation is necessary. In particular, it will be important to examine additional mouse models in which short-term plasticity is affected in isolation of other synaptic physiological abnormalities. Mice lacking various modulators of presynaptic plasticity and release are excellent candidates for such genetic manipulation. In future studies, it will be helpful to restrict the knockout of such targets to specific presynaptic neurons, perhaps in subregions of the hippocampal formation using conditional knockout approaches. Similarly, it will be important to expand the number of learning and memory paradigms used to characterize these mice. Working memory is one such understudied form of memory in which short-term plasticity might play a critical role. In addition to learning and memory paradigms, it will also be important to assess other aspects of behavior, such as sensorimotor gating, selective attention, and impulsivity.

4.36.6 Links between Long-Term Presynaptic Plasticity and Learning and Memory

The mechanisms involved in presynaptically expressed forms of LTP are being elucidated (Weisskopf et al., 1994; Huang et al., 1995; Weisskopf and Nicoll, 1995; Salin et al., 1996a,b; Tong et al., 1996; Castillo et al., 1997; Kapur et al., 1998; Lonart et al., 1998; Villacres et al., 1998; Yeckel et al., 1999; Henze et al., 2000; Kumar et al., 2001; Mellor and Nicoll, 2001; Poser and Storm, 2001; Castillo et al., 2002; Lonart, 2002; Schmitz et al., 2003; Ferguson et al., 2004; Wang et al., 2004). Although present at cerebellar parallel fiber-Purkinje cell synapses and at corticothalamic synapses, presynaptically expressed LTP has been most extensively studied at the dentate granule cell mossy fiber axon-to-CA3 pyramidal neuron synapse in the hippocampus (mfLTP). Recent advances in our understanding of how the CA3 region of the hippocampus may be involved in certain types of episodic memory, pattern completion, and pattern separation make understanding the role of this form of presynaptic plasticity particularly relevant to understanding hippocampal function.

Several molecular manipulations in mice have led to absent or reduced mfLTP with or without additional synaptic plasticity deficits. Results of behavioral analyses in these mice are mixed, but a relatively clear picture is emerging (see **Table 2**). The convergent evidence from several studies suggests that a lack of mfLTP does not affect traditional water maze, delay fear conditioning, or trace fear conditioning, but may have a subtle effect on episodic-like memory versions of the water maze (delayed matching to place) and perhaps working memory. This conclusion, however, is subject to alternative interpretations due to the limitations of traditional knockout studies mentioned earlier.

mfLTP seems to be required for more complex forms of spatial memory requiring cognitive flexibility or pattern recognition, a function consistent with current ideas about the role of plasticity in recurrent-collateral synapses in area CA3 of the hippocampus (Nakazawa et al., 2002; Nakazawa et al., 2003; Kesner et al., 2004). The behavioral ramifications of altering mfLTP have been studied in several lines of mice lacking mfLTP. An early study from Eric Kandel's group demonstrated that absence of the regulatory (R1β) or catalytic (Cβ1) subunit of PKA prevents mfLTP while sparing several learning and memory tasks, including the Morris water maze, fear conditioning, and radial arm maze (Huang et al., 1995). Subsequently, Hensbroek and collaborators showed that the absence of mfLTP, in Rab3A$^{-/-}$ mice tested with two different genetic backgrounds, did not affect delay fear conditioning or the Morris water maze (Hensbroek et al., 2003). Our own data and those of D'Adamo et al. confirmed this finding (D'Adamo et al., 2004; Powell et al., 2004). A more thorough behavioral characterization of the Rab3A$^{-/-}$ mice,

Table 2 Summary of long-term presynaptic plasticity mutants and behavioral results

	mfLTP	*CA1 LTP*	*Fear conditioning*	*Spatial memory*	*Other*	*Reference(s)*
PKA Cβ1−/−	↓	Normal E-LTP ↓ L-LTP	Normal	Normal water maze and Barnes maze	↓ CA1 LTD	Huang et al., 1995; Qi et al., 1996
PKA R1β**−/−**	↓	Normal E-LTP Normal L-LTP	Normal	Normal water maze and Barnes maze	↓ CA1 LTD	Brandon et al., 1995; Huang et al., 1995
Rab3A−/−	↓	Normal E-LTP	Normal	Normal water maze	↑ CA1 PPF	Castillo et al., 1997; Castillo et al., 2002; Powell et al., 2004
	↓		Normal Normal delay and trace fear conditioning	Normal water maze Normal initial water maze ↓ Reversal learning		Hensbroek et al., 2003 D'Adamo et al., 2004
Type I adenylyl cyclase−/−	↓	↓ E-LTP	–	↓ Water maze		Villacres et al., 1998;Wu et al., 1995
MGluR1−/−	↓	Normal E-LTP			↓ Cerebellar LTP ↓ Corticostriatal plasticity Cerebellar synaptic abnormalities	Bordi et al., 1997; Conquet et al., 1994;Gubellini et al., 2001; Lapointe et al., 2004
PAC1−/−	↓	–	↓ Contextual normal cue	Normal water maze	↓ Anxiety ↑ Locomotor activity Normal PP-dentate LTP	Otto et al., 2001a; Otto et al., 2001b

PKA cβ1, catalytic subunit of protein kinase A; PKA R1β, regulatory subunit of protein kinase A; E-LTP, early phase of long-term potentiation (lasting 1–3 h); L-LTP, late protein synthesis-dependent phase of long-term potentiation; MGluR1, metabotropic glutamate receptor type 1; PAC1, pituitary adenylyl cyclase receptor type 1; PP-dentate LTP, LTP of the perforant path to dentate granule cell in the hippocampal formation.

however, indicated deficits in the delayed match to place version of the Morris water maze and in working memory (D'Adamo et al., 2004). A subsequent study of Rab3A$^{-/-}$ mice supported normal spatial learning and contextual fear conditioning, though the authors found a deficit in cue-mediated fear conditioning (Yang et al., 2007). Overall, these data indicate that mfLTP is only required for more complex forms of spatial memory but not for initial learning of fear conditioning or the Morris water maze.

Other reports have suggested a more direct role for mfLTP in learning tasks. Mice lacking the metabotropic glutamate receptor (mGluR1) and type I adenylyl cyclase (AC1) also show subtle deficits in the Morris water maze (Conquet et al., 1994; Wu et al., 1995; Villacres et al., 1998). AC1 knockout mice also exhibit abnormalities in area CA1 LTP, which could explain their learning and memory deficits (Wu et al., 1995). Similarly, mGluR1 knockouts had severe motor abnormalities (Conquet et al., 1994), cerebellar synaptic abnormalities (Conquet et al., 1994), and synaptic plasticity abnormalities at corticostriatal and other synapses (Bordi et al., 1997; Gubellini et al., 2001; Gubellini et al., 2003; Lapointe et al., 2004). Another line of mice, PACAP (pituitary adenylyl cyclase activating peptide) type I receptor knockout mice, have absent mfLTP and normal water maze learning but impaired fear conditioning (Otto et al., 2001a,b). However, PACAP knockout mice also exhibit impairments in other emotional behaviors (Otto et al., 2001b). Because these molecules are upstream of the likely final common effectors of mfLTP, there are many opportunities for molecular divergence and effects on neuronal functions unrelated to mfLTP, making direct interpretation difficult.

The three molecular manipulations closest to the presumed final effectors of mfLTP expression, Rab3A and R1β or Cβ1 subunits of PKA, all lead to absent mfLTP with intact learning and memory on multiple paradigms. Three separate groups, in more than two different genetic backgrounds, have confirmed the results in Rab3A$^{-/-}$ mice. Among the remaining studies, only one of two learning and memory paradigms, either fear conditioning or water maze, was affected where both were tested, and the affected paradigm varied across studies. Thus, although it is possible that specific molecular accommodations account for these differences, the simplest interpretation is that mfLTP is involved in more complex, episodic-like spatial memory tasks. mfLTP does not appear to be consistently involved in classic water maze or delay or trace fear conditioning. Still, additional studies of existing mice will be helpful in both confirming the involvement of mfLTP in episodic-like memory as well as its lack of involvement in fear conditioning and water maze learning.

4.36.7 Role for Additional Presynaptic Proteins in Learning and Memory Behavior

4.36.7.1 RIM1α

RIM1α is an active zone protein primarily expressed in the brain that is involved in several aspects of presynaptic function (Castillo et al., 2002; Schoch et al., 2002; Calakos et al., 2004). RIM1α was identified as a putative Rab3A effector protein and found to be highly localized to the active zone region of central synapses (Wang et al., 1997). RIM1α interacts with multiple presynaptic active zone and synaptic vesicle proteins *in vivo* and is thought to act as a molecular scaffolding protein involved in organizing and modulating synaptic release machinery (**Figure 1**) (Wang Y et al., 1997, 2000, 2002; Betz et al., 2001; Coppola et al., 2001; Ohtsuka et al., 2002; Schoch et al., 2002; Takao-Rikitsu et al., 2004). Interactions *in vitro* occur between RIM1α and active zone components Munc13-1 (Betz et al., 2001), SNAP25, voltage-gated Ca^{2+} channels (Coppola et al., 2001), and the cytomatrix at the active zone-associated structural protein (CAST) (Takao-Rikitsu et al., 2004) as well as the synaptic vesicle–associated proteins Rab3A (Wang et al., 1997) and synaptotagmin 1 (Coppola et al., 2001). Additionally, RIM1α interacts with 14-3-3 (Sun et al., 2003), α-liprins (Schoch et al., 2002), and a family of RIM binding proteins (Wang et al., 2002). Its selective localization to presynaptic active zones and its interactions with multiple presynaptic molecules place RIM1α in a key position to modulate presynaptic release and plasticity.

Available evidence from acute hippocampal slices suggests that RIM1α modulates neurotransmitter release and presynaptically mediated plasticity. Mice lacking RIM1α (RIM1$\alpha^{-/-}$) display an ~50% reduction in probability of evoked neurotransmitter release (Pr) at the Schaffer collateral to CA1 pyramidal neuron synapse in hippocampal slices (Schoch et al., 2002). This decrease in Pr is accompanied by an increase in PPF in the same synapses. Prolonged stimulation at 14 Hz reveals a lack of normal synaptic

depression (Schoch et al., 2002). In addition, PTP, another presynaptically expressed form of short-term plasticity, is augmented in RIM1$\alpha^{-/-}$ mice (Schoch et al., 2002). Analysis of hippocampal synapses in RIM1$\alpha^{-/-}$ mice at the electron microscopic level revealed no significant ultrastructural abnormalities. Additionally, Western blot data analyzing multiple other synaptic proteins revealed only a reduction in Munc13-1 levels. Reduction of Munc13-1 in Munc13-1 heterozygous mice, however, does not lead to any of the synaptic physiology abnormalities seen in RIM1$\alpha^{-/-}$ mice (Schoch et al., 2002), so decreased Munc13-1 is unlikely to contribute to the RIM1α knockout phenotype.

A subsequent study of RIM1α-deficient hippocampal autapses in culture revealed that the RIM1$\alpha^{-/-}$ mice exhibit decreased Pr via a decrease in the readily releasable pool of synaptic vesicles (Calakos et al., 2004). Curiously, the decreased Pr in RIM1$\alpha^{-/-}$ hippocampal cultures is observed only during the initial 4–5 stimuli during sustained, 14-Hz stimulation (Calakos et al., 2004). Wild-type synapses exhibit depression of Pr during 14-Hz stimulation, whereas RIM1$\alpha^{-/-}$ synapses do not. Thus, the relationship between Pr and frequency of synaptic activation in the RIM1$\alpha^{-/-}$ mice is complex. In autaptic hippocampal cultures, at low frequencies, RIM1$\alpha^{-/-}$ mice exhibit decreased Pr, whereas at 14 Hz, and presumably higher frequencies, the Pr of RIM1$\alpha^{-/-}$ mice becomes similar to that of wild-type mice after the first four to five responses (Schoch et al., 2002). Nevertheless, loss of RIM1α affects presynaptically mediated forms of synaptic plasticity including PPF, PTP, and synaptic depression at 14 Hz.

RIM1$\alpha^{-/-}$ mice were also deficient in presynaptically expressed PKA-dependent forms of LTP. At the mossy fiber to CA3 pyramidal neuron synapse in the hippocampus, mfLTP is absent in RIM1$\alpha^{-/-}$ mice (Castillo et al., 2002). Similarly, presynaptically expressed LTP at the parallel fiber/Purkinje cell synapse in the cerebellum is absent in these mice (Castillo et al., 2002; Lonart et al., 2003). RIM1α is a PKA substrate *in vivo* and is primarily phosphorylated by PKA at serine 413 (Lonart et al., 2003), and PKA is the only known kinase for this site (Lonart et al., 2003). Phosphorylation of RIM1α at serine 413 is also required for presynaptically expressed LTP in cerebellar neurons. RIM1$\alpha^{-/-}$ cerebellar cultures lack presynaptic LTP, which can be rescued by expression of wild-type RIM1α. Expression of PKA phosphorylation site mutant RIM1αS413A fails to rescue presynaptic LTP in the same cultures (Lonart et al., 2003). Thus, phosphorylation of RIM1α by PKA is thought to be responsible for synaptic potentiation via increased Pr, or at least to be permissive for some other mechanism of increased Pr.

RIM1α's interaction with 14-3-3 protein and the small, synaptic vesicle associated GTPase Rab3A is also required for presynaptic LTP at least in cerebellar cultured neurons (Simsek-Duran et al., 2004). Thus presynaptically expressed forms of LTP are thought to require presynaptic activation of PKA and phosphorylation of RIM1α, which leads, via required interactions with 14-3-3 and Rab3A, to increased Pr (or RIM1α phosphorylation, and its interactions are permissive for a parallel mechanism to increase Pr).

The abnormalities in both short- and long-term plasticity in RIM1$\alpha^{-/-}$ mice led to the question of whether RIM1α might play a critical role in learning and memory. A broad behavioral analysis of RIM1$\alpha^{-/-}$ mice revealed abnormalities in learning and memory in both Morris water maze and fear conditioning (Powell et al., 2004). Despite the various synaptic physiology abnormalities and the widespread expression of RIM1α throughout the brain, RIM1$\alpha^{-/-}$ mice revealed normal motor coordination and anxiety-like behaviors (Powell et al., 2004).

The abnormalities in Morris water maze behavior imply abnormal hippocampus-dependent learning in the RIM1$\alpha^{-/-}$ mice. Although RIM1$\alpha^{-/-}$ mice are deficient in mfLTP, Rab3A$^{-/-}$ mice with mfLTP deficits, increased PPF, and abnormal synaptic depression exhibit normal learning and memory (Powell et al., 2004). Because mfLTP, PPF, and depression alone are unlikely to play a role on the RIM1α learning and memory deficits, the decrease in Pr in area CA1 seemed a likely candidate.

A decrease in Pr in isolation, however, does not necessarily alter learning and memory behavior. Syt1R233Q$^{+/+}$ mice have a similar decrease in Pr in CA1 as the RIM1$\alpha^{-/-}$ mice, but they exhibit normal learning and memory using the same tasks (Powell et al., 2004). Regardless of any molecular accommodations in these mice, the decreased Pr remains, suggesting that Syt1R233Q$^{+/+}$ mice can somehow accommodate at the synaptic or circuit level to dramatic alterations in their Pr setpoint.

Other possibilities remain to explain the behavioral abnormalities in RIM1α-deficient mice. These include increased PTP at excitatory synapses and altered PPD at inhibitory synapses. Furthermore,

the decreased Pr in RIM1$\alpha^{-/-}$ mice may differ from that of Syt1R233Q$^{+/+}$ mice. For example, it is possible that the synaptotagmin 1 mutation affects inhibitory and excitatory synapses equally, whereas the loss of RIM1α may affect excitatory more than inhibitory synapses. Such differential regulation of neurotransmitter release between excitatory and inhibitory synapses has been described for other presynaptic proteins such as Munc13-1 and Munc13-2 (Varoqueaux et al., 2002). In addition, it may be that the multiple synaptic abnormalities in RIM1$\alpha^{-/-}$ mice interact to lead to the behavioral deficits, though this conclusion requires excluding individual synaptic abnormalities as a cause. Finally, although LTP in area CA1 of the hippocampus was reportedly normal in RIM1$\alpha^{-/-}$ mice, this experiment was performed in the presence of $GABA_A$ antagonists using a very strong, 3-s, 100-Hz, LTP induction paradigm. Huang et al. (2005) showed that RIM1$\alpha^{-/-}$ mice exhibited defects in late phases of LTP in area CA1, potentially implicating abnormal long-term plasticity in the RIM1$\alpha^{-/-}$ behavioral deficits.

In fear conditioning, RIM1$\alpha^{-/-}$ mice were abnormal in both context-dependent and cue-dependent fear conditioning. The abnormal cue-dependent fear conditioning implicates RIM1α in amygdala function. Thus, it will be of particular interest to examine LTP in the amygdala, where some forms of LTP are thought to be NMDA receptor-independent. This is a particularly interesting proposition in light of recent data suggesting that some forms of heterosynaptic LTP in amygdala are presynaptically induced and expressed.

Although the loss of RIM1α leads to learning and memory deficits, many questions remain. Is RIM1α modulated during learning *in vivo*? Could RIM1α be phosphorylated at its PKA site in the hippocampus during learning? Does phosphorylation of this PKA site play an active role in learning and memory *in vivo* or is some more basic function of RIM1α merely permissive for normal LTP? What RIM1α binding proteins might also be important for learning and memory? Does the loss of RIM1α in a particular brain region correlate with the learning and memory deficits? These are questions currently under active investigation.

4.36.7.2 Munc13-3

Munc13 proteins make up a family of three presynaptic proteins important for synaptic vesicle priming (Brose et al., 2000). The Munc13-3 isoform is strictly localized to cerebellar neurons, granule and Purkinje neurons in particular (Augustin et al., 1999). Augustin et al. examined Munc13-3 knockout mice and observed *increased PPF* at parallel fiber-Purkinje cell synapses with no alteration in synaptic response to sustained 14-Hz stimulation (other synaptic parameters were not reported) (Augustin et al., 2001). In addition, spontaneous miniature inhibitory postsynaptic responses were unchanged in Munc13-3$^{-/-}$ mice. Analysis of cerebellar synapses at the electron microscopic level revealed normal synaptic density and normal synaptic ultrastructure. Examination of multiple additional synaptic proteins showed no compensatory changes in other synaptic proteins. This regionally selective, synaptic perturbation at cerebellar synapses led to a marked deficit in motor learning with no change in baseline coordination using the rotating rod apparatus. These motor learning abnormalities were reminiscent of similar abnormalities in the mGluR4 subunit of metabotropic glutamate receptor knockout mice that showed decreased PPF at parallel fiber-Purkinje cell synapses. Thus, it seems that alterations in PPF at this synapse critically impair motor learning without affecting baseline coordination (Augustin et al., 2001). This conclusion is, as always, tempered by the lack of information from other synapses and additional forms of synaptic plasticity such as LTP and LTD at these synapses.

4.36.7.3 GAP-43

Elegant experiments over the past several years were among the first to implicate the presynaptic growth associated protein (GAP-43, F1, B50) in normal learning and memory. These studies by Routtenberg and colleagues (2000) identified GAP-43 as a presynaptic protein kinase C (PKC) substrate that is phosphorylated during LTP in the dentate gyrus. They showed that GAP-43 is required for normal learning and memory as assessed in GAP43 heterozygous knockdown mice (Rekart et al., 2005). Indeed, transgenic overexpression of GAP43, but not the PKC phosphorylation site mutant form of GAP43, has been shown to increase both dentate gyrus LTP and learning (Routtenberg et al., 2000). It seems that PKC phosphorylation of presynaptic GAP-43 is required for normal learning *in vivo* and that GAP-43 can bidirectionally modulate learning and memory *in vivo*.

Several possible mechanisms for GAP-43's involvement in learning and memory have been proposed,

including direct modulation of presynaptic neurotransmitter release (Routtenberg et al., 2000). GAP-43 has been shown to interact directly with components of the synaptic release machinery including the SNARE complex proteins (SNAP-25, syntaxin, and synaptobrevin) and synaptotagmin (depicted in **Figure 1**) (Haruta et al., 1997). Some of these interactions seem to be dependent on PKC phosphorylation of GAP-43 (Haruta et al., 1997), making this interaction intriguing for the dynamic regulation of plasticity and memory. In fact, GAP-43 phosphorylation may lead to increased neurotransmitter release (Dekker et al., 1990; Heemskerk et al., 1990), whereas decreasing GAP-43 may decrease evoked neurotransmitter release (Ivins et al., 1993; Hens et al., 1995). Whereas presynaptic effects of GAP-43 may serve to enhance plasticity and memory, another possible mechanism is GAP-43's involvement in synaptic and circuit development, which might function to regulate plasticity and memory via developmental effects.

4.36.7.4 SNAP-25

Reduction of the SNARE complex protein SNAP-25 expression via antisense oligonucleotide infusion into CA1 impaired contextual fear conditioning and spatial memory (Hou et al., 2004). SNAP-25 antisense also reduces CA1 LTP, apparently without affecting presynaptic release or short-term plasticity (Hou et al., 2004). The authors suggest that the reduction in SNAP-25 is acting postsynaptically to reduce LTP as well as learning and memory (Hou et al., 2004). The precise mechanism whereby this SNARE complex protein affects learning and memory remains to be determined.

4.36.7.5 H-ras

Transgenic mice overexpressing a constitutively active form of the small, GTP binding protein H-ras (H-ras^{G12V}) exhibit increased PPF, increased LTP, and increased learning and memory. In a very thorough study by Kushner et al., transgenic, H-ras^{G12V}–overexpressing mice showed increased activation of the extracellular regulated kinase (ERK), increased ERK-dependent phosphorylation of the presynaptic protein synapsin I, and increased docked vesicles (Kushner et al., 2005). These changes were also associated with increased numbers of docked synaptic vesicles, increased PPF, increased LTP, and increased miniature excitatory postsynaptic current frequency in area CA1 of the hippocampus. These changes were associated with enhanced water maze and contextual fear conditioning learning and memory in these mice. By crossing the H-ras^{G12V} transgenic mice with synapsin I deficient mice, Kushner et al. were able to show that most of these changes were dependent on synapsin I, providing a compelling link between activation of H-ras, activation of ERK, phosphorylation of synapsin I, and enhanced learning and memory via a presynaptic mechanism (Kushner et al., 2005). Although the physiologic relevance of this mechanism *in vivo* has yet to be determined, these studies provide a clear link between presynaptic functional manipulations, LTP, and memory.

4.36.8 The Future of Presynaptic Plasticity and Learning and Memory

The study of molecular mechanisms involved in presynaptic plasticity is in its infancy. One major obstacle in understanding presynaptic mechanisms in synaptic plasticity is the relative difficulty in accessing the intracellular presynaptic compartment both electrophysiologically and pharmacologically owing to its small size and spatial isolation from the presynaptic cell body. Fortunately, techniques for imaging presynaptic vesicle release, recycling, and trafficking are making the presynaptic compartment more accessible than ever before. Furthermore, our understanding of molecular mechanisms of presynaptic neurotransmitter release and modulation of neurotransmitter release is advancing rapidly through the use of genetic tools to dissect the functions of individual presynaptic proteins and protein components (Sudhof, 2004). The genetic tools deciphering presynaptic function hold great promise for our future understanding of how activity-dependent forms of plasticity are mediated molecularly in presynaptic terminals.

Even further behind are studies implicating presynaptic plasticity and individual presynaptic proteins in cognitive function. A complete picture of how presynaptic short-term plasticity, long-term plasticity, or presynaptic signaling is involved in learning and memory has not yet emerged. Ongoing studies will continue to examine existing presynaptic protein knockout mice that have been extensively characterized electrophysiologically. Future studies will focus on restricted, conditional knockout approaches, modulation of presynaptic proteins during learning, and examining how such dynamic regulation of presynaptic proteins is involved in learning and memory.

With some exceptions, the existing studies stop short of understanding how dynamic modulation of presynaptic proteins might play a role in both synaptic plasticity and learning. It will be important to understand how presynaptic proteins are modified during synaptic plasticity and during learning tasks *in vivo*. More precise molecular manipulations in mice including point mutations that alter targets of these modifications should also prove informative.

Also, it is likely that presynaptic proteins and presynaptic plasticity are critical for learning and memory in specific brain regions and indeed particular neuronal subtypes. Clearly, following screening studies on existing knockout mice, regionally targeted, conditional knockout approaches will be critical for understanding how specific molecules fit into various memory circuits. For example, the use of conditional knockout mice combined with focal, virally mediated Cre recombinase expression is one novel way to approach this problem (Hommel et al., 2003).

The use of genetic manipulations in combination with presynaptic electrophysiologic and imaging studies will be critical to understanding the molecular basis of presynaptic plasticity.

All of these issues must be approached in a multidisciplinary fashion combining targeted, conditional genetic manipulations in mice, broader behavioral screening, thorough electrophysiological characterization, biochemical studies, and correlation with effects on synaptic plasticity. We are now in an excellent position to identify molecular mechanisms of presynaptic plasticity and to link these mechanisms to cognition, thereby identifying potential new targets in the treatment of cognitive disorders. We must include presynaptic plasticity and presynaptic molecular mechanisms to gain a complete picture of the molecular, cellular, and physiologic basis of learning and memory.

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4.37 Regulation of Synaptic Function by Endocannabinoids

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4.37.1 Introduction

The synapse is the fundamental functional unit for intercellular communication between neurons in many nervous systems. Communication across synapses in the brain involves chemical messengers (neurotransmitters, neuromodulators, and growth factors among them) that traverse the synaptic cleft to interact with cognate receptors. It is now appreciated that this communication is bidirectional, as neurochemicals released from both presynaptic and postsynaptic neurons can produce transsynaptic

signals (Stanton et al., 2004). In addition, neurochemical release can activate receptors on the same cell in a homotypic fashion.

A variety of compounds act as neurotransmitters, neuromodulators, and growth factors, ranging from amino acids and other small molecules to large polypeptides. In addition to functioning in the regulation of synapse formation and synaptic transmission, these synaptic neurochemicals have also been implicated in synaptic plasticity of the types thought to underlie in learning and memory (see other articles in this volume as well as Stanton et al., 2004).

In the present article the focus is on synaptic modulation and plasticity involving endocannabinoids (ECs) that act as retrograde messengers linking postsynaptic signals to presynaptic mechanisms of plasticity expression. Recent studies have implicated this EC signaling in plasticity at a wide range of synapses, and thus it seems to be a widespread mechanism for synaptic regulation (Alger, 2002; Gerdeman et al., 2002; Chevaleyre et al., 2006). The mechanisms of EC production and release, as well as regulation of the duration of EC, signaling will be discussed. In addition, the mechanisms activated by EC interaction with receptors and the role of these mechanisms in synaptic physiology will be considered. Finally, some discussion of emerging evidence that EC-dependent synaptic plasticity participates in learning and memory will be presented.

4.37.2 What is an Endocannabinoid?

In addition to serving as barriers to separate and compartmentalize cells and subcellular domains, cell membranes are also rich reservoirs of biomolecules that can be used for intracellular and intercellular communication. Lipids are a prominent membrane source of precursors for such signaling molecules. Metabolism of membrane lipids by phospholipases is a well-known mechanism for generation of intracellular second messengers (Hokin and Hokin, 1953; Fain and Berridge, 1978). Roles for lipid metabolites in neural intercellular communication are also well established. For example, prostaglandins and related lipid metabolites that act as autocrine and paracrine agents throughout the body also have such signaling roles in the nervous system (see Bazan, 2005, for review).

Endogenous cannabinoids, or ECs, are a class of lipid metabolites that was discovered relatively recently. These fatty acid derivatives are synthesized by modification and/or metabolism of arachidonate-containing membrane lipids. The two most prominent ECs are arachidonoylethanolamide (AEA or anandamide) (Devane et al., 1992) and 2-arachidonoyl glycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995). These hydrophobic messengers are now referred to as ECs because of their actions on the receptors for Δ9-tetrahydrocannabinol (THC), the major psychoactive component of cannabis-derived drugs such as marijuana. While AEA and 2-AG have actions at other molecular targets (van der Stelt and Di Marzo, 2005a; Oz, 2006), it is clear that cannabinoid (CB) receptors are among the major targets of these compounds.

4.37.2.1 Anandamide and 2-Arachidonoyl and Their Biosynthesis

Endocannabinoid synthesis involves a variety of biochemical pathways, some of which are well established, and others that are relatively novel. Production of AEA is thought to arise predominantly from phosphatidylethanolamine (PE) via a two-step process involving transacylation to produce an N-arachidonoyl-PE (NAPE) intermediate, followed by cleavage via phospholipase D (Schmid et al., 1990; Di Marzo et al., 1994). However, a new study suggests a novel AEA synthesis pathway involving phospholipase C (PLC) (Liu et al., 2006). Synthesis of 2-AG also involves well-known phospholipases. The predominant mechanism is PLC-mediated hydrolysis of an arachidonic acid–containing phospholipid to diacylglycerol (DAG) and subsequent deacylation at position 1 by DAG lipase (Mechoulam et al., 1995; Sugiura et al., 1995; Stella et al., 1997). Alternative synthesis pathways involving other phospholipases have also been proposed (Bisogno et al., 1999). Increased intracellular calcium can stimulate enzymes involved in the synthesis pathways for both AEA and 2-AG and appears to be an effective mechanism for 'on-demand' EC synthesis in neurons (Di Marzo et al., 1994; Bisogno et al., 1999).

4.37.2.2 Regulation of Synaptic Endocannabinoid Levels

Endocannabinoids are believed to be produced inside of cells, but the majority of their demonstrated actions involve signaling from extracellular sites. Thus, ECs are presumed to be released from cells, including

neurons, into the extracellular environment. There is good evidence for extracellular ECs in neural tissue, including neuronal cultures (Di Marzo et al., 1994) and brain dialyzate (Giuffrida et al., 1999). Increases in extracellular EC levels have been observed following stimulation of cell surface receptors and neuronal depolarization (Di Marzo et al., 1994; Giuffrida et al., 1999; Jung et al., 2005). The mechanisms by which hydrophobic ECs exit the cell and traverse the hydrophilic extracellular space are still not clear, with investigations focused on two potential mechanisms, simple diffusion or transporter/carrier-assisted movement through the membrane (Hillard and Jarrahian, 2000; Glaser et al., 2003; Fegley et al., 2004; McFarland and Barker, 2004; Ronesi et al., 2004; Moore et al., 2005). Extraction of these lipophilic messengers from the membrane may also require some sort of carrier protein in the extracellular space (Makriyannis et al., 2005a,b).

Termination of EC signaling may involve the proposed transmembrane transporter system mentioned above. Degradative enzymes also play key roles in creating a transmembrane EC gradient that favors uptake under most conditions. These enzymes also participate in termination of EC signaling. Anandamide can be degraded by fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996) or by cyclooxygenase-2 (COX-2) (Kozak et al., 2002), although the FAAH pathway seems to predominate in neurons (Glaser et al., 2003). The degradation of 2-AG appears to be mediated by both COX-2 (Kozak et al., 2002) and monoglyceride lipase (MGL) (Dinh et al., 2002).

4.37.3 CB1 Receptors

4.37.3.1 Cannabinoid Receptors and Signaling

The actions of CB drugs and ECs on CB receptors account for many of their effects in the nervous system. Within the brain, the CB1 receptor appears to be the predominant receptor subtype (Matsuda et al., 1990; Freund et al., 2003; Mackie, 2005), although recent evidence indicates the presence of CB2 receptors in rodent brain (Van Sickle et al., 2005; Gong et al., 2006). The CB1 receptor is a member of the classical family of G-protein-coupled receptors that includes the muscarinic acetylcholine (ACh) and adrenergic receptors (Matsuda et al., 1990). As such, CB1 receptor activation produces modulatory effects via its ability to activate G-proteins. It appears that the Gi/o G-proteins are the major subtypes coupled to CB1 (Howlett et al., 1986; Matsuda et al., 1990). Activation of Gi/o is generally thought to be 'inhibitory,' because this G-protein inhibits adenylyl cyclase activity through its α subunit and produces inhibitory neuronal signals such as activation of K^+ channels and inhibition of Ca^{2+} channels via liberation of the associated β/γ subunits (Clapham and Neer, 1997). The majority of CB1 receptor–mediated responses observed in neurons to date appear to involve such inhibitory mechanisms (Freund et al., 2003), with notable exceptions (e.g., Glass and Felder, 1997). Through heterotrimeric G-protein signaling, CB1 receptor activation can impact acute electrophysiological properties of neurons and produce longer-lasting biochemical changes that could subtly influence neuronal function. Indeed, CB1 activation can stimulate signaling molecules, such as extracellular-signal-related kinase (ERK), that alter protein phosphorylation and gene expression (Wartmann et al., 1995; Davis et al., 2003). Thus, CB1 receptors are poised to produce transient and long-lasting effects on neurophysiology.

4.37.3.2 CB1 Receptor Pharmacology

In addition to Δ9-tetrahydrocannabinol (Δ9-THC) and ECs, a number of synthetic agonists for CB1 receptors have been created (Pertwee, 2005; Reggio, 2005). These compounds generally display high efficacy at the receptor. Synthetic CB1 antagonists have also been produced (Pertwee, 2005; Reggio, 2005). These compounds appear to have the capacity to act as inverse agonists, reducing intrinsic receptor activity under conditions where such activity is high (Pertwee, 2005). However, it is not yet clear if this action is relevant to their pharmacological effects, as ECs are likely to be present in tissue and perhaps even in single cells. In this case effects of these compounds in the absence of explicitly added agonist or tissue stimulation could reflect pure antagonism of EC actions.

4.37.3.3 Neuronal CB1 Receptors Are Mainly Presynaptic

Within the central nervous system (CNS), CB1 receptor immunoreactivity is found mainly at presynaptic terminals (Freund et al., 2003; Mackie, 2005). However, there is also immunological evidence for low levels of postsynaptic receptor expression in a few brain regions, such as the striatum (Tsou et al.,

1997). Sporadic reports of postsynaptic CB1 signaling have appeared. For example, CB1 receptors on neocortical low-threshold-spiking interneurons produce an inhibitory postsynaptic potential involving activation of a K^+ channel (Bacci et al., 2004). However, the bulk of physiological and neurochemical evidence also supports a predominantly presynaptic role for CB1 receptors (Alger, 2002; Mackie, 2006). These presynaptic receptors generally inhibit neurotransmitter release. Two mechanisms have been predominantly suggested to underlie this presynaptic inhibition, activation of K^+ channels and modulation of voltage-gated Ca^{2+} channels (Mackie and Hille, 1992; Henry and Chavkin, 1995; Mackie et al., 1995; Pan et al., 1996; Bacci et al., 2004; Guo and Ikeda, 2004). While it is not yet clear which of these mechanisms predominates in synaptic modulation at all synapses, evidence from direct studies of calcium dynamics at cerebellar synapses and the calyx of Held favors inhibition of calcium channels (Brown et al., 2004; Kushmerick et al., 2004). In addition, a recent paper indicates that CB1-mediated synaptic depression is itself modulated via frequency-dependent changes in the function of N-type calcium channels (Foldy et al., 2006). This issue will be discussed in more detail later in this article. Regardless of the mechanism, it is clear that CB1 activation inhibits neurotransmitter release on an acute timescale. This mechanism can account for the short-lasting forms of synaptic modulation that will be discussed below. However, it appears that additional mechanisms need to be invoked to explain CB1 participation in long-term synaptic plasticity.

4.37.4 Depolarization-Induced Suppression of Synaptic Transmission

4.37.4.1 Depolarization-Induced Suppression of Excitation and Inhibition

Transient synaptic depression involving EC activation of CB1 receptors underlies several forms of short-term synaptic plasticity at excitatory glutamatergic and inhibitory gamma-aminobutyric-acid (GABA)ergic synapses (see Alger, 2002; Chevaleyre et al., 2006, for review). The first of these to be discovered were depolarization-induced suppression of excitation (DSE) and inhibition (DSI) (Llano et al., 1991; Pitler and Alger, 1992). Postsynaptic depolarization of cerebellar Purkinje neurons or CA1 hippocampal pyramidal neurons leads to a decrease in glutamatergic (cerebellum, Llano et al., 1991) and GABAergic (hippocampus, Pitler and Alger, 1992) transmission onto the postsynaptic neuron. Several pieces of evidence in these studies indicated that this synaptic depression involved decreased glutamate or GABA release from presynaptic terminals. More recent findings indicate that CB1 receptors are expressed presynaptically, but not postsynaptically, at synapses that show DSI and DSE (see Freund et al., 2003; Mackie, 2005, for review).

Examination of DSE/I in hypothalamus is providing information that could prove relevant for understanding EC roles in feeding and bodily homeostasis. In particular, the role of the EC system in feeding behavior has led investigators to postulate actions of these neuromodulators in the hypothalamic feeding circuitry (Giuliani et al., 2000; Di Marzo et al., 2001). Recent papers from three laboratories have confirmed that retrograde EC signaling modulates neurotransmitter release in hypothalamus (Di et al., 2003; Hentges et al., 2005; Jo et al., 2005). The EC effects include DSE at synapses onto magnocellular neurons in the supraoptic nucleus (SON) (Di et al., 2003) and DSI at synapses onto lateral hypothalamic neurons that express melanin-concentrating hormone (Jo et al., 2005). In the arcuate nucleus GABAergic inhibitory inputs to proopiomelanocortin (POMC)-expressing neurons appear to be under tonic EC suppression (Hentges et al., 2005). This is not true of the neurons that do not express POMC. The tonic disinhibition appears to be due to postsynaptic EC production, as increases in inhibitory transmission produced by CB1 antagonists are blocked by postsynaptic loading of calcium chelators. Thus, like many other instances of tonic EC signaling in brain, the tone is due to endogenous agonist activation of CB1 receptors rather than autonomous CB1 receptor activity (cf. Zhu and Lovinger, 2005).

Various forms of EC-dependent DSE and DSI have now been observed in several other brain regions including basolateral amygdala (BLA) (Zhu and Lovinger, 2005), brainstem (Mukhtarov et al., 2005), dentate gyrus (Isokawa and Alger, 2005), neocortex (Trettel and Levine, 2003; Bodor et al., 2005), striatum (Narushima et al., 2006), substantia nigra (Yanovsky et al., 2003), and the ventral tegmental area (VTA) (Melis et al., 2004a), in addition to the cerebellum and CA1 region of the hippocampus. **Table 1** summarizes our current state of knowledge concerning synapses that exhibit DSE/I and the

Table 1 Characteristics of DSE/DSI and STD in different brain regions[a]

Brain region	*DSE/DSI receptors*	*Ion channels*	*Expression involved*	*Involved measure*
Basolateral amygdala (Zhu and Lovinger, 2005)	DSI	mGluR, CB1		sIPSC frequency
Brainstem, hypoglossal nucleus (Mukhtarov et al., 2005)	DSI (glycinergic synapses)		Postsynaptic VGCCs & NMDARs	eIPSCs, paired-pulse responses, synaptic failures, mIPSCs
Calyx of Held (Kushmerick et al., 2004)	STD	mGluR, CB1	Postsynaptic VGCCs, presynaptic VGCCs	eEPSCs, presynaptic calcium levels, presynaptic capacitance
Cerebellum climbing fibers (Maejima et al., 2005; van Beugen et al., 2006)	STD	mGluR, CB1	Postsynaptic VGCCs, presynaptic VGCCs	eEPSCs, presynaptic calcium levels, prevents PF-LTP
Cerebellum parallel fibers (Llano et al., 1991; Kreitzer and Regehr, 2001; Brown et al., 2003)	DSE, STD	mGluR, CB1	N, P/Q, R-type VGCCs	eIPSCs, sIPSCs, presynaptic calcium transients
Cerebellum parallel fiber-stellate synapses (Rancillac and Barbara, 2005)	STD	CB1		eEPSCs, synaptic failures
Cerebellum inhibitory inputs (Galante and Diana, 2004)	STD	mGluR, CB1		eIPSCs, sIPSCs
Hippocampus (see Alger, 2002, for review; Heinbockel et al., 2005)	DSI, STD	mAChR, mGluR, CB1	Postsynaptic VGCCs, presynaptic N-type VGCCs	eIPSCs, sIPSCs, anandamide uncaging
Hypothalamus, SON (Di et al., 2003)	DSE	Glucocorticoid receptor	Postsynaptic VGCCs	mEPSCs
Hypothalamus, arcuate nucleus (Hentges et al., 2005)	EC tone	CB1		eIPSCs, mIPSCs
Hypothalamus, lateral nucleus (Jo et al., 2005)	DSI		Postsynaptic VGCCs	eIPSCs, sIPSCs
Sensory cortex (Trettel and Levine, 2003)	STD	mAChR	Postsynaptic VGCCs	eISPCs, mIPSCs, paired-pulse responses
Striatum (Narushima et al., 2006)	DSE, STD	mGluR, mAChR, CB1	Postsynaptic VGCCs	eEPSCs, paired-pulse responses
Striatum (Yin and Lovinger, 2006)	STD	D2R, mGluR, CB1		eEPSCs, paired-pulse responses
Substantia nigra (Yanovsky et al., 2003)	DSI	mAChR		eIPSCs, sIPSCs
VTA (Melis et al., 2004a)	DSE	CB1	Postsynaptic VGCCs	eEPSPs, eEPSCs, mEPSCs
VTA (Melis et al., 2004b)	STD	mGluR, CB1		eESPCs
VTA (Riegel and Lupica, 2004)	STD	CB1	Postsynaptic VGCCs	$GABA_B$R-mediated eIPSCs

[a]Abbreviations: CB1, cannabinoid 1 receptor; D2R, D2 receptors; DSE/I, depolarization-induced suppression of excitation/inhibition; eEPSC/P, evoked excitatory postsynaptic current/potential; eIPSC, evoked inhibitory postsynaptic current; $GABA_B$R, gamma-aminobutyric acid type B receptor; LTD, long-term depression; mAChR, muscarinic acetylcholine receptor; mEPSC, miniature excitatory postsynaptic current; mGluR, metabotropic glutamate receptor; NMDAR, *N*-methyl-D-aspartate receptor; sIPSC, spontaneous inhibitory postsynaptic current; STD, short-term depression; VGCC, voltage-gated calcium channel; VTA, ventral tegmental area; mIPSC, miniature excitatory postsynaptic current.

mechanisms implicated in these forms of synaptic plasticity.

4.37.4.2 Mechanisms of Depolarization-Induced Suppression of Excitation and Inhibition Induction

Surprisingly little is known about the mechanisms linking depolarization to the postsynaptic EC release apparently needed for DSE/I, despite the fact that DSI and DSE were the first forms of synaptic plasticity shown to involve retrograde EC signaling. Increased postsynaptic intracellular calcium is implicated in both DSI and DSE, as postsynaptic infusion of calcium chelators blocks both forms of plasticity (Llano et al., 1991; Pitler and Alger, 1992), and uncaging-induced increases in postsynaptic calcium induce DSI (Wang and Zucker, 2001; Wilson and Nicoll, 2001). Voltage-gated calcium channels are almost certainly involved in this calcium increase, although the identities of the postsynaptic calcium channels involved in DSE/I have not been fully elucidated. The steps linking the calcium increase to production and release of the EC messenger are less clear. Calcium can activate the phosphotransferases and phospholipases implicated in AEA synthesis, as well as the lipases necessary for 2-AG production (Di Marzo et al., 1994; Bisogno et al., 1999). However, treatment with compounds that block PLC and DAG lipase do not alter DSI in hippocampus (Edwards et al., 2006), despite other evidence that 2-AG is involved in this form of plasticity (Dinh et al., 2002; Kim and Alger, 2004). In hippocampus, DSI is unaffected by gene-targeted knockout of PLCβ1 (Hashimotodani et al., 2005), and similar results were obtained using PLCβ4 knockout mice for cerebellar DSI (Maejima et al., 2005). Both the enzyme inhibitors and PLCβ isoform knockouts prevent other forms of EC-mediated synaptic depression in the same neurons in which they fail to affect DSI or DSE, as will be discussed later in this article. The inability of lipase inhibition to affect DSI/E has led to the suggestion that *de novo* synthesis of the EC retrograde signal is not necessary for plasticity induced by depolarization alone. One idea put forward by Alger and colleagues is that a 'mobilization' step is all that is needed to activate retrograde signaling by preformed ECs in DSE/I (Edwards et al., 2006). However, the biochemical or cell biological nature of such a step is a matter of speculation at this point. Perhaps a fatty acid carrier protein of some sort is involved.

4.37.4.3 Presynaptic Depression during Depolarization-Induced Suppression of Excitation and Inhibition

The postsynaptic release of the EC retrograde messenger during induction of DSE or DSI leads to activation of presynaptic CB1 receptors that inhibit neurotransmitter release (Kreitzer and Regehr, 2001; Maejima et al., 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). The aforementioned mechanisms set into motion by activation of Gi/o-type heterotrimeric G-proteins, namely activation of K^+ channels and inhibition of Ca^{2+} channels, could both inhibit neurotransmitter release. Activation of K^+ channels can reduce the magnitude or duration of presynaptic depolarization, while inhibition of voltage-gated calcium channels directly reduces excitation/secretion coupling. In addition, some Gi/o-coupled receptors inhibit steps involved in vesicle fusion (reviewed in Dunwiddie and Lovinger, 1993). Which of these mechanisms contributes to presynaptic inhibition in DSE and DSI is a matter of debate that has not been resolved at most synapses. Experiments in which CB1 receptors are activated by synthetic agonists indicate that these receptors can activate K^+ channels and inhibit Ca^{2+} channels (Mackie and Hille, 1992; Mackie et al., 1995; Pan et al., 1996; Guo and Ikeda, 2004). Most of the existing evidence indicates that CB1 receptors do not reliably inhibit transmission at a site downstream of these channels (Hoffman and Lupica, 2001; Katona et al., 2001; Zhu and Lovinger, 2005), with notable exceptions (Gerdeman and Lovinger, 2001). Examination of DSE at the parallel fiber (PF) synapses onto cerebellar Purkinje cells has generated convincing evidence that Ca^{2+} channel modulation is the predominant mechanism of inhibition (Brown et al., 2004). Similar studies were also carried out at a specialized sensory synapse known as the calyx of Held (Kushmerick et al., 2004). This synapse consists of a single large presynaptic terminal that is wrapped around the majority of the postsynaptic cell body. The large presynaptic element affords electrophysiologists the opportunity to record ion current in a 'whole-terminal' configuration. Using this recording approach, Kushmerick and coworkers (2004) showed that CB1 activation produces a decrease in synaptic transmission that is accompanied by a decrease in the presynaptic calcium current in the calyx terminal. However, mechanisms of DSE and DSI at other synapses remain to be explored, and it has been suggested that activation of K^+ channels plays a

role in DSE and DSE expression in the cerebellum and hippocampus (Daniel and Crepel, 2001; Varma et al., 2002; Diana and Marty, 2003). **Figure 1(a)** presents a schematic diagram of the molecular events believed to be involved in DSE and DSI.

4.37.4.4 Regulation of the Timing and Duration of Depolarization-Induced Suppression of Excitation and Inhibition

Evidence collected to date suggests that the duration of DSE/I is determined by the length of time that the EC is present and activating CB1 receptors within the synapse. Early experiments with compounds that block cellular uptake of ECs indicated that enhancing EC levels in the slice preparation produced a depression of transmission that occluded DSI (Wilson and Nicoll, 2002). Furthermore, inhibiting the EC-degrading enzymes COX-2 and monoglycerol lipase (MGL) prolongs the duration of DSI, most likely through an increase in the duration of 2-AG presence in the synapse (Kim and Alger, 2004; Makara et al., 2005). Kreitzer and Regehr (2001) demonstrated that DSE at parallel-fiber-Purkinje neuron synapses is well correlated in time with a decrease in the presynaptic calcium transient. Furthermore, the decreased duration of DSE at higher temperatures (Kreitzer and Regehr, 2001) could be explained by increased uptake or enzymatic degradation of the EC and fits well with the idea that DSE/I time course is determined by the synaptic lifetime of the EC messenger.

An elegant series of experiments from Alger and colleagues also supports the idea that DSI follows the time course of EC release and synaptic actions (Heinbockel et al., 2005). These investigators used a photoactivatable form of AEA to precisely time the production of this EC and examine the effect on transmission. Suppression of transmission was rapidly induced with this paradigm, and the time course of this 'uncaging-induced' DSI suggested that it persisted as long as the free EC was present. These lines of evidence all indicate that DSE/I directly result from transient increases in synaptic levels of the EC messenger that activates CB1 receptors, leading to inhibition of presynaptic calcium transients and the resultant depression of neurotransmitter release.

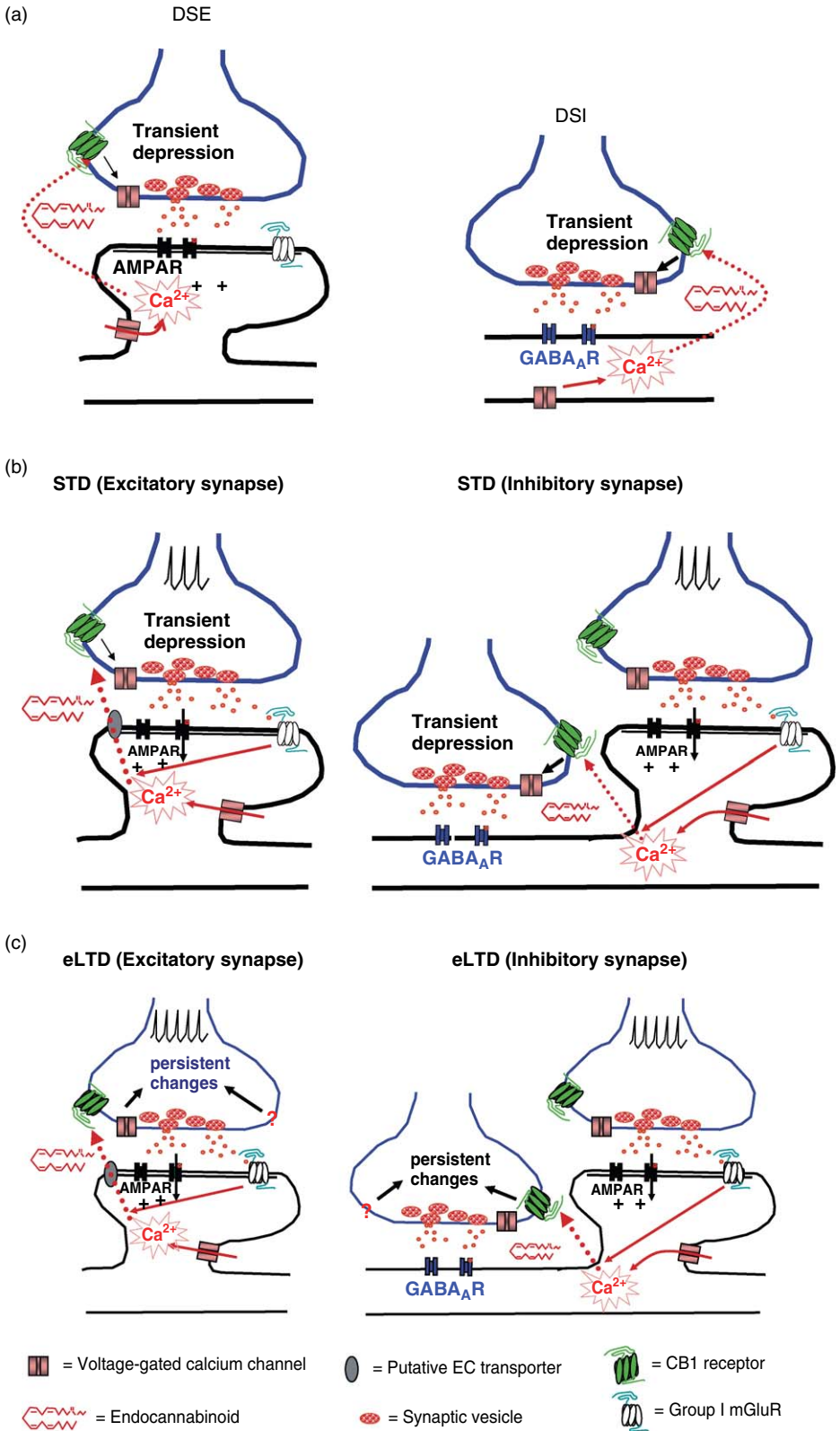
The photoactivation experiments, coupled with data from purely electrophysiological studies, also provide information about the rate of onset of EC-mediated synaptic depression. The time to onset of inhibition following AEA photoactivation is in the range of 50–175 ms, while the latency from depolarization to the onset of inhibition is about 400 ms. These findings indicate that steps between depolarization and EC release take somewhere between 225 and 350 ms, and the estimated time from the rise in postsynaptic calcium to EC release is somewhere between 75 and 190 ms (Heinbockel et al., 2005).

4.37.5 Synaptically Driven, Endocannabinoid-Mediated Short-Term Depression

4.37.5.1 Short-Term Depression Induced by Activation of Metabotropic Receptors

While retrograde EC signaling leading to presynaptic modulation was shown to be inducible by artificial, and often prolonged, postsynaptic depolarization, it was important to determine if DSI and DSE could be induced by physiological mechanisms such as synaptic stimulation. In the late 1990s Alger and colleagues demonstrated that activation of muscarinic ACh receptors and metabotropic glutamate receptors (mGluRs) enhanced and mimicked DSI, respectively, in the hippocampus (Morishita et al., 1998; Martin and Alger, 1999). This finding provided a powerful clue as to the type of molecular pathway that could stimulate EC signaling. Subsequently, it has been shown that this depression is stimulated by activation of group I mGluRs (Edwards et al., 2006). Similar group I mGluR-stimulated short-term depression (STD) has now been observed in cerebellum (Galante and Diana, 2004), and several other mechanisms involving G-protein-coupled, metabotropic receptors have been linked to EC retrograde signaling in various brain regions, as discussed below. With the discovery of the EC retrograde-signaling role in DSI/DSE, it became apparent that signals which would enhance postsynaptic intracellular calcium or stimulate phospholipases might be good stimuli for induction of retrograde messenger formation and action.

Synaptic depression produced by mGluR activation and mediated by EC retrograde signaling has also been observed at the calyx of Held (Kushmerick et al., 2004). This form of depression is blocked by a CB1 antagonist, and immunochemical analysis of this giant synapse indicated that CB1 receptors are localized on presynaptic but not postsynaptic elements of the calyx. Recordings from the presynaptic terminal showed inhibition of the presynaptic calcium current associated with mGluR-induced retrograde synaptic


(a)
DSE
Transient depression
AMPAR
Ca2+
DSI
Transient depression
GABAAR
Ca2+
(b)
STD (Excitatory synapse)
STD (Inhibitory synapse)
Transient depression
AMPAR
Ca2+
Transient depression
AMPAR
GABAAR
Ca2+
(c)
eLTD (Excitatory synapse)
eLTD (Inhibitory synapse)
persistent changes
?
AMPAR
Ca2+
persistent changes
?
AMPAR
GABAAR
Ca2+
= Voltage-gated calcium channel
= Putative EC transporter
= CB1 receptor
= Endocannabinoid
= Synaptic vesicle
= Group I mGluR

depression. Measurement of presynaptic terminal capacitance was used to estimate the magnitude of vesicle fusion/neurotransmitter secretion during synapse activation, and it was determined that CB1 activation reduced the capacitance increase. These findings constitute the most direct evidence to date that retrograde signaling to presynaptic CB1 receptors reduces calcium currents and vesicle fusion in a presynaptic terminal.

4.37.5.2 Short-Term Depression Induced by Synaptic Activation

Synaptic depression mediated by mGluRs can also be produced by synaptic activation. Stimulation of PF inputs to cerebellar Purkinje neurons produces a short-lasting depression of excitatory transmission that is blocked by both mGluR1 and CB1 antagonists (Brown et al., 2003; Maejima et al., 2005). This synaptically induced STD can synergize with DSI in a manner that is dependent on the relative timing of the two stimuli and the resultant increase in postsynaptic intracellular calcium (Brown et al., 2003). A similar form of STD can also be induced at climbing fiber–Purkinje cell synapses (Maejima et al., 2005). Brief high-frequency stimulation of PF inputs to stellate neurons reveals a CB1-dependent STD at this synapse as well (Rancillac and Barbara, 2005).

Two intriguing forms of EC-dependent STD are observed in the ventral tegmental area (VTA), a brain region with known roles in reward and addiction processes. Excitatory glutamatergic inputs to dopaminergic neurons in VTA display an activity-dependent form of STD that involves EC retrograde signaling (Melis et al., 2004b). This STD of excitatory transmission is blocked by mGluR1, but not mGluR5, antagonism. Conditions that promote enhanced excitation and bursting activity in VTA neurons lead to inhibition of both excitatory glutamatergic and inhibitory GABAergic transmission (Riegel and Lupica, 2004). These forms of STD are actually promoted by mGluR antagonists. The mGluRs in this case appear to be presynaptic group III receptors that are normally involved in inhibiting glutamatergic transmission. When these receptors are blocked, glutamatergic drive is enhanced and helps to stimulate postsynaptic EC production. The inhibitory synapses that are targeted by the retrograde EC action in this case are those which primarily activate $GABA_B$-type receptors. Thus, the net effect of the EC signal is to produce disinhibition of a slow inhibitory postsynaptic potential (IPSP) that would normally limit VTA neuronal excitability.

Within the sensory and auditory neocortices EC-dependent STD induced by synaptic activation has also been observed (Trettel and Levine, 2003; Trettel et al., 2004). This synaptic depression occurs at GABAergic inhibitory synapses between interneurons and 'right-arrow' pyramidal cells in superficial cortical layers. The interneuron subtype involved in this transmission is strongly stimulated by activation of muscarinic ACh receptors. Trains of as few as three action potentials could evoke this form of STD, indicating that EC signaling can be triggered by brief bursting events within the physiological range of cortical neuron firing.

Figure 1 Endocannabinoid-dependent depolarization-induced suppression of excitation (DSE) and inhibition (DSI), short-term depression (STD), and long-term depression. (a) Schematic diagram of mechanisms involved in DSE at excitatory (left) and DSI at inhibitory (right) synapses. Postsynaptic depolarization leading to activation of voltage-gated calcium channels stimulates endocannabinoid (EC) production and/or release. EC activation of CB1 (cannabinoid 1) receptors produces a transient decrease in neurotransmitter release, and inhibition of presynaptic voltage-gated calcium channels is one mechanism strongly implicated in this presynaptic depression. (b) Schematic diagram of mechanisms involved in homosynaptic STD at excitatory synapses (left) and heterosynaptic STD at inhibitory synapses (right). Activation of group I metabotropic glutamate receptors (mGluRs) is a common mechanism involved in postsynaptic EC production in STD. Some forms of STD require postsynaptic depolarization and calcium channel activation, while others do not. A postsynaptic membrane transport system has been implicated in EC release for STD at excitatory synapses in the ventral tegmental area (Melis et al., 2004b), but the role of this mechanism in other forms of STD has not been examined. ECs activate presynaptic CB1 receptors that inhibit neurotransmitter release, presumably via mechanisms similar to those involved in DSE/I. (c) Schematic diagram of mechanisms involved in homosynaptic EC-dependent LTD (eLTD) at excitatory synapses (left) and heterosynaptic eLTD at inhibitory synapses (right). Activation of postsynaptic mGluRs and depolarization-induced activation of postsynaptic voltage-gated calcium channels is a common theme in eLTD induction. Transporter-mediated EC release has been implicated in eLTD at synapses in striatum (Ronesi et al., 2004) and somatosensory cortex (Bender et al., 2006). Maintained expression of eLTD appears to require combined activation of presynaptic CB1 receptors and additional mechanisms that are not yet well understood (although, see Kreitzer and Malenka, 2005). For example, presynaptic, NMDA receptors appear to synergize with CB1 receptors in the induction of LTD at synapses in visual cortex (Sjostrom et al., 2003). $GABA_A$R, gamma-aminobutyric acid type A receptor; AMPAR, alpha-amino-3-hyroxyl-5-methyl-4-isoxazolepropionate receptor.

Recent studies suggest some evidence that the EC responsible for synaptically induced STD is 2-AG. Blockade of 2-AG synthesis prevents STD in hippocampus (Edwards et al., 2006), cerebellum (Galante and Diana, 2004), and VTA (Melis et al., 2004b). The molecular mechanisms linking mGluR activation to 2-AG production may involve activation of phospholipase C and DAG lipase (Jung et al., 2005). There is also evidence that EC release from postsynaptic VTA neurons involves a transmembrane transport system (Melis et al., 2004b). Similar evidence has been gathered for EC-dependent LTD (Ronesi et al., 2004; Bender et al., 2006), a type of plasticity that will also be discussed later in this article.

The duration of EC-dependent STD suggests that the ultimate expression mechanism is similar to that of DSE/I, namely presynaptic CB1 activation leading to inhibition of calcium channels and depression of neurotransmitter release. However, there is as yet little experimental evidence directly supporting this conclusion. Mechanisms involved in STD at excitatory and inhibitory synapses are schematized in **Figure 1(b)**.

4.37.5.3 Dopamine-Induced Short-Term Depression in Striatum

In the striatum, dopamine acts on D2 receptors to stimulate formation of AEA (Giuffrida et al., 1999). This D2 receptor activation of EC signaling has recently been implicated in depression of glutamatergic corticostriatal transmission (Kreitzer and Malenka, 2005; Yin and Lovinger, 2006). Activation of D2 receptors in concert with cortical input at frequencies above 20 Hz induces STD of glutamatergic synaptic input onto striatal medium spiny neurons (MSNs) (Yin and Lovinger, 2006). The mechanisms underlying this synaptic depression include synaptic activation of postsynaptic mGluR1, release of calcium from intracellular stores, and activation of postsynaptic PLC. Expression of D2-stimulated STD is associated with increased paired-pulse ratio, indicative of a presynaptic decrease in probability of neurotransmitter release.

As all of these mechanisms bring to mind EC retrograde signaling, examining the role of ECs and CB1 receptors in this form of STD seemed like a logical course of action. Indeed, STD produced by combined D2R activation and 20-Hz afferent stimulation is blocked by CB1 antagonism and is absent in CB1−/− mice (Yin and Lovinger, 2006). Pharmacological studies indicate that CB1 receptor activation takes place subsequent to D2 activation. The mechanism through which D2 receptors participate in EC production is not yet known. Given the similarity of the mechanisms involved in this corticostriatal STD to those involved in STD at other synapses (e.g., mGluR activation, PLC activation), it is tempting to speculate that D2 receptors have a modulatory role similar to their role in LTD (Kreitzer and Malenka, 2005; Wang et al., 2006, to be discussed in a subsequent section of this article). This may be one example of a mechanism through which other neurotransmitters can modulate the core mGluR–EC signaling process necessary for STD.

4.37.5.4 Physiological Roles of Depolarization-Induced Suppression of Excitation and Inhibition and Short-Term Depression

The roles of EC-dependent DSE/I and STD in circuit function and behavior are not yet fully known. The temporary pauses in synaptic transmission produced by these mechanisms could certainly influence circuit throughput. While DSE would be likely to decrease activity of postsynaptic neurons, DSI and STD at inhibitory synapses have the potential to produce disinhibition that would amplify the effect of excitatory transmission. Short-term depression of transmission could also play a role in biasing circuitry toward one set of outputs in preference to another. In this context, dopamine-mediated STD in striatum could affect the output of MSNs contributing to the two major basal ganglia pathways, the direct and indirect pathways (Gerfen, 1992). Patterns of alteration at different synapses have the potential to influence action patterns at the level of the whole organism. Disinhibition produced by DSI and STD at GABAergic synapses might participate in circuit timing and generation of patterned activity. As discussed later, these forms of disinhibition provide a time window for enhanced likelihood of long-term potentiation. Endocannabinoids may promote the storage of information through such a mechanism. Transient inhibition of excitatory transmission might also be neuroprotective, as excess glutamatergic transmission is often neurotoxic. Indeed, CB1 receptor antagonists have been shown to be neuroprotective (see Mechoulam, 2002; van der Stelt and Di Marzo, 2005b, for review).

Table 1 summarizes data on EC-dependent STD induced by agonist application and/or synaptic

activation in different brain regions. Information on common and divergent mechanisms and measures of STD expression are also provided in this table.

4.37.6 Endocannabinoid-Mediated Long-Term Synaptic Depression

In addition to producing transient depression of transmission, ECs also play key roles in certain long-lasting forms of synaptic plasticity. Retrograde EC signaling has generally been implicated in long-term synaptic depression (LTD), and this topic will be discussed in detail. **Figure 1(c)** shows schematic diagrams of the mechanisms that are common to most forms of EC-dependent LTD at excitatory and inhibitory synapses. It is worth noting that activation or inhibition of CB1 receptors was shown to influence long-term potentiation (LTP) induction prior to the discovery of EC-dependent LTD. For example, Stella et al. (1997) showed that 2-AG suppresses LTP induction, while Auclair et al. (2000) showed that CB1 activation suppresses and CB1 antagonism promotes LTP in rat prefrontal cortex (PFC). Working in the cerebellum, Levenes et al. (1998) also showed that CB agonists suppress LTD induction. Such modulatory roles for ECs are part of a growing pattern of findings indicating that ECs participate in determining the types of long-term plasticity evoked at synapses in several brain regions.

4.37.6.1 Homosynaptic Endocannabinoid-Dependent Long-Term Depression at Glutamatergic Synapses

While the earlier studies hinted that ECs and CB1 could modulate long-lasting synaptic plasticity, evidence that these molecules directly participate in the induction of LTD began to appear in 2002. The work began with investigators examining excitatory synaptic transmission onto MSNs in the dorsal and ventral striatum (the latter is also known as the nucleus accumbens). It was established in the early 1990s that high-frequency activation of excitatory glutamatergic inputs to MSNs in dorsal striatum produces a long-lasting decrease in synaptic efficacy termed striatal LTD (Calabresi et al., 1992a,b; Lovinger et al., 1993; Walsh, 1993). This form of LTD requires postsynaptic induction mechanisms, but expression appears to involve a presynaptic decrease in neurotransmitter release probability, indicating the involvement of a retrograde signal in the induction process (Choi and Lovinger, 1997a,b). Studies over the last decade have established that striatal LTD requires activation of group I mGluRs (Gubellini et al., 2001; Sung et al., 2001), the L-type voltage-gated calcium channel (most likely the CaV1.3 isoform, Calabresi et al., 1992b; Wang et al., 2006), and dopamine D2 receptors (Calabresi et al., 1992a,b; Tang et al., 2001; Kreitzer and Malenka, 2005; Wang et al., 2006). As we have already seen, these molecules are implicated in postsynaptic production of ECs.

Combined electrophysiological, pharmacological, and genetic approaches were used by Gerdeman et al. (2002) to establish a role for ECs and CB1 in striatal LTD. These investigators showed that LTD induction was blocked by a CB1 antagonist and was absent in gene-targeted CB1−/− mice. Experiments involving manipulation of postsynaptic calcium signals and filling the postsynaptic neuron with ECs provided evidence that the compounds are produced in the postsynaptic neuron and can signal in a retrograde manner to inhibit glutamate release (Gerdeman et al., 2002; Ronesi et al., 2004). Based on these findings, the authors suggested that ECs constitute the retrograde signal in striatal LTD, and that LTD induction involves presynaptic CB1R activation.

Shortly thereafter, a report indicating a similar role for ECs and CB1 in LTD of excitatory prelimbic cortex inputs to MSNs in the nucleus accumbens was published (Robbe et al., 2002). Manzoni and colleagues described LTD activated by moderate-frequency (13 Hz) activation of afferent fibers to accumbens MSNs. This LTD shared many of the properties already established for striatal LTD, namely, dependence on activation of an mGluR (mGluR5 in this case) and the necessity of an increase in postsynaptic intracellular calcium. A presynaptic mechanism of expression was also shared between the two forms of LTD. The similarity between LTD in the two striatal subregions that was the focus of the Robbe et al. (2002) paper was the involvement of ECs and the CB1 receptor. This was demonstrated using CB1 antagonists as well as CB1−/− mice. This study was the first to indicate that the long-lasting expression of LTD was independent of sustained CB1 receptor activation. This was demonstrated by applying the CB1 antagonist well after LTD was established, at which time it had no effect on the depressed synaptic response. Similar observations were subsequently made in hippocampus (Chevaleyre and Castillo, 2003) and dorsal

striatum (Ronesi et al., 2004). These important findings indicate that CB1 receptor activation is only important for the initiation of LTD.

There were, however, noticeable differences in the induction mechanisms of EC-dependent LTD (henceforth eLTD) in the two striatal subregions. Most notable was the absence of a role for dopamine and its receptors in the accumbens (Robbe et al., 2002). This finding was interesting in light of the observed role of dopamine in eLTD in the dorsal striatum (Calabresi et al., 1992a,b; Tang et al., 2001; Kreitzer and Malenka, 2005; Wang et al., 2006) and the fact that both striatal subregions contain abundant dopaminergic afferents. These findings also appear to have provided the first clue that dopamine modulates striatal LTD rather than being strictly necessary for this form of plasticity. Another interesting difference between eLTD in dorsal striatum and nucleus accumbens is the role of depolarization. The LTD induced by high-frequency stimulation in dorsal striatum depends on depolarization (Choi and Lovinger, 1997a), but it is unclear if depolarization is necessary for accumbens eLTD. Furthermore, the role of postsynaptic calcium channels, implicated in dorsal striatal LTD, is not yet clear in accumbens. These findings suggest that multiple mechanisms can participate in activation of EC release necessary for LTD induction, even at synapses onto very similar sets of neurons.

Subsequent to publication of the findings of Robbe et al. (2002), additional reports of eLTD induced by moderate-frequency stimulation in striatum appeared. Hoffman et al. (2003) essentially replicated the findings of Robbe et al. (2002) in nucleus accumbens, although 10-Hz stimulation was used. Ronesi and Lovinger (2005) working in dorsal striatum also found eLTD induced by 10-Hz stimulation, and this form of LTD had notable similarities and differences relative to accumbens eLTD. Like the eLTD described by Robbe et al. (2002), the synaptic depression induced by 10-Hz stimulation in dorsal striatum was independent of postsynaptic depolarization. Furthermore, the magnitude of 10 Hz–induced LTD was reduced only slightly by L-type calcium channel blockers. The similarities to LTD in accumbens do not extend to involvement of neurotransmitter receptors, however. No role was found for mGluRs in 10 Hz–induced dorsal striatal LTD, while D2 receptors appear to participate in LTD induction. At present the cellular source of ECs involved in 10 Hz–induced dorsal striatal LTD is unclear, given that postsynaptic manipulations do not influence LTD induction. This is a clear difference, relative to accumbens LTD, and signals the need for more research to determine the similarities and differences in the LTD mechanisms in these two related brain regions.

Recent work on PF inputs to cerebellar stellate neurons has revealed key elements of the postsynaptic signaling involved in a somewhat unusual form of eLTD (Soler-Llavina and Sabatini, 2006). This synapse is made by glutamatergic inputs impinging on dendritic shafts. Trains of 30-Hz stimuli produce an LTD that requires increased postsynaptic calcium arising from calcium influx through alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate receptors (AMPARs), presumably of the type that lack GluR2 and are therefore highly calcium permeable. Expression of this type of LTD also depends on activation of group I mGluRs and CB1 receptors, and the final expression mechanism appears to be presynaptic. High-resolution calcium-imaging techniques were used to show that the calcium signal necessary to initiate LTD is confined to a segment of dendrite no more than 10 μm along the axial dendritic length, accounting for the synapse specificity of eLTD. These findings provide a good mechanistic basis for the localized nature of mechanisms involved in eLTD induction. Alternative mechanisms by which EC production takes place during LTD initiation are also highlighted in this study. The source of calcium, AMPARs, is different from that involved in most forms of eLTD, and there is no need for a dendritic spine-like apparatus. This is not too surprising, as the inhibitory synapses that show eLTD in BLA and hippocampus are not localized to dendritic spines (Marsicano et al., 2002; Chevaleyre and Castillo, 2003). The supporting role of mGluRs in EC production, but not stimulation of the intracellular calcium transient, also links this form of LTD to STD found at other cerebellar synapses (Brenowitz and Regehr, 2005).

4.37.6.2 Heterosynaptic Endocannabinoid-Dependent Long-Term Depression at Gamma-Aminobutyric Acid–ergic Synapses

The characterization of eLTD in striatal subregions heralded a rapidly growing appreciation of the fact that EC-dependent long-term plasticity is a widespread mechanism for synaptic modification. Following the initial descriptions of eLTD in dorsal and ventral striata, a number of studies appeared in rapid succession which established eLTD at both inhibitory and excitatory synapses in several forebrain regions. At

GABAergic synapses in amygdala and hippocampus, LTD involving ECs has now been firmly established and is sometimes known as LTDi (the i refers to inhibitory synapses) (Marsicano et al., 2002; Chevaleyre and Castillo, 2003). Marsicano et al. (2002) described LTD at GABAergic inhibitory synapses onto principal neurons in the basolateral amygdala (BLA) as part of a study implicating ECs in extinction of fear conditioning. These investigators found that LTD induced by sustained low-frequency (1 Hz) stimulation was absent in BLA slices from CB1−/− mice and in wild-type mouse slices treated with a CB1 antagonist. This eLTD was accompanied by an increase in paired-pulse facilitation, indicative of a decrease in neurotransmitter release probability. It is worth noting that the original report of this form of eLTD did not present evidence for a retrograde signaling role of ECs. However, considerable evidence for such a role was obtained in a subsequent study (Azad et al., 2004).

Investigators examining GABAergic inhibitory transmission in hippocampus were also beginning to unearth evidence for eLTD. The discovery that the mechanism underlying DSI involved EC retrograde signaling onto terminals of GABAergic basket cells in the hippocampal CA1 region spurred Chevaleyre and Castillo to begin to investigate potential EC-dependent eLTD at these synapses (Chevaleyre and Castillo, 2003). The first report from this group indicated that eLTD could be induced by high-frequency stimulation and presented evidence that this long-lasting form of synaptic depression involved retrograde EC signaling. Like the eLTD observed at excitatory synapses, eLTD in hippocampus is dependent on activation of mGluRs, perhaps a combination of mGluR1 and mGluR5. A long-lasting depression similar to that induced by afferent stimulation could also be induced by application of the group I mGluR agonist, (S)-3,5-dihydroxyphenylglycine (DHPG). Chevaleyre and Castillo (2003) also presented evidence that mechanisms involved in induction of eLTD differed from those implicated in DSI. For example, blockade of enzymes implicated in EC production prevented eLTD without altering DSI. In this form of eLTD, CB1 receptors were necessary for induction but not sustained expression of plasticity, similar to what had been seen at excitatory synapses in nucleus accumbens (Robbe et al., 2002). Also similar to other forms of eLTD was the evidence for a presynaptic site of eLTD expression. The authors discussed the possibility that eLTD would secondarily enhance excitatory synaptic transmission, possibly enhancing LTP and promoting information storage (see discussion to follow).

4.37.6.3 Endocannabinoid-Dependent Long-Term Depression in Neocortex

In the sensory cortex, it was known that different forms of long-lasting synaptic plasticity could be induced by pairing pre- and postsynaptic activation with different timing protocols, phenomena called spike-timing-dependent plasticity (STDP). Activation of the postsynaptic neuron prior to presynaptic action potential firing was known to produce LTD at excitatory glutamatergic synapses between thick-tufted neurons in layer V of the visual cortex (Sjostrom et al., 2001). Elegant experiments involving paired recordings from synaptically connected pre- and postsynaptic neurons revealed that induction of STDP-LTD involved postsynaptic activation, while expression involved a decrease in neurotransmitter release probability (Sjostrom et al., 2001, 2003). These investigators found that this STDP-LTD was blocked by CB1 receptor antagonists, and that it could be induced by pairing application of AEA or the synthetic analog arachidonyl-2-chloroethylamide (ACEA) with afferent stimulation. Interestingly, coincident activation of presynaptic *N*-methyl-D-aspartate-type glutamate receptors (NMDARs) was required for STDP-LTD at these synapses. This led the investigators to propose a model in which synergistic effects of presynaptic CB1 and NMDAR activation lead to STDP-LTD at these excitatory synapses. It remains to be determined what signaling pathways are necessary for expression of this form of eLTD.

A subsequent series of studies have confirmed and extended the role of ECs in LTD at cortical synapses. Feldman and colleagues have examined synapses between layer 4 and layer 2/3 neurons in S1 somatosensory cortex that exhibit STDP-LTD which relies on EC-dependent mechanisms similar to those implicated in visual cortical eLTD (Bender et al., 2006). These investigators have implicated postsynaptic increases in calcium, most likely involving both T- and L-type voltage-gated calcium channels, in this LTD. Other postsynaptic mechanisms implicated in LTD induction at this synapse include mGluR5 activation, release of calcium from intracellular stores associated with IP3 receptors, and an AMT-like EC transporter. Blockade of CB1 receptors prevented eLTD at these S1 cortical synapses, and LTD was induced by combined afferent stimulation and

anandamide application. The major difference in LTD mechanisms in S1 versus visual cortex is the role of NMDARs. In S1, cortical blockade of NMDAR function does not appear to abolish LTD induction, whereas activation of presynaptic NMDARs is implicated in visual cortical LTD (Sjostrom et al., 2003; Bender et al., 2006). It is not yet clear if another presynaptic receptor or channel serves a role similar to presynaptic NMDARs in LTD within S1.

4.37.6.4 Endocannabinoids and Cerebellar Long-Term Depression

The LTD observed at PF synapses between granule cells and Purkinje neurons was the first form of long-lasting synaptic depression described (Ito and Kano, 1982) and remains one of the best characterized forms of synaptic plasticity (see Ito, 2001, for review). Years of research have revealed that both the induction and expression mechanisms of PF-LTD involve postsynaptic mechanisms. Induction requires postsynaptic activation of AMPA-type ionotropic receptors, as well as mGluR1. Postsynaptic increases in calcium, involving both voltage-gated calcium channels and release of calcium from intracellular stores, are also implicated in LTD induction at this synapse. There is strong evidence that expression of PF-LTD involves decreased surface expression of AMPARs, with no evidence for any change on the presynaptic side. Thus, it was surprising when Safo and Regehr (2005) reported that CB1 activation is required for induction of PF-LTD. This finding was doubly puzzling because CB1 receptors appear to be expressed solely on axon terminals at this synapse. This finding led to the hypothesis that cerebellar LTD involves a sequence of retrograde and anterograde signaling mechanisms. It was hypothesized that postsynaptic activation leads to production and release of an EC retrograde messenger that then triggers production and/or release of a chemical signal from the presynaptic terminal that acts on the postsynaptic cell to trigger the final steps in LTD expression. The identity of this anterograde signaling molecule is not yet clear, but nitric oxide (NO) has been implicated in PF-LTD and may thus serve such a role (Shibuki and Okada, 1991; Safo and Regehr, 2005).

However, there are a few elements of this mechanistic scenario that must be reconciled with previous findings regarding PF-LTD. Experiments conducted using acutely isolated Purkinje neurons or neurons grown in cell culture indicate that LTD can be induced in the absence of presynaptic inputs (Narasimhan and Linden, 1996). This suggests that LTD may rely on purely postsynaptic mechanisms that would not require a retrograde signal. However, it is possible that the mechanisms involved in the LTD observed in isolated neurons are different from those taking place during LTD induction in the slice preparation, even though the final expression mechanism appears to be the same in both situations. In addition, the proposed anterograde signal, NO, poses some complications. Recent data indicate that parallel fibers are not the source of the NO involved in cerebellar LTD (Shin and Linden, 2005). Thus, a scenario in which retrograde EC signaling leads to anterograde NO signaling at the same synapse is not likely to occur. Clearly, further studies are needed to clarify the EC role in cerebellar LTD.

4.37.6.5 Amphetamine-Induced Long-Term Depression in Amygdala

Working in the BLA in brain slices, Gean and colleagues found that application of amphetamine produces LTD at glutamatergic synapses that shares important mechanistic similarities with eLTD in other brain regions (Huang et al., 2003). Expression of this drug-induced LTD is associated with increased paired-pulse facilitation and decreased frequency of miniature excitatory postsynaptic currents (mEPSCs), consistent with a presynaptic role in expression. Neurochemical measurement of glutamate release also supported the conclusion that this form of LTD involves decreased neurotransmitter release. Importantly, amphetamine-induced LTD in BLA is blocked by CB1 receptor antagonists. Postsynaptic calcium chelation reduced LTD, indicating some role for postsynaptic activation on LTD induction. This study suggests that drugs of abuse are yet another trigger for eLTD.

4.37.7 Endocannabinoids and Long-Term Potentiation

The disinhibition produced by DSI enhances excitatory synaptic transmission. This has been demonstrated in the hippocampal CA1 region. In the absence of receptor antagonists, stimulation of Schaffer collateral/commissural afferents produces a fast-EPSP/IPSP sequence that reflects glutamatergic transmission followed by feedforward and feedback GABAergic inhibitory transmission. Reducing the inhibitory component is known to enhance the magnitude and duration of the excitatory drive, as

well as the probability of eliciting an action potential (Wagner and Alger, 1996). Carlson et al. (2002) have shown that a DSI-inducing depolarization enhances excitatory synaptic transmission elicited by a train of stimuli. This enhancement facilitates induction of LTP at the glutamatergic Schaffer collateral/commissural synapses via a mechanism that involves CB1 receptors (**Figure 2**).

Chevaleyre and Castillo have also gathered evidence that ECs participate in a 'metaplastic' enhancement of LTP (**Figure 2**) (Chevaleyre and Castillo, 2004). Metaplasticity refers to alterations in the threshold for induction of synaptic plasticity due to prior synaptic experience (Abraham and Bear, 1996). This can often take the form of modulation of one form of plasticity by another. At the basket cell–pyramidal cell synapses in hippocampus, Chevaleyre and Castillo (2004) found that the induction of EC-dependent LTD enhanced the induction of LTP at excitatory synapses onto the same pyramidal neurons. A weak theta-burst stimulus that was normally insufficient to induce LTP could do so after prior induction of eLTD using 10-Hz stimulation. Thus, EC signaling can promote LTP induction in either the short term, during DSI, or the long term, during expression of eLTD. This adds a level of modulation onto the basic plasticity of excitatory synapses in hippocampus. It is likely that similar mechanisms will also be found in other brain regions where plasticity at excitatory synapses is accompanied by EC-dependent synaptic depression at inhibitory synapses. Indeed, enhancement of LTP was observed in CB1−/− mice in the BLA that also show loss of eLTD at inhibitory synapses (Marsicano et al. 2002).

One line of evidence that appears to argue against EC facilitation of LTP comes from a recent study by Slanina et al. (2005). These authors found that CB1 antagonists enhanced LTP at excitatory synapses onto hippocampal CA1 pyramidal neurons, in contrast to the effects seen by others (Carlson et al., 2002; Chevaleyre and Castillo, 2004). The enhancement of LTP is attributed to relief from EC-mediated inhibition of excitatory transmission. The EC inhibition is most likely due to activation of CB receptors on glutamatergic presynaptic terminals in the CA1 region. While there has been much speculation about CB and EC actions at these excitatory synapses, and it has been suggested that a non-CB1 receptor mediates these effects (Hajos et al., 2001; Hoffman et al., 2005), recent evidence strongly suggests that CB1 receptors are indeed also present and active in this system (Domenici et al., 2006; Katona et al., 2006; Kawamura et al., 2006). Thus, the scenario suggested by Slanina et al. (2005) is certainly plausible.

Within the cerebellum, EC release and CB1 activation induced by climbing fiber (CF) activation also have an LTP-suppressing role (van Beugen et al., 2006). It appears that ECs released postsynaptically after CF activation of the Purkinje neuron act on PF terminals to decrease adenylyl cyclase activity that is necessary for LTP induction. The suppression of LTP persists even when PF-LTD is blocked, indicating that loss of potentiation is not simply a result of enhancement of competing LTD. This LTP suppression could, however, underlie promotion of PF-LTD by removing competing LTP. This remains to be determined.

The emerging evidence that ECs can both promote and reduce LTP suggests a complicated picture. The net effect of EC release and CB1 activation likely depends on the level and location of EC release as well as which synapses contain CB1 receptors within a given brain region. The factors that determine whether EC actions on inhibitory or excitatory transmission predominate, and the impact of these factors on LTP induction, need to be explored. The relationship between strength of afferent activation and subsequent EC release at postsynaptic elements associated with excitatory and inhibitory synapses also needs further characterization. It appears that the EC/CB1 system provides flexible neuromodulation that can produce differential mediation or modulation of synaptic plasticity in response to different patterns of neural activity, even within a single brain region. This flexibility in EC-dependent plasticity likely underlies the different roles of the neuromodulator in learning and memory, as will be discussed in the following section of this article.

4.37.8 Endocannabinoid and CB1 Receptor Roles in Learning and Memory

4.37.8.1 Exogenous Cannabinoid Agonists Impair Memory

Endocannabinoids and CB1 receptors have been implicated in a number of neural circuit functions and behaviors. These include feeding behavior, responses to stress, nociception, and several others. This discussion will focus on the roles of these molecules in learning and memory. Traditionally, evidence for a connection between the CB system and learning and memory came mainly from the actions of CB drugs and Δ9-THC itself (**Figure 2(a)**). It has long been

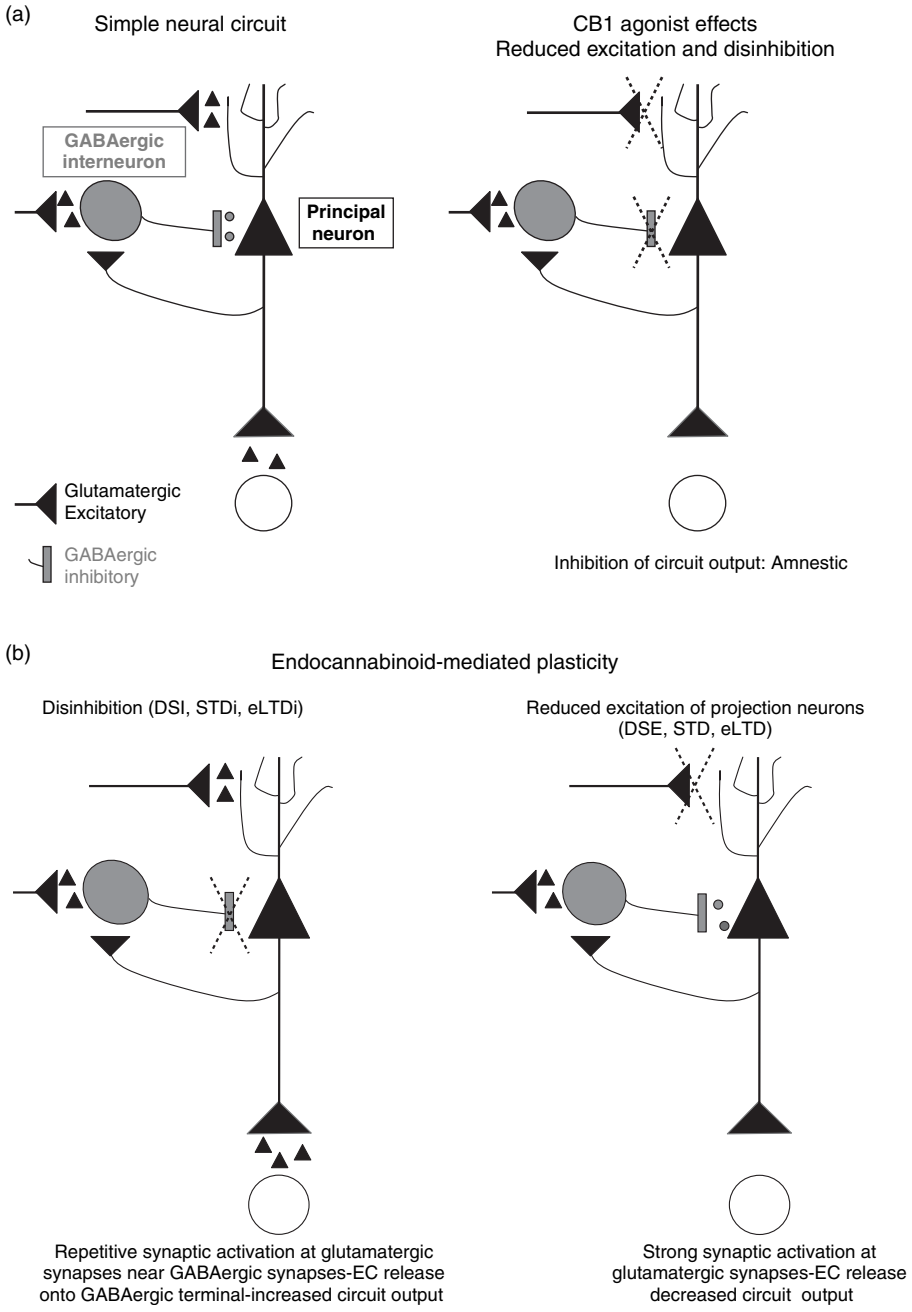


Figure 2 Effects of cannabinoid drugs and endocannabinoids on excitatory and inhibitory transmission and potential roles in information storage. (a) Schematic illustration of the arrangement of a principal neuron and an inhibitory interneuron that participates in both feedforward and feedback inhibition, similar to the arrangement of pyramidal and basket cells in the hippocampal CA1 region (left). Cannabinoid agonist drugs including Δ9-THC activate cannabinoid (CB) receptors on both excitatory and inhibitory terminals, reducing net circuit output and synaptic plasticity (right). This would account for impairment of learning and memory produced by these drugs. (b) Given the same circuit arrangement, postsynaptic activation that is sufficiently strong and localized to excitatory synapses located near inhibitory synapses will have a net disinhibitory effect. This would lead to increased circuit output and promote long-term potentiation. In this way endocannabinoid (EC) signaling could promote learning and memory. Sufficiently strong postsynaptic activation localized to excitatory synapses with CB1-containing presynaptic terminals would produce EC-dependent presynaptic depression that would tend to reduce circuit output. This might contribute to EC roles in impairment of information storage. GABA, gamma-aminobutyric acid; DSI, depolarization-induced suppression of inhibition; STDi, short-term depression (the i refers to inhibitory synapses); eLTDi, EC-dependent long-term depression (inhibitory synapses).

known that these drugs have a generally disruptive effect on learning and memory, and that some of the amnestic cannabis actions resemble effects of hippocampal lesions (Miller and Branconnier, 1983; Hampson and Deadwyler, 1998; Sullivan, 2000; Wilson and Nicoll, 2002).

4.37.8.2 Endocannabinoids and Memory Impairment

With the discovery of the CB1 receptor followed by the discovery of ECs, and finally the synthesis of CB1 antagonists, interest in examining the role of this receptor in learning and memory rapidly grew. The CB1 antagonist SR141716A (rimonabant) was found to reverse the amnestic effects of CB1 agonists or β-amyloid (Mallet and Beninger, 1998; Mazzola et al., 2003). The antagonist itself did not appear to have any effect on established memory in either the instrumental learning task used by Mallet and Beninger (1998) or the passive avoidance task used by Mazzola et al. (2003). However, effects on learning were not explicitly examined.

Additional evidence supports the idea that ECs can have memory-inhibiting function. For example, Shiflett et al. (2004) found that rimonabant given in the bird hippocampus improved memory for food storage locations but enhanced proactive interference for new location memories. This compound also improved rodent performance in a social recognition task (Terranova et al. 1996). Takahashi et al. (2005) have shown that the CB1 antagonist/partial agonist facilitates learning and reverses scopolamine-induced amnesia in rats using an elevated T-maze paradigm. The circuitry involved in this learning paradigm is not well defined. Using a radial arm maze task incorporating a significant training-retention delay, presumably a task that involves the hippocampus, Wolf and Leander (2003) found improved retention after rimonabant treatment. Improvement in the radial arm maze task was also observed by Lichtman (2000). Thus, there is ample evidence that EC activation of CB1 receptors impairs memory under certain conditions.

4.37.8.3 Endocannabinoid Roles in Promoting Learning and Memory

The development of gene-targeted mice lacking the CB1 receptor produced an opportunity to further examine the role of the EC system in brain information storage. Marsicano et al. (2002) used CB1−/− mice to implicate EC signaling in extinction of fear conditioning. A reduction in extinction similar to that observed in the CB1−/− mice was also seen upon injection of a CB1 antagonist, and evidence for increased EC levels in BLA during extinction-inducing trials was also observed. The eLTD observed at inhibitory BLA synapses was also lost in the CB1 knockout mouse. Studies in other laboratories have confirmed that loss of CB1 function reduces extinction in this aversively motivated task (Suzuki et al., 2004) and in the Morris water maze task, where CB1−/− mice show increased perseveration (Varvel and Lichtman, 2002; Varvel et al., 2005). However, a subsequent study by Marsicano, Lutz, and coworkers has added an interesting twist to the story (Kamprath et al., 2006). It appears that lack of CB1 receptor function also prevents habituation to the stimuli that produce fear conditioning. Thus, it is possible that the loss of extinction simply reflects an inability to habituate, perhaps reflecting an inability to adjust to sensory stimuli. It remains to be seen if there is a specific EC role in extinction.

A recent study has also implicated the EC system in aversive Pavlovian conditioning involving BLA inputs to the prefrontal cortex (Laviolette and Grace, 2006). These investigators have used a novel paradigm involving pairing of olfactory cues with a noxious stimulus in awake or lightly anesthetized rats. This pairing method produces conditioned freezing to the olfactory cue in unanesthetized rats and associated changes in the firing frequency and the incidence of bursting activity of medial PFC neurons observed during *in vivo* electrophysiological recordings in the anesthetized state. Treatment with a CB1 antagonist reduces conditioned freezing, while agonist treatment promotes learning to a normally subthreshold stimulus. Furthermore, these drugs are active when applied directly into the PFC, implicating this brain region as a site of important CB1 function. Much remains to be determined about the EC role in this form of learning, including the cellular localization of the CB1 receptors involved.

4.37.8.4 Possible Roles of Long-Term Depression and Long-Term Potentiation in Cannabinoid and Endocannabinoid Effects on Memory

Activation of CB1 receptors with exogenous agents also inhibits the induction of LTP in the hippocampal CA1 subregion (Nowicky et al., 1987; Terranova

et al., 1996; Stella et al., 1997; Misner and Sullivan, 1999), which could contribute to memory impairment produced by these compounds. Both synthetic and naturally occurring CB1 agonists are effective in this paradigm. The ability of CB1 activation to both promote and impair LTP may seem inconsistent at first glance. However, it must be kept in mind that the LTP-promoting effects are mainly observed during activation of EC signaling stimulated by physiological means, while LTP impairment has been observed primarily during application of exogenous agonists (**Figure 2**). Factors including the level of receptor occupancy and duration of receptor activation likely play a role in determining the net effect of CB1 activation.

Physiological activation of EC signaling primarily selectively depresses inhibitory synaptic transmission, presumably because these terminals are more sensitive to such depression (**Figure 2(b)**). The relatively high density of CB1 receptors on inhibitory terminals likely accounts for this increased sensitivity (Freund et al., 2003; Mackie, 2005). This disinhibitory effect primes LTP induction, as has been shown for many conditions that reduce GABAergic inhibition (see discussion above). In contrast, application of high concentrations of CB1 agonists depresses glutamatergic excitatory transmission in addition to synaptic inhibition. This reduced excitation suppresses the glutamatergic drive necessary for LTP induction and thereby prevents LTP induction (Misner and Sullivan, 1999). Such differences could explain the differential effects of EC signaling and CB drugs on learning and memory. Brief activation of CB1 receptors during physiological signaling localized on inhibitory synapses could produce disinhibition and enhance LTP. Strong and prolonged activation of receptors could depress excitatory transmission, leading to diminished LTP (**Figure 2(b)**). A potential role for disinhibitory effects of ECs/CB1 in memory is supported by de Oliveira Alvares et al. (2006), who demonstrated that CB1 blockade prevents LTP in the CA1 region *in vivo*. The antagonist also impairs memory on a single-trial step-down avoidance task. These data are intriguing, but the role of the hippocampus is not yet clear (although see Whitlock et al., 2006), and thus it is premature to conclude too much from this study.

The totality of evidence suggests that ECs have a complex involvement in learning and memory. The memory-promoting versus memory-impairing effects of this system may relate to the neural circuitry involved in a particular memory or neuronal locus of expression of the CB1 receptor. One idea is that EC-dependent DSI, STD, or LTD at inhibitory synapses made by interneurons promotes memory by enhancing LTP and increasing information flow through a circuit. In contrast, EC-dependent DSE, STD, or LTD at excitatory synapses onto projection neurons might impair memory by preventing or impairing LTP and reducing net circuit output (**Figure 2**). However, this is undoubtedly too simple a formulation, because one can think of instances in which depression of excitatory transmission might participate in memory formation (e.g., by inhibiting output from inhibitory projection neurons or sharpening signal/noise ratios for relevant stimuli). It is more likely that the net effect of all the ECs and CB1 receptors within the relevant circuitry is what determines the effect on information storage. Given that the EC/CB1 system is neuromodulatory, the extent of receptor activation may also be important. Subtle influences on the circuitry may push relevant synaptic and neuronal activity above or below thresholds necessary for plasticity and information storage.

Clearly more information is needed about which EC-releasing neurons and CB1 receptors are involved in the different types of information storage. More brain region- and neuronal-specific knockouts are needed, and these should be coupled with brain region–specific receptor activation and blockade. In addition, a wider range of behavioral tests needs to be employed to examine effects on learning, consolidation, extinction, relearning, and other relevant aspects of information storage that involve different neuronal circuitry. We are near the beginning of our quest to characterize EC roles in brain information storage, and much interesting work lies ahead.

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4.38 Transsynaptic Signaling by NO during Learning-Related Synaptic Plasticity

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4.38.1 Introduction

The relationship between pre- and postsynaptic mechanisms of plasticity has been one of the more controversial areas of neuroscience. On the one hand, studies from a variety of systems including hippocampus and *Aplysia* have suggested that protein and RNA synthesis–dependent long-term (hours to days) synaptic plasticity can involve changes in the number or structure of synapses, which by its nature requires coordinated pre- and postsynaptic alterations. On the other hand, short-term (up to about 1 h) plasticity and the early phases of long-term plasticity have been thought to involve a completely different type of mechanism, covalent modification of existing proteins in either postsynaptic (hippocampus) or presynaptic (*Aplysia*) structures (Kandel, 2001; Malinow and Malenka, 2002). However, this dichotomy was questioned by the discovery of intermediate (2–3 h) forms of plasticity in both hippocampus (Winder et al., 1998) and *Aplysia* (Ghirardi et al., 1995), and in recent years it has been challenged by additional evidence from electrophysiological and imaging studies.

At least under some conditions, pre- as well as postsynaptic physiological mechanisms have been found to contribute to early-phase long-term potentiation (LTP) in hippocampus (Arancio et al., 1995, 1996, 2001; Malgaroli et al., 1995; Ryan et al, 1996; Choi et al., 2003; Zakharenko et al., 2003; Ninan and Arancio, 2004; Wang et al., 2005; Lu and Hawkins, 2006; Ninan et al., 2006) and to a variety of types of short-term plasticity in *Aplysia* including homosynaptic potentiation (Bao et al., 1997; Jin and Hawkins, 2003), heterosynaptic facilitation by serotonin (5HT) (Chitwood et al., 2001; Jin et al., 2004; Li et al., 2005), activity-dependent facilitation (Bao et al., 1998), and the synaptic plasticity during behavioral sensitization (Antonov et al., 2006) and conditioning (Antonov et al., 2003, 2004). In many cases inhibitors injected into the pre- and postsynaptic neurons have more than additive effects (Bao et al., 1997, 1998; Arancio et al., 2001; Antonov et al., 2003, 2004, 2006; Jin and Hawkins, 2003; Wang et al., 2005; Lu and Hawkins, 2006), suggesting that the pre- and postsynaptic mechanisms are not independent but, rather, act synergistically. Furthermore, in some cases postsynaptic inhibitors have been shown to affect presynaptic properties, suggesting transynaptic signaling (Antonov et al., 2003).

One type of mechanism that is known to have these properties is the growth of new synapses or the structural modification of existing synapses (Cohen-Cory, 2002). Consistent with such a mechanism, imaging studies have shown that even the earliest phases of hippocampal LTP are accompanied by pre- and postsynaptic structural alterations. Tens of minutes after the induction of LTP there is an outgrowth of new pre- and postsynaptic processes (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Nikonenko et al., 2003), and even earlier (minutes), there are increases in spine size (Matsuzaki et al., 2004), clusters of postsynaptic glutamate receptors (Shi et al., 1999), and clusters of presynaptic

vesicle-associated proteins and sites where the pre- and postsynaptic clusters colocalize (Antonova et al., 2001). Moreover, the presynaptic alterations appear to depend on retrograde signaling from the postsynaptic cells (Nikonenko et al., 2003; Wang et al., 2005). Similarly, intermediate-term facilitation by 5HT in *Aplysia* is accompanied by increases in clusters of the postsynaptic protein GluR1 (glutamate receptor 1) and the presynaptic protein synaptophysin within hours (Kim et al., 2003; Li et al., 2004), and homosynaptic potentiation is accompanied by aggregation of synaptophysin into new clusters or puncta within minutes (Jin et al., 2003), as occurs during LTP in hippocampal neurons.

4.38.2 Transsynaptic Messengers

Collectively, these results suggest that the early phases of learning-related synaptic plasticity can engage a coordinated sequence of pre- and postsynaptic functional and structural changes that may be early steps in the formation of new synapses, as occurs during synaptic development (Sanes and Lichtman, 1999; Cohen-Cory, 2002). According to this view, early- and late-phase and pre- and postsynaptic mechanisms of plasticity may not be fundamentally different and independent, as previously thought, but, rather, may have features in common (structural alterations) that are involved in the transition from short- to long-term plasticity.

The growth of new synapses during development is thought to involve a variety of anterograde and retrograde messengers, which may serve different functional roles and act cooperatively (Scheiffele, 2003). Consistent with the idea that synaptic plasticity may engage similar mechanisms, LTP involves many of the same messenger molecules including adhesion molecules such as neural cell adhesion molecule (NCAM) (Luthi, 1994), integrins (Staubli et al., 1998; Chan et al., 2003, 2006; Kramar et al., 2006), cadherins (Tang et al., 1998; Bozdagi et al., 2000), and ephrins (Contractor et al., 2002; Armstrong et al., 2006); secreted molecules such as platelet activating factor (del Cerro et al., 1990; Clark et al., 1992; Wieraszko et al., 1993), tissue plasminogen activator (Baranes et al., 1998), brain-derived neurotrophic factor (BDNF) (Kang and Schuman, 1995; Thoenen, 1995; Korte et al., 1996; Zakharenko et al., 2003; Pang et al., 2004), and neuregulin (Kwon et al., 2005); and freely diffusible molecules such as arachidonic acid (Lynch et al., 1989; Williams and Bliss, 1989; Williams et al., 1989), carbon monoxide (Stevens and Wang, 1993; Zhuo et al., 1993), reactive oxygen species (Klann and Thiels, 1999), and nitric oxide.

4.38.3 Early Studies of the Role of Nitric Oxide in Long-Term Potentiation

In this chapter I focus on nitric oxide (NO), which plays a role in late-stage developmental processes and synaptogenesis in a variety of systems (Hindley et al., 1997; Van Wagenen and Rehder, 1999; Packer et al., 2003; Schwarte and Godfrey, 2004; Sunico et al., 2005; Zhang et al., 2005). A previous review (Hawkins et al., 1998) summarized the evidence to that date that NO also plays a role in LTP. Briefly, both neuronal NO synthase (nNOS) and endothelial NO synthase (eNOS) were thought to be present in hippocampal pyramidal neurons (Dinerman et al., 1994; O'Dell et al., 1994; Wendland et al., 1994). At least under some conditions, LTP in hippocampal slices is blocked by NO scavengers, NO synthase (NOS) inhibitors, or knockout of nNOS and eNOS (Bohme et al., 1991; O'Dell et al., 1991; Schuman and Madison, 1991; Haley et al., 1992; Son et al., 1996). LTP can also be mimicked by exogenous NO paired with weak presynaptic activity (in the presence of an *N*-methyl-D-aspartate (NMDA) receptor antagonist to prevent potentiation by the activity alone) (Zhuo et al., 1993). The requirement for pairing with presynaptic activity is thought to preserve the input specificity of LTP with a diffusible messenger such as NO, which might otherwise spread from active neurons and produce potentiation in inactive neighboring neurons.

Experiments on cultured hippocampal neurons indicated that NO acts specifically as a retrograde messenger during potentiation (Arancio et al., 1996). Long-lasting potentiation in culture is blocked by injection of a NOS inhibitor into the postsynaptic but not the presynaptic neuron, or by injection of an NO scavenger into either neuron. The potentiation can be mimicked by release of caged NO in either neuron, paired with weak presynaptic activity. Moreover, an extracellular NO scavenger blocks potentiation by release of caged NO in the postsynaptic neuron, but not in the presynaptic neuron. These results indicate that NO is produced in the postsynaptic neuron, diffuses across the extracellular space, and acts in the presynaptic neuron to produce potentiation.

Additional evidence suggested that NO acts through cyclic guanosine monophosphate (cGMP)

and cyclic GMP-dependent protein kinase (cGK), as it does in many other systems. Soluble guanylyl cyclase (sGC) and cyclic GMP-dependent protein kinase I (cGKI) are present in hippocampal pyramidal neurons (Matsuoka et al., 1992; Verma et al., 1993; Kingston et al., 1996). LTP in hippocampal slices is blocked by inhibitors of sGC or cGK and mimicked by membrane-permeable cGMP analogs paired with weak activity (Zhuo et al., 1994a; Boulton et al., 1995). Similarly, potentiation in cultured hippocampal neurons is blocked by injection of a peptide inhibitor of cGK into the presynaptic neuron and mimicked by injection of cGMP or cGKI into the presynaptic neuron paired with weak activity (Arancio et al., 1995, 2001).

These results made a strong case that the NO-cGMP-cGK pathway can play a role in retrograde signaling during hippocampal LTP. However, some of the results were not replicated in other labs, and the roles of NO, cGMP, and cGK remained controversial. Experiments since 1998 have addressed many of the areas of controversy and, in addition, have made progress in new directions.

4.38.4 Areas of Controversy

A likely reason for different results in different labs is that LTP is a complex phenomenon involving many signaling pathways, with the relative importance of the different pathways depending on a host of experimental variables such as species, strain, age, temperature, induction protocol, slice or culture preparation, saline composition, and so on. A number of studies have tried to define the conditions under which NO, cGMP, and cGK are more or less important (e.g., Son et al., 1998; Lu et al., 1999; Blackshaw et al., 2003; Kleppisch et al., 2003; for discussion, see Lu and Hawkins, 2002), but each of those studies can account for only some of the published data. Thus they support the general idea that experimental variables are important, but they have not provided a simple explanation that can account for all of the differences in published results. Recent studies have suggested that the dynamics of signaling may also be an important variable. For example, NO may play a tonic, as well as phasic signaling role in LTP (Bon and Garthwaite, 2003). In addtition, cGMP and cGK may activate phosphodiesterases that feed back to decrease cGMP levels *below* baseline (Montfort et al., 2002). That result may explain why brief application of cGMP analogs is more effective than prolonged application in producing potentiation (Son et al., 1998).

Another area of controversy has been whether NO, cGMP, and cGK have the correct cellular and subcellular localization to play a role in retrograde signaling during LTP. Recent imaging studies have shown that NO is released during the induction of LTP by tetanic stimulation in hippocampal slices (von Bohlen et al., 2002; Takata et al., 2005). Furthermore, nNOS is concentrated in postsynaptic densities, and sGC and cGKI are concentrated in presynaptic varicosities in hippocampal neurons, consistent with a role in retrograde signaling (Arancio et al., 2001; Burette et al., 2002; Wang et al., 2005). However, sGC and cGKI are also found in postsynaptic densities, suggesting that the NO-cGMP-cGK pathway may have postsynaptic actions as well. In addition, recent studies have shown that eNOS is *not* present in hippocampal neurons, as previously thought, but, rather, is restricted to endothelial cells (Blackshaw et al., 2003). However, NO generated by eNOS in the vascular endothelium produces tonic depolarization of axons (Garthwaite et al., 2006), which might explain both the role of eNOS in LTP (Kantor et al., 1996; Son et al., 1996) and the tonic role of NO (Bon and Garthwaite, 2003). Recent experiments on eNOS knockout animals have supported that idea (Hopper and Garthwaite, 2006).

4.38.5 Molecular and Functional Consequences of NO Signaling

There has also been progress in describing the molecular and functional consequences of NO signaling in hippocampal neurons. Postsynaptically generated NO produces increases in presynaptic cGMP and vesicle cycling, indicative of increased transmitter release, in cultured hippocampal neurons (Micheva et al., 2003). Long-lasting potentiation in culture is also accompanied by an increase in presynaptic vesicle cycling that is blocked by inhibitors of NOS or cGK and mimicked by application of an NO donor or cGMP analog paired with weak activity (Ninan and Arancio, 2004; Ninan et al., 2006). The effects of the NO donor or cGMP analog are blocked by an inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), suggesting that CamKII acts downstream of the NO-cGMP-cGK pathway.

Somewhat surprisingly, very similar results have been reported for long-term *depression* (LTD) in hippocampus, which is accompanied by a decrease in presynaptic vesicle cycling that is thought to involve

retrograde signaling by the NO-cGMP-cGK pathway (Stanton et al., 2001, 2003). In that case, cGK is thought to act by stimulating production of cADP ribose, the endogenous ligand for ryanodine receptors, leading to enhanced Ca^{2+}-induced Ca^{2+} release from intracellular stores and activation of CamKII in the presynaptic neurons (Reyes and Stanton, 1996; Stanton and Gage, 1996; Reyes-Harde et al., 1999a,b). Consistent with that idea, imaging studies have shown that NO produces a long-lasting increase in presynaptic Ca^{2+} transients (Martinez-Serrano et al., 1996). The idea that the NO-cGMP-cGK pathway acts in part by enhancing Ca^{2+} signaling may help to explain how that pathway can be involved in both potentiation and depression, because Ca^{2+} is also thought to be involved in both types of plasticity with different thresholds (for discussion, see Lu and Hawkins, 2002). Thus, although NO or cGMP analogs produce potentiation when paired with brief, high-frequency activity (50 Hz, 0.5 s), they produce long-term depression when paired with longer, low-frequency activity (0.25 Hz, 100 s) (Zhuo et al., 1994b). In both cases, the activity does not produce any plasticity by itself, and NO might act by increasing Ca^{2+} levels above the threshold for that form of plasticity. NO also contributes to both LTP and LTD induced by different patterns of stimulation in cerebellum (Shibuki and Okada, 1991; Lev-Ram et al., 2002).

In addition to acting indirectly by modulating Ca^{2+} levels, NO can regulate transmitter release directly by s-nitrosylation of N-ethylmaleimide sensitive factor (NSF), which is thought to disassemble soluble NSF attachment protein receptor (SNARE) complexes during exocytosis (Matsushita et al., 2003). Interestingly, NO-mediated s-nitrosylation of NSF has also been shown to contribute to a postsynaptic mechanism, membrane insertion of GluR2-containing alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors during LTP in both hippocampus (Huang et al., 2005) and cerebellum (Kakegawa and Yuzaki, 2005).

4.38.6 Role of NO in Early Microstructural Alterations

The NO-cGMP-cGK pathway is also involved in microstructural alterations during LTP including the formation of new presynaptic filopodia, which begin to appear around 10 min (Nikonenko et al., 2003), and new puncta of synaptic proteins, which begin to appear in as little as 1 min (Wang et al., 2005). The increase in puncta is blocked by inhibitors of NOS or cGK and mimicked by exogenous NO or a cGMP analog. Furthermore, cGMP levels are elevated, and cGK is activated and phosphorylates an endogenous substrate, vasodilator-stimulated phosphoprotein (VASP), during potentiation. Somewhat surprisingly, these events occur on both sides of the synapse, and cGMP and cGK are involved in the increases in postsynaptic GluR1 puncta as well as presynaptic synaptophysin puncta and sites where the pre- and postsynaptic puncta colocalize. Consistent with that finding, the NO-cGMP-cGK pathway is also involved in clustering of postsynaptic Ach receptors at the neuromuscular junction (Jones and Werle, 2004; Schwarte and Godfrey, 2004). Furthermore, a few studies have suggested that NO, cGMP, and cGK may play physiological roles in the postsynaptic as well as presynaptic neuron during long-lasting potentiation in hippocampal neurons (Son et al., 1998; Lu et al., 1999; Arancio et al., 2001; Lu and Hawkins, 2002). Thus, NO may activate sGC and cGK simultaneously in both the pre- and postsynaptic neurons, perhaps engaging similar mechanisms and producing coordinate microstructural changes on the two sides of the synapse (**Figure 1**).

The early microstructural changes could in turn be some of the first steps in a sequence of events that leads to the growth of new synapses. That idea suggests a possible novel role for a diffusible messenger such as NO, which is to signal the *de novo* formation of synapses at new locations in response to activation of NMDA receptors at existing synapses. Potentiation is accompanied by a rapid increase in presynaptic puncta that colocalize with puncta of the postsynaptic proteins GluR1, NR1, or PSD95 and therefore might be triggered by very local retrograde signaling, for example, through adhesion molecules (Antonova et al., 2001). However, potentiation is also accompanied by an increase in presynaptic puncta that do not colocalize with any of these postsynaptic puncta but nevertheless require NMDA receptor activation, suggesting the involvement of a messenger that diffuses from the NMDA receptors to the location of the new puncta. Inhibitors of the NO-cGMP-cGK pathway block the increases in synaptophysin puncta that are either colocalized or not colocalized with postsynaptic puncta, suggesting that NO can act as such a messenger both very locally and at some distance (Wang et al., 2005). Furthermore, NMDA receptors and the NO-cGMP-cGK pathway also play roles in

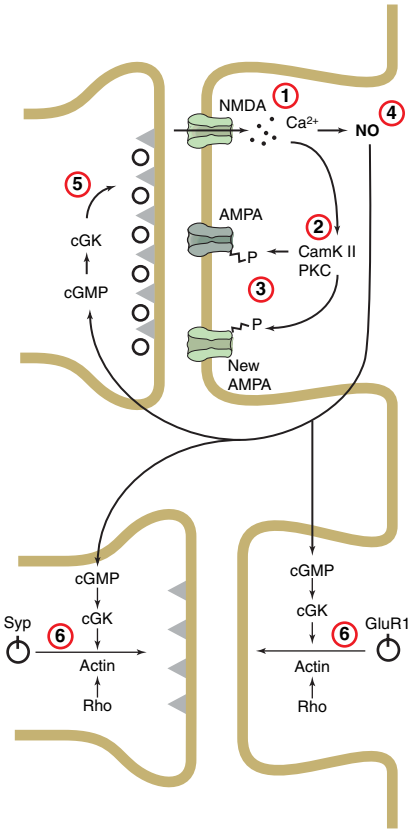


Figure 1 Role of NO in hippocampal long-term potentiation (LTP). Early-phase LTP in the CA1 region of hippocampus involves (1) Ca^{2+} influx through postsynaptic *N*-methyl-D-aspartate (NMDA) receptor channels, (2) activation of protein kinases including CamKII and PKC, and (3) increased conductance of existing AMPA receptor channels and membrane insertion of new alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors. Postsynaptic Ca^{2+} also (4) stimulates production of NO, which acts through cGMP and cGK to (5) enhance presynaptic transmitter release and (6) recruit pre- and postsynaptic molecules, including synaptophysin (Syp) and the GluR1 subunit of AMPA receptors, to new synaptic sites. See the text for details.

the formation of new GluR1 puncta that are either colocalized or not colocalized with synaptophysin puncta and, therefore, may form in new locations as well.

4.38.7 Role of NO in Late-Phase Long-Term Potentiation

In addition to playing roles in E-LTP, NO, cGMP, and cGK are also involved in late-phase LTP (L-LTP), which requires gene regulation and RNA and protein synthesis (Lu et al., 1999). NO can regulate genes directly by binding to a heme group in a nuclear receptor (Reinking et al., 2005). Alternatively, the NO-cGMP-cGK pathway can phosphorylate and activate the transcription factor CREB (cAMP response element binding protein) either directly (Gudi et al., 1996) or indirectly by activating other kinases such as protein kinase A (PKA), mitogen-activated protein kinase (MAPK), or CamK. CREB phosphorylation during L-LTP is blocked by inhibitors of either PKA or cGK and mimicked by application of either a membrane-permeable cAMP analog or a cGMP analog paired with weak activity (Lu et al., 1999). The cGMP-induced L-LTP and CREB phosphorylation are blocked by a high concentration of ryanodine, which blocks Ca^{2+}-induced Ca^{2+} release, suggesting that cGMP and cGK may act indirectly by enhancing postsynaptic Ca^{2+} release during the induction of L-LTP (Lu and Hawkins, 2002).

4.38.8 Roles of NO in Other Forms of Learning-Related Synaptic Plasticity

NO is also involved in LTP in a variety of other brain areas including amygdala (Schafe et al., 2005) and neocortex (Hardingham and Fox, 2006), where it may act as a retrograde messenger. In addition, NO contributes to both LTD and LTP in cerebellum, where it is thought to play a different role. Cerebellar LTD can be induced by prolonged low-frequency stimulation of presynaptic parallel fibers paired with either climbing fiber stimulation or direct depolarization of postsynaptic Purkinje neurons. In either case, the depression is blocked by an NO synthase inhibitor or extracellular NO scavenger and mimicked by substituting exogenous NO for the parallel fiber stimulation, suggesting that NO is involved and travels through the extracellular space (Shibuki and Okada, 1991; Lev-Ram et al., 1995). LTD can also be induced by uncaging NO in a Purkinje neuron paired with depolarization of that neuron (Lev-Ram et al., 1995). In that case, the depression is blocked by an intracellular but not by an extracellular NO scavenger, suggesting that NO acts directly in the postsynaptic Purkinje neuron to produce LTD.

Low-frequency stimulation of parallel fibers without postsynaptic depolarization can induce LTP, rather than LTD (Lev-Ram et al., 2002). Like LTD, the LTP is blocked by an NO synthase inhibitor or

extracellular NO scavenger and mimicked by exogenous NO. Uncaging NO in a Purkinje neuron without depolarization can also induce LTP, which (again like LTD) is blocked by an intracellular but not by an extracellular NO scavenger. These results suggest that NO can act directly in the postsynaptic neuron to produce either LTD or LTP, depending on whether the NO is paired with depolarization. In addition to this NO-dependent LTP, somewhat higher-frequency stimulation of parallel fibers can induce a different form of LTP that is thought to be entirely presynaptic and depend on cAMP rather than NO (Salin et al., 1996). However, NO also appears to be involved in the heterosynaptic spread of that form of LTP to neighboring presynaptic parallel fibers (Jacoby et al., 2001). For LTD and both forms of LTP, NO is generally thought to come from the presynaptic parallel fibers (Shibuki and Kimura, 1997), in which case it would act as either an anterograde or lateral messenger. Alternatively, NO may come from other cell types (Shin and Linden, 2005), in which case it would act as a paracrine messenger.

NO also plays a role in learning-related synaptic plasticity in *Aplysia*. NO synthase is present and active in the *Aplysia* central nervous system (Moroz et al., 1996; Bodnarova et al., 2005; Moroz, 2006), and NO is involved in a variety of cellular (Mothet et al., 1996; Koh and Jacklet, 1999; Sung et al., 2004) and behavioral effects, including conditioning of feeding behavior (Katzoff et al., 2002) and siphon withdrawal (Antonov et al., 2004). Classical conditioning of siphon withdrawal in a semi-intact preparation involves activity-dependent facilitation of monosynaptic excitatory postsynaptic potentials (EPSP) from siphon sensory neurons to siphon motor neurons in the abdominal ganglion, as well as enhanced membrane resistance and evoked firing of the sensory neurons (Antonov et al., 2001, 2003). The behavioral and cellular effects are blocked by bathing the ganglion in a membrane-impermeant NO scavenger, oxymyoglobin, suggesting that NO is involved and travels through the extracellular space. In addition, the facilitation and changes in sensory neuron membrane properties are blocked by injecting oxymyoglobin into the sensory neuron, suggesting that NO acts directly in that neuron. Facilitation of the EPSP is also reduced by injecting oxymyoglobin into the motor neuron, but not by injecting a NOS inhibitor into the motor neuron. Consistent with these physiological results, NADPH diaphorase staining suggests that NO synthase is not present in either the motor neurons or sensory neurons but is present in interneurons in the ganglion (Moroz, 2006). Thus, NO appears to play a paracrine role, coming from interneurons and acting in both the sensory and motor neurons to contribute to facilitation of the EPSP between them during conditioning.

4.38.9 Conclusions

Studies in different systems have suggested that NO can play a variety of different pre- and postsynaptic roles during learning-related synaptic plasticity, including retrograde signaling, anterograde signaling, lateral signaling, and paracrine signaling. In most of these cases, however, NO is thought to coordinate pre- and postsynaptic functional changes. In the case of hippocampal LTP, NO is also thought to contribute to coordinated pre- and postsynaptic structural alterations, which may be early steps in the formation of new synapses. Transsynaptic messengers like NO may be involved in similar structural alterations during other forms of synaptic plasticity as well. The idea that even the early phases of synaptic plasticity can recruit messengers that are also involved in the early stages of synapse formation might help to reconcile some of the seemingly conflicting evidence for pre- versus postsynaptic mechanisms of plasticity, because synapse formation would require both.

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4.39 Action Potentials in Dendrites and Spike-Timing-Dependent Plasticity

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4.39.1 Overview

Over the past 50 years it has become clear that individual neurons are sophisticated signal-processing devices rather than simple summing elements. The computational power of neurons originates from the morphological and physiological properties of their dendrites, which are the neuronal processes that receive most synaptic input. The electrical properties of these dendrites determine how inputs are integrated

and whether they lead to the generation of an action potential (AP) in the axon initial segment. Furthermore, dendritic excitability and synaptic potentials are themselves modified by neuronal output, an effect that is largely mediated by AP propagation or initiation in dendrites. This plasticity enables tuning of synaptic integration and continuous updating of the functions of individual neurons within the neuronal network. In this chapter we give an overview of the active properties of dendrites and discuss their influence on synaptic integration, circuit dynamics, and synaptic plasticity.

4.39.2 Introduction: Electrically Active Dendrites

Much of our understanding of dendritic properties and dendritic function originates from early theoretical work in which cable equations were used to describe the filtering and interaction of postsynaptic potentials (PSPs) in electrically passive dendrites (Rall, 1959, 1962, 1964, 1967; Rall et al., 1967; Rall and Rinzel, 1973; Jack et al., 1975). While passive models were building the framework for our current understanding of dendritic integration, evidence from both extracellular and intracellular recordings was accruing that dendrites are not merely passive but 'active' structures, i.e., that they contain voltage-gated conductances (extracellular: Cragg and Hamlyn, 1955; Fatt, 1957a,b; Andersen, 1960; Fujita and Sakata, 1962; Andersen and Lømo, 1966; Llinas et al., 1968, 1969; Rall and Shepherd, 1968; Wong and Prince, 1978; Llinas and Sugimori, 1979; Miyakawa and Kato, 1986; Turner et al., 1989; Herreras, 1990; intracellular: Spencer and Kandel, 1961; Fujita, 1968; Llinas and Hess, 1976; Wong et al., 1979; Llinas and Sugimori, 1980; Turner et al., 1991; Kim and Connors, 1993). The potential importance of active dendritic properties in shaping synaptic integration was recognized by early theoreticians (e.g., Jack et al., 1975), but it was not until infrared differential interference contrast (IR-DIC) optics were used to visualize dendrites in brain slices in combination with patch pipette recordings (Stuart et al., 1993; Stuart and Sakmann, 1994) that membrane potential recording from thick dendrites of the CNS became a routine procedure and active dendritic properties were studied in detail and their importance was firmly established (Johnston et al., 1996; Häusser et al., 2000; Magee, 2000; Reyes, 2001; Gulledge et al., 2005).

It is now clear that many neurons express a wide array of voltage-gated conductances in their dendrites, including Na^+, K^+, Ca^{2+} and nonselective cation channels (Johnston et al., 1996; Reyes, 2001; Migliore and Shepherd, 2002; Gulledge et al., 2005). The presence of these channels enables various forms of dendritic action potentials (**Figure 1**), which have several consequences for dendritic function. For instance, in hippocampal and neocortical pyramidal neurons, dendritic channels enable the local initiation of spikes within the dendritic tree ('dendritic spikes'). As a consequence, dendritic branches can act as independent (or semi-independent) integrative units (Gasparini et al., 2004; Gasparini and Magee, 2006), thereby expanding the computational power of the dendritic tree (Häusser and Mel, 2003; London and Häusser, 2005).

Voltage-gated channels also support retrograde propagation ('backpropagation') of action potentials (APs) into the dendritic trees of many types of neurons (Stuart and Sakmann, 1994; Stuart et al., 1997b; Waters et al., 2005). Since dendrites also contain voltage-gated Ca^{2+} channels, both backpropagation and dendritic spike initiation can evoke Ca^{2+} influx into dendrites and also into dendritic spine heads (dendrites: Jaffe et al., 1992; Markram et al., 1995; Schiller et al., 1995, 1997; Magee et al., 1995; dendritic spines: Jaffe et al., 1994; Yuste and Denk, 1995; Denk et al., 1996; Sabatini and Svoboda, 2000; Sabatini et al., 2002). Intracellular Ca^{2+} exerts a variety of effects on intracellular signaling pathways, depending on peak concentration, exact localization, and time course of the Ca^{2+} transient. In particular, Ca^{2+} signals that are associated with the propagation (or initiation) of APs in dendrites are essential for some forms of synaptic plasticity, including spike-timing-dependent plasticity (Magee and Johnston, 1997, 2005; Markram et al., 1997; Sjöström and Nelson, 2002; Frick et al., 2004; Nevian and Sakmann, 2004; Golding et al., 2002; Holthoff et al., 2004; Kampa et al., 2006). Action potential backpropagation and spike-timing-dependent plasticity (STDP) are two of the most intensely studied features of active dendrites and are the focus of this chapter.

4.39.3 Action Potentials in Dendrites

4.39.3.1 Variations on a Theme: Action Potential Backpropagation in Different Dendrites

In passive dendrites (i.e., those lacking voltage-dependent conductances) any perturbation of the membrane

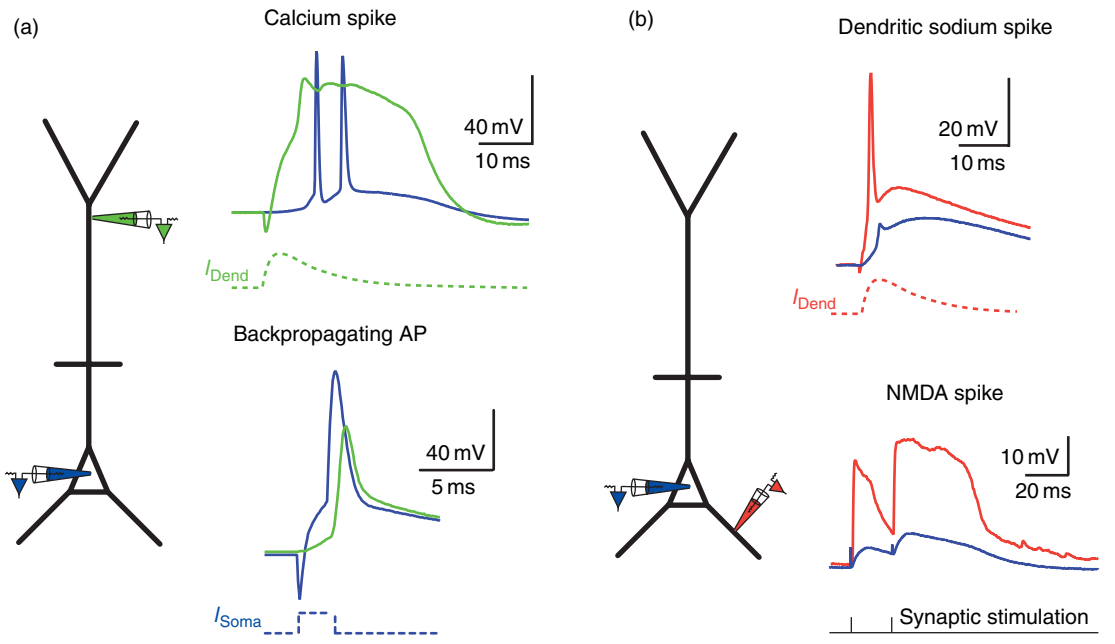


Figure 1 Diversity of dendritic excitability. Illustration of the different types of action potentials observed in dendrites of neocortical pyramidal neurons using multiple-electrode recordings. Electrodes schematically indicate the site of recordings from the soma (blue), distal apical dendrite (green), or basal dendrites (red). Membrane voltage recordings are shown in the corresponding colors. (a) Somatically evoked action potentials backpropagate actively and decrementally into the apical (and basal) dendritic tree (lower traces; example from a layer 2/3 pyramidal neuron). Apical dendrites of layer 5 pyramidal neurons can, in addition, generate calcium spikes mediated by voltage-dependent Ca^{2+} channels (upper traces; evoked in this case by local dendritic depolarization). (b) Sodium action potentials can be elicited locally in both apical and (as shown here) basal dendrites (upper part). Dendritic sodium spikes are greatly attenuated as they propagate toward the soma, thus not necessarily reaching threshold at the soma. Strong synaptic activation can result in the initiation of a *N*-methyl-D-aspartate (NMDA) spike (lower part). Adapted from Waters J, Larkum M, Sakmann B, and Helmchen F (2003) Supralinear Ca^{2+} influx into dendritic tufts of layer 2/3 neocortical pyramidal neurons *in vitro* and *in vivo*. *J. Neurosci.* 23: 8558–8567; Larkum ME, Zhu JJ, and Sakmann B (1999b) A new cellular mechanism for coupling inputs arriving at different cortical layers. *Nature* 398: 338–341; and Nevian T, Larkum ME, Polsky A, and Schiller J (2007) Properties of basal dendrites of layer 5 pyramidal neurons: A direct patch-clamp recording study. *Nat. Neurosci.* 10: 206–214.

potential will spread some measurable distance along the dendrite, but will be attenuated, i.e., cause a lesser perturbation in a neighboring region of the dendrite. The space constant of attenuation is typically on the order of a hundred micrometers or less. This is short compared to the length of most dendrites. In many types of neurons, dendritic ion conductances greatly extend the spatial spread of membrane potential changes, enabling propagation of voltage signals throughout much of the dendritic tree. In particular, dendritic Na^+ channels enable propagation of axonally initiated APs back into the dendritic tree.

4.39.3.1.1 Single action potentials

The extent of AP backpropagation varies in different cell types. In a few dendrites, such as those of dopaminergic substantia nigra neurons (Häusser et al., 1995), the apical dendrites of mitral cells in the olfactory bulb (Bischofberger and Jonas, 1997; Chen et al., 1997) and hippocampal interneurons (Martina et al., 2000), AP backpropagation is reminiscent of axonal propagation in that an AP propagates through the dendritic tree without a substantial decrease in amplitude. In contrast, backpropagation of axosomatically initiated APs approximates passive spread in the dendrites of cerebellar Purkinje neurons, even though local calcium spikes can occur in these dendrites (Llinas and Sugimori, 1980; Stuart and Häusser, 1994). However, in most dendrites examined to date, dendritic ion channels increase the spatial spread of an AP, but fail to maintain its full amplitude as it propagates through the dendritic arbor. Such 'decremental' backpropagation is observed in the apical dendrites of layer 5 and layer 2/3 pyramidal neurons (Kim and Connors, 1993; Stuart et al., 1993, 1997a; Stuart and Sakmann, 1994; Williams and Stuart, 2000b; Larkum et al., 2001; Waters et al., 2003), hippocampal CA1 pyramidal

cells (Andreasen and Lambert, 1995; Spruston et al., 1995), spinal motor neurons (Larkum et al., 1996), and thalamocortical neurons (Williams and Stuart, 2000a). In addition, decremental attenuation has recently been observed in the basal dendrites of layer 5 pyramidal neurons (Nevian et al., 2007) and oblique dendrites of hippocampal CA1 pyramidal neurons (Gasparini et al., 2007).

The inability of most dendrites to support full-amplitude AP propagation can be attributed primarily to a lower density of Na^+ channels in dendrites than in axons. Hence, compared to axons most dendrites can be considered weakly excitable, but even this weak excitability is sufficient to greatly facilitate the spread of APs into the dendritic tree.

Backpropagating APs are often described as 'global' retrograde signals, conveying information about the neuron's output to the sites of synaptic input in the dendritic tree. In light of the preceding discussion, the idea that AP backpropagation is a global signal obviously requires qualification. AP backpropagation generally is not global in that an AP typically does not propagate throughout the whole dendritic tree, but backpropagating APs are also not confined to small dendritic compartments. In this respect AP backpropagation contrasts with dendritically initiated spikes, which produce localized depolarizations that may be restricted to a subregion of the dendritic tree, such as a single branch (Golding and Spruston, 1998; Wei et al., 2001). Hence backpropagation of single APs is not truly global, but is widespread rather than localized.

In dendrites that support decremental backpropagation, the amplitude of a single backpropagating AP can become small enough in distal dendrites that it fails to evoke regenerative activation of Na^+ channels. AP backpropagation is passive distal to this point (Stuart and Häusser, 2001; Bernard and Johnston, 2003). In the part of the dendritic tree where propagation is passive, additional depolarization, such as that from an excitatory postsynaptic potential (EPSP), can restore active propagation, greatly enhancing the amplitude of the backpropagating AP and its conduction into the distal dendrite (Stuart and Häusser, 2001; Bernard and Johnston, 2003).

4.39.3.1.2 Trains of action potentials

Backpropagation of APs is affected differentially during trains of APs, depending on the frequency of the APs in the burst. At low frequencies (<50 Hz) the dendritic amplitudes of late APs in a train are reduced in hippocampal CA1 and neocortical layer 5 pyramidal neurons (Spruston et al., 1995; Stuart et al., 1997b). This accommodation of dendritic AP amplitude arises from a slow recovery of Na^+ channels from inactivation (Colbert et al., 1997). The effect seems to be cell-type specific, as accommodation is minimal in layer 2/3 pyramidal cells (Larkum et al., 2007). At higher AP frequencies, an opposing effect can be observed: bursts of APs can produce more distal depolarization than single APs in dendrites with decremental propagation (e.g., Spruston et al., 1995; Svoboda et al., 1999; Waters et al., 2003; Kampa and Stuart, 2006). With an increase in AP halfwidth during propagation, APs can merge in the dendrite. In effect, the APs act cooperatively to activate dendritic Na^+ and Ca^{2+} channels and depolarize the distal dendrite. Hence bursts of APs – rather than single APs – can provide a global retrograde signal. For example, in layer 5 pyramidal neurons a burst at sufficiently high frequency can evoke a calcium spike (see the section titled 'Calcium spikes'), so that the distal dendrite remains depolarized for tens of milliseconds. This depolarization then propagates forward to the soma and evokes further spiking (Larkum et al., 1999a).

4.39.3.2 Determinants of Action Potential Propagation

The degree of AP amplitude attenuation and the spatial extent of backpropagation are determined by several factors, including dendritic morphology, active dendritic conductances, neuromodulation, and coincident synaptic activity.

4.39.3.2.1 Morphology

Dendritic diameter, spine density and branching patterns affect the biophysical properties of a dendritic tree such as impedance and capacitance. These properties affect AP propagation, but distinguishing these effects from those on dendritic ion channels is difficult. The most informative studies on morphology to date have been compartmental simulations, based on histological reconstructions of single neurons. Compartmental modeling has revealed that dendrites can be interconverted between supporting no propagation, supporting decremental propagation, and supporting nondecremental propagation by changing solely their morphology (Vetter et al., 2001). Furthermore, some morphologies are more sensitive to changes in channel density and distribution than others (Vetter et al., 2001). In pyramidal neurons, the

location of oblique branches also influences backpropagation along the apical dendrite (Schaefer et al., 2003a). In short, dendritic morphology appears to determine to what extent a dendrite will be dependent on its complement of ion channels to support backpropagation. In those dendrites where gross morphology is not dominant, fine morphology and ion channels together fine-tune the degree of AP backpropagation.

Dendritic morphology also contributes to the difference in forward and backward propagation of spikes. Dendrites taper, becoming thinner with distance from the soma. As a result, backpropagating APs travel from regions with relatively low to relatively high impedance and relatively high to relatively low capacitance (Goldstein and Rall, 1974; Koch, 1999). These factors favor backward propagation as each successive patch of membrane in the AP's path along the dendrite depolarizes more readily than the previous one. Spikes traveling in the opposite direction (dendritically initiated spikes traveling toward the soma) suffer the reverse effect, with each successive patch of membrane depolarizing less readily than the previous one, and the AP is therefore heavily attenuated during forward propagation. As a result, AP backpropagation is typically stronger than forward propagation in the same dendrite (Vetter et al., 2001). This effect of morphology contributes to the frequent failure of dendritic spikes to propagate to the soma in dendrites with robust AP backpropagation (e.g., Golding and Spruston, 1998).

Impedance mismatch also makes backpropagating APs less prone to branch-point failure than forward propagating APs. Branch-point failure is normally the result of impedance mismatch between parent and daughter branches and occurs where the sum of the impedances of the daughter branches is lower than the impedance of the parent branch, giving a smaller depolarization in the daughter branches than in the parent dendrite (Koch, 1999). This situation arises in axons and also during the forward propagation of APs in dendrites, where the AP passes from a small-diameter distal dendritic branch into a larger-diameter proximal branch. In contrast, during backpropagation the AP travels from large- to small-diameter dendritic branches, i.e., from lower to higher input impedance branches. Branch-point failure is therefore unlikely during backpropagation of single APs. However, partial branch-point failure has been observed during the backpropagation of bursts of APs in hippocampal pyramidal neurons (Spruston et al., 1995). In this instance backpropagation was inferred from imaging an intracellular fluorescent calcium indicator. Throughout much of the apical dendritic tree the amplitude of the AP-evoked Ca^{2+} signal following a burst was substantially larger than that following a single AP, but in some branches the burst-evoked was comparable with the single AP-evoked Ca^{2+} signal (Spruston et al., 1995). Various potential explanations for this result have been suggested (Migliore, 1996), including failure of some APs in a burst to propagate through axonal branch points. This is likely attributable to the redistribution of ions during bursts of APs (Koch, 1999) and a higher density of A-type K^+ channels in the branch in which full propagation of bursts of APs fails to occur (see the following section titled 'Active conductances').

4.39.3.2.2 Active conductances

Dendritic channel densities are generally studied with whole-cell recordings and with cell-attached or excised patches. In addition, imaging of Ca^{2+} and Na^+ concentration changes using fluorescent indicator dyes can provide information about the distribution of ion channels. However, the influence of other factors such as surface-to-volume ratio and Ca^{2+} extrusion mechanisms largely precludes quantification of channel density using these imaging methods. Hence patch-clamp recordings have provided the most valuable quantitative information, although they also face a number of problems. Not only are dendritic recordings difficult to obtain, but formation of a GΩ seal can stretch the membrane in the patch, making estimates of the area of membrane in the patch approximate. This decreases the accuracy of estimates of channel density from patch clamp recordings. Seal formation can also affect channel properties (Clark et al., 1997; Fenwick et al., 1982). Nonetheless, cell-attached recordings probably provide the most accurate estimates of channel distributions, since they avoid the pronounced space-clamp problems inherent in whole-cell recordings from dendrites (but see Schaefer et al., 2003b).

A wealth of information on dendritic channel densities has been derived from these electrophysiological techniques (Reyes, 2001; Migliore and Shepherd, 2002). In most neurons the distributions of Na^+ channels, K^+ channels, and sometimes Ca^{2+} channels are the most important determinants of AP backpropagation. This is clearly illustrated in compartmental models. For example, the dendritic excitability of motor neurons can be reproduced with a dendritic

Na^+ current density approximately three orders of magnitude lower than the estimated axonal Na^+ channel density (Lüscher and Larkum, 1998). Estimates of dendritic Na^+ channel densities in various neurons correlate with the strength of propagation, from near zero in the dendrites of Purkinje neurons (that do not support AP backpropagation), around 40–60 $pS\,\mu m^{-2}$ in the apical dendrites of CA1 and neocortical pyramidal neurons (that support decremental AP backpropagation) to approximately 100 $pS\,\mu m^{-2}$ in the apical dendrites of olfactory mitral cells and hippocampal interneurons (that support nondecremental AP backpropagation; Migliore and Shepherd, 2002).

Potassium channel density and distribution have been investigated in detail only in pyramidal neurons of the CA1 region in the hippocampus and layer 5B pyramidal neurons in the neocortex (Hoffman et al., 1997; Bekkers, 2000a,b; Korngreen and Sakmann, 2000). In both cell types transient (A-type) K^+ currents are important regulators of AP backpropagation. In hippocampal, but not neocortical pyramidal neurons, A-type current density increases with distance from the soma (Hoffman et al., 1997). A-type current activates so rapidly upon depolarization by an AP that it can significantly limit the amplitudes of backpropagating APs, and suppression of dendritic A-type current substantially enhances AP backpropagation in hippocampal CA1 pyramidal neurons (Hoffman et al., 1997; Johnston et al., 2000).

Na^+ and A-type K^+ currents act synergistically in the apical dendrites of hippocampal pyramidal neurons to limit backpropagation during bursts of APs. During bursts the depolarization provided by each AP causes both inactivation of dendritic Na^+ channels and activation of dendritic A-type K^+ channels (Colbert et al., 1997). The resulting reduction in Na^+ current and shunting of the membrane together reduce the amplitudes of the subsequent APs. As a result, later APs in a burst propagate less readily into the dendritic tree than does the first AP, and this is seen as pronounced accommodation in the dendritic AP amplitude during a burst (Callaway and Ross, 1995). Similar accommodation occurs in the apical dendrites of some layer 5B neocortical pyramidal neurons (Stuart et al., 1997a; Larkum et al., 2001), but not in layer 2/3 neocortical pyramidal neurons (Larkum et al., 2007). Presumably these differences reflect differences in Na^+ or, more likely, K^+ channel expression patterns between these pyramidal neurons.

The distribution of Ca^{2+}channels has been described in less detail, but in hippocampal pyramidal neurons Ca^{2+} channel subtype expression changes along the apical dendrite, although total Ca^{2+} channel density remains constant (Magee and Johnston, 1995a). There is also indirect evidence for a dendritic 'hotspot' in Ca^{2+} channel density in the apical dendrites of layer 5 neocortical pyramidal neurons (Yuste et al., 1994; Schiller et al., 1997; Helmchen et al., 1999; Schaefer et al., 2003b).

4.39.3.2.3 Synaptic activity

In the preceding sections we have discussed the extent of AP backpropagation and the manner in which AP backpropagation is influenced by dendritic morphology and the presence of ion channels. These parameters are relatively static for any one neuron, changing only over relatively prolonged timescales. However, AP backpropagation will also be affected by excitatory and inhibitory synaptic activity, which can strongly alter dendritic properties on the millisecond timescale. In addition, neuromodulatory synapses can regulate AP backpropagation.

Both EPSPs and inhibitory postsynaptic potentials (IPSPs) can affect dendritic membrane potential and input resistance, and both can modulate AP backpropagation (Tsubokawa and Ross, 1996; Stuart and Häusser, 2001; Lowe, 2002; Xiong and Chen, 2002; Pérez-Garci et al., 2006). Since the chloride reversal potential is in most neurons close to the resting membrane potential (e.g., around −70 mV for layer 5 neocortical pyramidal neurons; Luhmann and Prince, 1991; Gulledge and Stuart, 2003), IPSPs exert their effects primarily by shunting the membrane. The time window over which this shunt is effective is the duration of the conductance change underlying the IPSP (approximately 10–20 ms; Destexhe et al., 2003; Koch, 1999). In contrast to IPSPs, the reversal potential for EPSPs is far from the resting membrane potential, and both shunting and depolarization will therefore shape the interaction of AP and EPSP. However, the conductance change of an EPSP is briefer than that of an IPSP (AMPAR-mediated EPSP lasts about 5 ms; Häusser and Roth, 1997; Koch, 1999; Magee and Cook, 2000). For both EPSPs and IPSPs the evoked voltage change is longer than the conductance change. Furthermore, conductance changes are more spatially localized in dendrites than are the resulting voltage changes (Rall, 1967; Koch et al., 1990; Williams, 2004). Hence the time window over which an EPSP can shunt a backpropagating AP is brief, whereas the time course over which the resulting depolarization can interact with the backpropagating AP is more prolonged.

The dominant effect of EPSPs is to enhance AP backpropagation, acting synergistically with a backpropagating AP to depolarize the dendritic membrane beyond threshold for Na^+ channel activation (Stuart and Häusser, 2001). EPSPs are therefore particularly effective in the distal dendrite, where AP amplitude is small and may not activate Na^+ channels (Stuart and Häusser, 2001; Bernard and Johnston, 2003). In addition, the combined depolarization of EPSP and backpropagating AP can initiate additional spiking in the dendrite (Magee and Johnston, 1997; Larkum et al., 1999a,b; Larkum and Zhu, 2002).

Backpropagation of APs is also influenced by neuromodulators, for example via muscarinic acetylcholine receptors (McCormick, 1992; McCormick et al., 1993). The effects of neuromodulators on AP backpropagation have been examined in most detail in hippocampal CA1 pyramidal neurons, where muscarinic agonists enhance the backpropagation by inhibiting K^+ channels (Tsubokawa and Ross, 1997; Hoffman and Johnston, 1999). Modulatory pathways may also effect dendritic spikes. For example, inhibitory inputs can cause a long-lasting blockade of the dendritic Ca^{2+} spike in the apical dendrite of layer 5 pyramidal neurons via metabotropic receptors of the $GABA_{B1b}$ isoform (Pérez-Garci et al., 2006). Most likely metabotropic action in this case involves inhibition of Ca^{2+} channels, although activation of K^+ channels may also contribute (Pérez-Garci et al., 2006).

4.39.3.3 Action Potential Backpropagation *in vivo*

Ongoing, background synaptic activity is higher *in vivo* than in brain slices (Paré et al., 1997, 1998). This has led to the suggestion that AP backpropagation may be different *in vivo* from that in slices (Steriade, 2001a,b). In principle, synaptic activity *in vivo* could enhance, suppress, or have little effect on AP backpropagation, depending on the spatiotemporal pattern of synaptic activity and the numbers of active excitatory, inhibitory, and neuromodulatory synapses. As already discussed, the dominant effects of EPSPs and IPSPs are enhancement and suppression of AP backpropagation, respectively. Hence one would expect the ratio of excitation to inhibition to be the principal factor in determining the effect of ongoing synaptic activity on AP backpropagation. The total amount of synaptic activity may also be important, since EPSPs and IPSPs both are generated by the opening of receptor-gated channels and therefore necessarily increase membrane conductance.

4.39.3.3.1 AP backpropagation in anaesthetized animals

There is now a substantial body of information available examining AP backpropagation in anaesthetized animals. Two early studies concluded that AP backpropagation was suppressed in the neocortex of the anaesthetized rat (Svoboda et al., 1997, 1999). However, the majority of studies concluded otherwise, indicating that AP backpropagation occurs in the hippocampus (Buzsáki et al., 1996), olfactory bulb (Charpak et al., 2001; Debarbieux et al., 2003), and neocortex (Waters et al., 2003; Waters and Helmchen, 2004) of anaesthetized animals.

A direct comparison of AP backpropagation in brain slices and in anaesthetized animals is available for only one cell type; the layer 2/3 pyramidal neuron from somatosensory neocortex (**Figure 2**). In layer 2/3 pyramidal neurons AP backpropagation is supported by Na^+ channels in the slice preparations and is similar in slices and during periods of little synaptic activity ('Down states') in anaesthetized rats, implying that AP backpropagation is also actively supported by dendritic Na^+ channels *in vivo* (Waters et al., 2003). Comparison of dendritic Ca^{2+} transients evoked by backpropagating APs during relatively quiet Down states and during periods with synaptic background activity ('Up states') revealed that ongoing synaptic activity slightly enhanced AP backpropagation (Waters and Helmchen, 2004).

The enhanced AP propagation during Up states in layer 2/3 neurons might suggest that, in these neurons, the ratio of excitatory to inhibitory synapses active during periods of pronounced background activity is greater than one. Consistent with this idea, the estimated numbers of synapses active during Up states was relatively low, producing only a small (<10 nS) conductance change at the soma (Waters and Helmchen, 2006). Furthermore, the ratio of active excitatory to inhibitory synapses was high (around one in ten synapses was inhibitory; Waters and Helmchen, 2006). These estimates were indirect, being derived from a combination of input resistance measurements and compartmental modeling. They indicate that ongoing, background synaptic activity in anesthetized rats has little effect on AP backpropagation principally because activity is sparse and mostly excitatory.

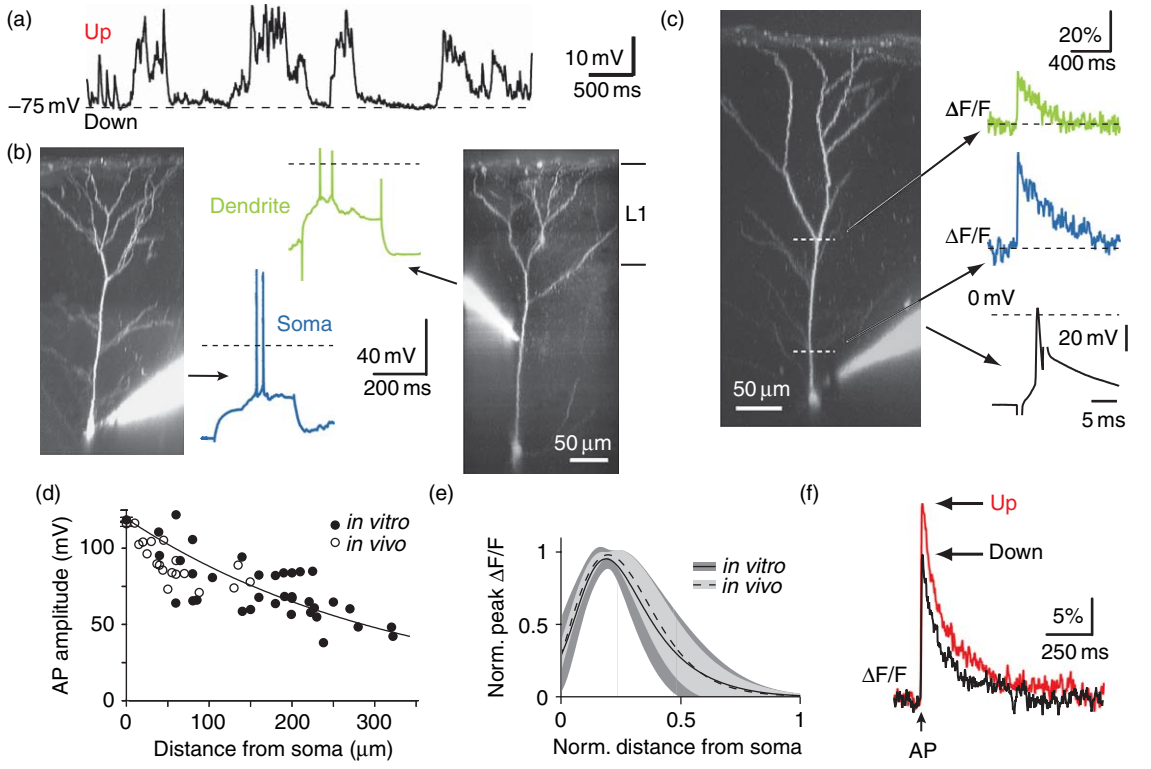


Figure 2 Action potential backpropagation. (a) Example of ongoing subthreshold membrane potential fluctuations *in vivo,* recorded with a whole-cell patch pipette from the apical dendrite of a layer 2/3 pyramidal neurons in a urethane-anesthetized rat. Spontaneously occurring synaptic inputs cause membrane potential fluctuations between so-called Up and Down states. (b) Fluorescence side-projections of two layer 2/3 pyramidal neurons in somatosensory cortex from anaesthetized rats. Each neuron was filled with a fluorescent indicator via the whole-cell recording pipette located on the soma (left) or the dendrite (right). Action potential recorded from the soma (blue) and dendrite (green) are also shown. (c) Calcium transients in the proximal (blue) and distal (green) apical dendrite evoked by single APs backpropagating into in the apical dendrite of a layer 2/3 neuron in a urethane-anesthetized rat. Capacitive artifacts on the AP trace are truncated. (d) Backpropagating AP amplitudes as a function of distance from the soma, determined with whole-cell recordings from the apical dendrites of layer 2/3 pyramidal neurons both in brain slices (*in vitro*) and in anesthetized rats (*in vivo*). (e) Spatial profile of single AP-evoked dendritic Ca^{2+} transients (normalized to maximum amplitude) along the apical dendrites of layer 2/3 pyramidal neurons both *in vitro* and *in vivo*. (f) Comparison of single AP-evoked Ca^{2+} transients in the distal apical dendritic trunk of a layer 2/3 pyramidal neuron during Up and Down states. Figure parts adapted from Waters J, Larkum M, Sakmann B, and Helmchen F (2003) Supralinear Ca^{2+} influx into dendritic tufts of layer 2/3 neocortical pyramidal neurons *in vitro* and *in vivo*. *J. Neurosci.* 23: 8558–8567; and Waters J and Helmchen F (2004) Boosting of action potential backpropagation by neocortical network activity *in vivo*. *J. Neurosci.* 24: 11127–11136.

Whether ongoing background synaptic activity is similar in other preparations and other types of neurons is unclear. Similar results have been reported for deep-layer pyramidal neurons in ferret visual cortex (McCormick et al., 2003; Shu et al., 2003; Haider et al., 2006). In contrast, results from deep-layer pyramidal neurons in cat neocortex are more consistent with the idea that the majority of active synapses during background activity are inhibitory (Destexhe et al., 2003). The reason(s) for this discrepancy are unclear. Anesthesia, species, and age differences and neocortical region studied are all possible contributory factors. However, one prominent difference is the rate of spiking in some studies, which may confound the interpretation of differences in input resistances between Up and Down states in terms of the numbers of active synapses (Waters and Helmchen, 2006).

4.39.3.3.2 AP backpropagation in awake animals: what should we expect?

High levels of ongoing synaptic activity also occur in awake animals (Steriade et al., 2001; Destexhe et al., 2003), and extracellular recordings suggest that AP

backpropagation is not completely suppressed in the hippocampus or neocortex in awake, behaving animals (Buzsáki and Kandel, 1998; Quirk et al., 2001). However, it is unclear how AP backpropagation in awake animals compares to that in brain slices and in anesthetized animals. Small changes in active properties, which might not be readily apparent in extracellular recordings, could have profound effects on AP waveform and spatial spread and, therefore, on time windows and the dendritic locations of synaptic plasticity.

The effect of synaptic input on both AP backpropagation and dendritic spike initiation depends strongly on the spatiotemporal pattern of synaptic activation (Stuart and Häusser, 2001; Gasparini and Magee, 2006; Pérez-Garci et al., 2006). Nonetheless, in light of the available extracellular data and data from anesthetized animals, showing robust AP backpropagation in the presence of synaptic activity, it is tempting to speculate that ongoing background excitatory and inhibitory synaptic activity have little effect on AP backpropagation in awake animals. However, AP backpropagation may be more strongly influenced by appropriately timed evoked activity, such as from sensory input, attentional processing, or motor activity. In addition, neuromodulatory input may be an important factor in awake animals. Many neuromodulators have strong effects on dendritic excitability in slice preparations. Neuromodulatory activity is relatively sparse in anesthetized animals, but neuromodulatory activity varies in many brain regions with behavior. In all probability, therefore, AP backpropagation will be modulated by synaptic activity in at least some behavioral states. Intracellular measurements describing the extent of AP backpropagation in awake (and ideally behaving) animals are required to resolve this issue (Lee et al., 2006).

4.39.3.4 Local Dendritic Action Potentials

Another consequence of active dendritic conductances is that regenerative potentials can be initiated in dendritic branches. These local APs propagate forward toward the soma and either remain subthreshold or trigger sodium APs at the soma. The conditions under which local dendritic APs are generated are still unclear, but it is presumed that they require synchronous and clustered synaptic inputs. Since they are typically associated with substantial dendritic Ca^{2+} influx, dendritic spikes may contribute to synaptic plasticity (Golding et al., 2002; Holthoff et al., 2006). Three types of local dendritic APs have been distinguished. They differ with respect to the shape of the voltage waveform and the underlying currents.

4.39.3.4.1 Sodium spikes

Due to the presence of voltage-dependent Na^+ channels in the dendrites of most cell types, fast APs can be initiated in the dendrites themselves. The idea of local dendritic APs, which then summate at the soma, is an old one (Spencer and Kandel, 1961), but a convincing demonstration of dendritic spikes had to await the development of direct dendritic recordings. In multiple cell types in brain slices, current injection into the dendrite can produce fast APs that are sensitive to tetrodotoxin (TTX) (Stuart et al., 1997a; Golding and Spruston, 1998; Larkum et al., 2001; Wei et al., 2001). These fast dendritic APs can also be triggered by synaptic stimulation (Turner et al., 1991; Golding and Spruston, 1998; Gasparini et al., 2004) and attenuate while propagating forward toward the soma. If they remain subthreshold at the soma they are often referred to as 'isolated' dendritic spikes.

Sodium spikes were first described in the apical dendrites of hippocampal and neocortical pyramidal neurons (Regehr, 1993; Stuart et al., 1997) and for mitral cell dendrites in the olfactory bulb (Chen et al., 1997). More recently evidence has accumulated that they can also occur in thinner dendrites such as the basal and oblique dendrites of pyramidal cells (Milojkovic et al., 2005; Losonczy and Magee, 2006; Nevian et al., 2007). It is likely that many thin dendrites are capable of generating APs, although it is unclear under what conditions they occur.

4.39.3.4.2 Calcium spikes

The activation of other channels, in particular Ca^{2+} channels, can cause more complex dendritic spikes that are typically characterized by a longer-lasting plateau phase. A clear example is the large Ca^{2+} spike in the apical dendrite in layer 5 neocortical pyramidal neurons, which can last 20–30 ms (Schiller et al., 1997; Larkum and Zhu, 2002). Calcium spikes in layer 5 apical dendrites in most cases are suprathreshold at the soma, meaning that they initiate one or more somatic Na^+ APs. In turn, Ca^{2+} spikes can be generated by applying high-frequency stimuli above a critical frequency of around 100 Hz to the soma (Larkum et al., 1999a). In this case depolarizations caused by the individual stimuli summate in the dendrite until sufficient numbers of Ca^{2+}

channels are activated for the depolarization to become regenerative.

A key signature of Ca^{2+} spikes is the large concomitant Ca^{2+} influx (Yuste et al., 1994; Schiller et al., 1997; Helmchen et al., 1999). Calcium imaging therefore can help to identify dendritic zones in which dendritic Ca^{2+} spikes might occur. For example, *in vivo* calcium imaging demonstrated the occurrence of nonlinear Ca^{2+} influx in distal dendrites of layer 2/3 neurons in anesthetized animals (Waters et al., 2003). A similar nonlinear behavior has been reported for basal dendrites of layer 5 pyramidal neurons (Kampa et al., 2006). It remains to be determined, however, whether these dendritic Ca^{2+} signals are caused by pure Ca^{2+} spikes or by more complex, mixed depolarizing currents.

4.39.3.4.3 NMDA spikes

Another mode of dendritic regenerativity involves the voltage dependence of the *N*-methyl-D-aspartate receptor (NMDAR). Schiller et al. (2000) noticed that glutamate uncaging at dendritic sites can elicit plateau potentials at the soma that depend nonlinearly on stimulus intensity. Similar results were obtained with focal synaptic stimulation, and these potentials depend on NMDAR activation (Schiller and Schiller, 2001). NMDA spikes result in plateau potentials with large dendritic depolarizations (around 30 mV). These spikes are less attenuated *en route* to the soma than subthreshold EPSPs and therefore represent a mechanism by which dendrite-to-soma coupling is greatly enhanced (Nevian et al., 2007).

4.39.3.5 Potential Physiological Functions

Why are dendrites excitable? What are potential functions of APs in dendrites? Are they essential for proper circuit function *in vivo?* Several functions can be attributed to APs in dendrites. These functions can be divided into two classes according to whether they predominantly act in a 'forward' manner, shaping the integration of synaptic inputs, or whether they provide a 'feedback' signal to the dendrites. In addition, one has to differentiate between effects occurring on disparate timescales. For example, APs in dendrites largely determine the spread of membrane potential changes on a millisecond time scale but they also can lead to short-term and long-term changes of dendritic excitability and of synaptic inputs.

First, voltage-dependent currents in the dendrites can help distal synaptic currents to contribute to membrane potential changes at the soma. For example, dendritic Na^{+} and Ca^{2+} channels can amplify synaptic potentials (Magee and Johnston, 1995b; Schwindt and Crill, 1995; Lipowsky et al., 1996; Gillessen and Alzheimer, 1997; Oviedo and Reyes, 2002). In contrast, transient A-type K^{+} currents in dendrites can counteract rapid and strong dendritic excitation, acting as a brake on dendritic excitability (Hoffman et al., 1997). Hence dendritic channels can modulate the summation of synaptic potentials in both directions.

Dendritic integration is transformed once local dendritic APs are initiated. As has been demonstrated for several neuronal cell types, dendrites can act as local, nonlinear thresholding devices (Polsky et al., 2004; London and Häusser, 2005). Parts of the dendritic tree or even single dendritic branches thus may form independent or semi-independent integration zones (Häusser and Mel, 2003; Poirazi et al., 2003). Conceptually, this finding is significant because it means that dendritic branches, rather than single neurons, may constitute the functional building blocks of synaptic integration. In network models each individual neuron may have to be treated as a small, but complicated 'neural network'. In addition, local spikes may contribute, via concomitant Ca^{2+} influx, to synaptic plasticity (as discussed below). Despite the demonstration of local dendritic spikes in many neurons in brain slice preparations, a confirmation of their existence *in vivo* is still lacking. A possible explanation for this lack of evidence is that the initiation of local spikes has stringent requirements for clustered and synchronized synaptic inputs (Gasparini and Magee, 2004). It remains unclear under what conditions and during which specific behavioral states these requirements might be fulfilled. Likewise, the function of dendritic spikes in circuit computations and behavior remains unknown.

Complex interactions are expected to occur between backpropagating APs, synaptic input, and dendritic spikes. Backpropagating APs, for example, can lower the threshold current required to generate a dendritic Ca^{2+} spike in neocortical layer 5 pyramidal neurons (Larkum et al., 1999a). Furthermore, dendritic APs can increase the probability of initiating an additional AP at the axon/soma. As a result, the ability of a neuron to initiate spikes in several locations can influence the neuron's spiking output. Hence synaptic input onto the distal dendrites of a layer 5 neocortical pyramidal neuron can act

synergistically with perisomatic input to generate a burst of APs (Schiller et al., 1997; Larkum et al., 1999b; Williams and Stuart, 1999; Schaefer et al., 2003b). The basal/oblique and the apical/tuft dendritic trees of pyramidal cells receive synaptic input from different sources. For example in somatosensory cortex, basal/oblique and apical/tuft dendrites receive ascending thalamic input from different nuclei, corresponding to specific sensory (lemniscal) and more diffuse (paralemniscal) pathways, respectively. As a result of the ability of layer 5B neurons to generate dendritic Ca^{2+} spikes, excitation might be particularly effective in those pyramidal neurons that receive coincident activation of their tufts located in layer 1 and of their basal dendrites in layer 5 during sensory stimuli. Hence it has been suggested that dendritic spiking enables pyramidal neurons to act as cellular coincidence detectors, associating input from different projections which arrive in different layers of the cortex.

Like individual APs, trains of APs are reliably conveyed via the axonal arbor toward the cell's target neurons. At axonal boutons, short-term facilitation of release can render the transmission of AP bursts more effective than the transmission of single APs (Lisman, 1997; Williams and Stuart, 1999). At synapses with short-term depression, this effect will be less pronounced (Markram et al., 1998a,b; Reyes et al., 1998; Thomson, 2003). Individual pyramidal neurons are connected to different classes of postsynaptic cells via synapses that are either facilitating or depressing, depending on the target cell type (e.g., Reyes et al., 1998; Rozov et al., 2001). Thus by inducing axosomatic AP bursts, backpropagating APs in neocortical pyramidal neurons contribute to short-term changes in synaptic transmission. Thus they dynamically regulate the direction of signal flow through neuronal circuits.

Backpropagating APs can also release transmitter in dendrites that form dendro-dendritic synapses. Here, backpropagating APs have a similar function to axonally propagating APs, triggering dendritic Ca^{2+} influx, which then leads to the release of fast-acting transmitter. For example, in the olfactory bulb, dendritic GABA release from granule cells, dendritic glutamate release from mitral cells onto granule cells, and mitral cell self-excitation are influenced by the extent of backpropagation (Isaacson, 1999; Margrie et al., 2001; Salin et al., 2001; Lowe, 2002; Xiong and Chen, 2002; Christie and Westbrook, 2003; Egger et al., 2003). As a consequence, lateral inhibition, which is a potential cellular correlate of contrast enhancement for odor discrimination (Yokoi et al., 1995; Shepherd and Greer, 1998; Urban, 2002; Margrie and Schaefer, 2003), relies on the spread of APs in these dendrites. Similarly, AP backpropagation in some neocortical interneurons (Kaiser et al., 2001, 2004) may evoke dendritic release of GABA onto axonal boutons of pyramidal cells (Zilberter et al., 1999), and backpropagating APs in pyramidal neurons may control the release of glutamate or cannabinoid from the dendrite (Zilberter, 2000; Kreitzer and Regehr, 2002; Sjöström et al., 2003; Trettel and Levine, 2003). These two local feedback loops could regulate the AP activity of both interneurons and pyramidal cells to maintain stable patterns of APs in local cortical circuits.

Another function of backpropagating APs could be the stabilization of nascent synapses. During postnatal development, neuronal circuits in many CNS regions are still in the process of shaping their longer-term connectivity, often in an experience-dependent manner. With elevated levels of spontaneous activity in young animals, backpropagating APs could not only contribute to synchronizing a developing network via dendro-dendritic gap junctions (Peinado, 2001; Peinado et al., 1993), but might also evoke dendritic release of trophic factors which could serve as attractors for axonal growth cones or as a signal for axon collateralization (McAllister, 1999; Poo, 2001). The dependence of synapse growth and stabilization on backpropagating APs, which transmit a modified copy of the axonal AP pattern to the dendrites, could help establish cell assemblies that fire together. NMDA-type glutamate receptors are probably key elements in this process, as they are abundant in postsynaptic structures early in development (Crair and Malenka, 1995; Washbourne et al., 2002). Backpropagating APs could lead to enhanced, highly localized Ca^{2+} influx into dendrites via postsynaptic NMDA receptors through a transient relief of the Mg^{2+} block of NMDARs (Mayer et al., 1984). This putative function of AP backpropagation in refining the specificity of neuronal circuits by coincident pre- and postsynaptic APs in neuronal assemblies during early postnatal development has yet to be studied in detail.

Finally, a backpropagating AP, as a widespread signal to the dendritic arbor, reports output activity to sites of synaptic input so that localized long-term changes in the efficacy of excitatory synapses may be induced. Such changes in synaptic strength depend on precise timing relationships between pre- and postsynaptic AP activity during a relatively narrow

time window on the order of about 100 ms (for review see Linden, 1999; Dan and Poo, 2006). This spike-timing-dependent plasticity (STDP) will be addressed in detail in the second part of this chapter.

4.39.4 Spike-Timing-Dependent Plasticity

4.39.4.1 Plasticity of Synapses and Memory Formation

The cellular basis of memory formation and information storage is thought to depend on long-term changes in synaptic efficacy. These changes depend on activity patterns in the connected neurons (Bliss and Collingridge, 1993; Moser et al., 1998; Morris, 2006; Whitlock et al., 2006). As proposed by Donald Hebb, the repeated activation of one neuron by other connected neurons results in the strengthening of the connections between them (Hebb, 1949). This form of synaptic plasticity, called long-term potentiation (LTP), was first demonstrated in the hippocampus using tetanic stimulation (Bliss and Lomo, 1973). The inverse process, weakening of a synaptic connection, occurs under different conditions, as observed in the form of long-term depression (LTD) following repeated low-frequency synaptic stimulation (Dudek and Bear, 1992; Mulkey and Malenka, 1992). Similar to some forms of behavioral learning and memory formation *in vivo*, the activity-dependent modification in synaptic strength is associative and input specific (Stanton and Sejnowski, 1989).

Changes in synaptic strength can also be accompanied by cellular and subcellular structural modifications. The structure of a synapse is dynamic, and new synaptic connections may be established and old ones degraded. On a more subtle level, the expression of LTP and LTD may be correlated with dendritic spine growth and shrinkage, respectively. The morphological changes associated with synaptic plasticity are summarized elsewhere (Harris, 1999; Lüscher et al., 2000; Muller et al., 2000; Yuste and Bonhoeffer, 2001; Kasai et al., 2003; Nimchinsky et al., 2002; Oertner and Matus, 2005). Here we focus on the role of active dendrites in the induction of changes in synaptic efficacy.

4.39.4.1.1 Timing matters: spike-timing-dependent plasticity

The rule for modifying synaptic weights proposed by Hebb (1949) suggested that coincident activity in two neurons (Hebb states: "cell A is near enough to excite cell B") is a prerequisite for the modification in synaptic strength. More recently it was found for excitatory synaptic connections that the precise timing and the order of pre- and postsynaptic APs can determine the magnitude and the direction of changes in synaptic weight (Markram et al., 1997; Bi and Poo, 1998; Feldman, 2000). For example, a postsynaptic AP that follows a presynaptic AP within a critical time window of 5–40 ms can result in LTP, whereas a postsynaptic AP preceding a presynaptic AP within a time window of 1–100 ms produces LTD, with a sharp transition from maximal LTP to maximal LTD at around zero time delay (**Figure 3**). This form of synaptic modification has been termed spike-timing-dependent plasticity (STDP). STDP is considered one plausible model for the induction of local synaptic modifications, which could account for experience-driven changes of connectivity in neuronal circuitry (Song and Abbott, 2001; Senn, 2002; Dan and Poo, 2006). In the following sections we will discuss the mechanisms that underlie the spike timing-dependence of synaptic plasticity, the shape of the curve describing the time dependence of STDP, the variability between cell types, and the dependence on the location of the synapse within the dendritic tree. First, we focus on the induction phase of STDP, during which the electrical activity pattern is translated into a biochemical signal that controls the subsequent expression of changes in synaptic efficacy (Derkach et al., 2007).

4.39.4.1.2 Induction protocols

The parameter space for pairing protocols that lead to changes in synaptic efficacy by near coincident pre- and postsynaptic APs is large. One can vary not only the timing interval between pre- and postsynaptic APs but also the number of spikes, their frequency, the number of pairings, and their repetition rate. In most cases a repeated number of pairings on the order of 30 to 100 is required for STDP, although single pairing–induced plasticity has been described recently (Holthoff et al., 2004). The specific requirements for STDP induction might reflect a need for a repeated dendritic depolarization and a rise in intracellular Ca^{2+} concentration. Downstream signaling cascades are activated and integrate the biochemical signals generated by individual pairings (Lisman and Spruston, 2005). One prominent example is the activation of calcium/calmodulin-dependent protein kinase II (CamKII) (Lisman, 1989; Lisman et al., 2002). CamKII is required for the induction of LTP.

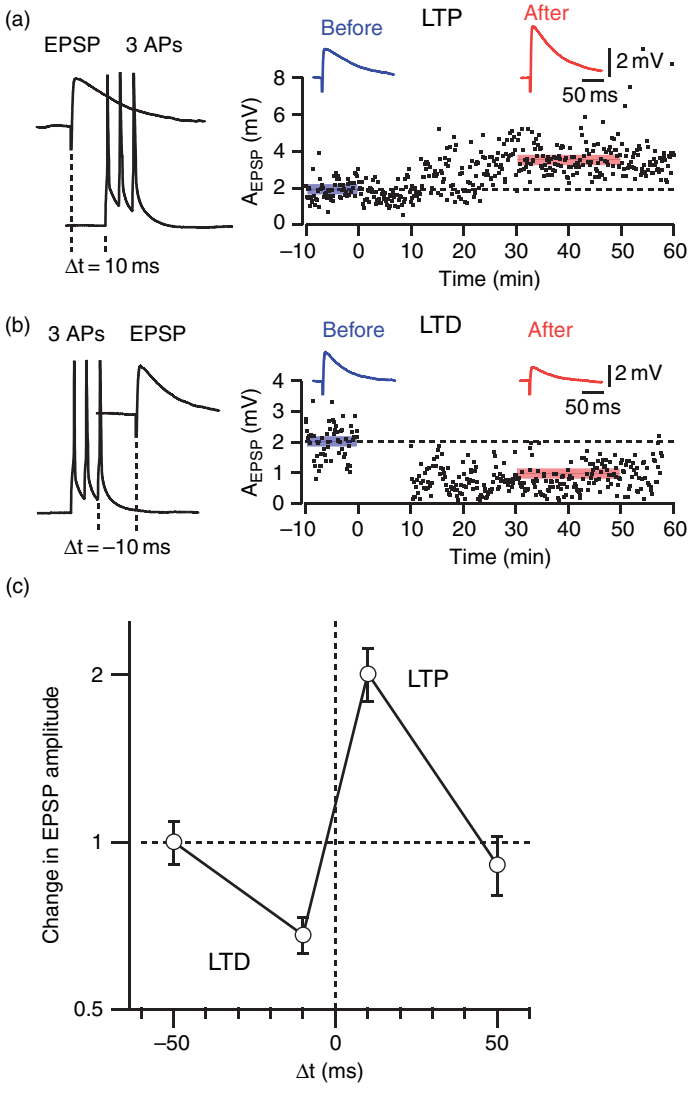


Figure 3 Spike-timing-dependent plasticity. Illustration of the influence of spike-timing on synaptic plasticity. (a) Pairing a burst of three backpropagating APs (50 Hz) following an EPSP by 10 ms results in the induction of LTP. The pairing protocol is depicted on the left. The right graph shows the peak EPSP amplitude over time. Mean EPSPs before (blue) and after (red) pairing are shown on the top. Mean amplitudes before and after are indicated by blue and red bars in the graph. (b) A burst of three backpropagating APs preceding an EPSP by 10 ms results in the induction of LTD. (c) STDP curve for pairing a burst of three backpropagating APs with an EPSP. Vertical line indicates onset of the EPSP (note definition of Δt in A and B). Horizontal line corresponds to no change in synaptic strength. Adapted from Nevian T and Sakmann B (2006) Spine Ca^{2+} signaling in spike-timing-dependent plasticity. *J. Neurosci.* 26: 11001–11013.

It becomes constitutively activated by autophosphorylation after binding several calmodulin molecules. Since Ca^{2+} transients evoked by a single pairing are brief, CamKII can integrate repeated pairings, provided they occur with an appropriate temporal separation (Lisman and Spruston, 2005). This may be one reason why the interval between pairings of individual pre- and postsynaptic spikes is critical (Sjöström et al., 2001). Pairing a presynaptic AP with a subsequent postsynaptic AP at repetition rates below 10 Hz failed to induce LTP in pairs of layer 5 pyramidal neurons, but low-frequency stimulation with reversed temporal order induced LTD (Markram et al., 1997; Sjöström et al., 2001). At repetition frequencies between 10 and 20 Hz LTP or LTD were observed, depending on the relative timing between APs and EPSPs. At a repetition frequency of 40 Hz, only LTP was induced, independent of the

relative timing. The increased frequency of spike pairs results in increased temporal summation of postsynaptic depolarization, which enhances synaptic Ca^{2+} influx for each spike and results in temporal integration of the Ca^{2+} transients. These findings demonstrate the sensitivity of STDP to additional factors beyond AP timing (Wittenberg and Wang, 2006).

The observation that LTP is not induced by repeated low-frequency pairing of single EPSPs followed by single APs has been supported by a number of studies (Sjöström et al., 2001; Wittenberg and Wang, 2006; Nevian and Sakmann, 2006). Hippocampal CA1 pyramidal neurons show a developmental switch between P14 and P23 from single-AP-induced LTP to a requirement of postsynaptic burst-firing to induce LTP (Meredith et al., 2003). In contrast, in other preparations repeated low-frequency pairing of single APs was shown to be sufficient to induce STDP (Bi and Poo, 1998; Feldman, 2000; Froemke and Dan, 2002). These contrasting effects of low-frequency pairing may reflect different degrees of Ca^{2+} accumulation in these neurons. The differences could result from variations in the local depolarization caused by an EPSP, the efficiency of AP backpropagation, or the interaction of EPSP and AP. Such variations could result in different Ca^{2+} transients and may render some synapses more sensitive to single-spike pairing. Nevertheless, bursts of postsynaptic APs following synaptic activation result in a robust induction of LTP.

An important question is how effectively spike-timing-dependent changes in synaptic efficacy can be driven by 'natural' spike patterns observed *in vivo*. Dual to quadruple AP pairings have been used to derive rules for how trains of spikes interact (Wang et al., 2005). Furthermore, variation of the pairing frequency and number of pairings have pronounced effects on the resulting STDP curves ranging from LTP-only to bidirectional and LTD-only learning rules (Wittenberg and Wang, 2006). Various interpretations have been suggested, including the views that the timing of the first AP in each burst is dominant in synaptic modification (Froemke and Dan, 2002), that synaptic weight changes are linearly summated for all pre- and postsynaptic spike pairs (van Rossum et al., 2000), and that potentiation and depression compete for the net change in synaptic strength (Wang et al., 2005). Whether a universal 'set of rules' can be derived from such experiments is still unclear because the interactions between EPSPs and backpropagating APs are variable and strongly nonlinear.

4.39.4.1.3 Diversity of spike-timing-dependent changes in synaptic efficacy

STDP occurs in many neuronal cell types in different regions of the brain. The first reports showed the importance of the timing of backpropagating APs in increasing or decreasing synaptic efficacy in connected pairs of layer 5 pyramidal neurons in somatosensory cortex (Markram et al., 1997), in pairs of hippocampal CA1 pyramidal neurons (Magee and Johnston, 1997), and in CA3 to CA1 pyramidal connections in hippocampal slice cultures (Debanne et al., 1998). In cultures of dissociated hippocampal neurons, changes in synaptic strength depend on the relative timing of single presynaptic APs and single postsynaptic APs, with an effective time window for the induction of LTP and LTD of 20 ms (Bi and Poo, 1998). An asymmetric time window for STDP was also found in layer 4 to layer 2/3 connections between excitatory neurons in somatosensory cortex with a longer time window for the induction of LTD (40–100 ms) as compared to LTP (20 ms) (Feldman, 2000; Celikel et al., 2004; Bender et al., 2006). Presumed layer 2/3 pyramidal-to-pyramidal connections in visual (Froemke and Dan, 2002), somatosensory (Nevian and Sakmann, 2006), and entorhinal cortices can also be modified bidirectionally. Interestingly, layer 4 spiny stellate neurons in somatosensory cortex show only LTD, independent of the timing sequence of pre- and postsynaptic spikes (Egger et al., 1999). STDP has been demonstrated also in perforant path synapses to granule cells in the dentate gyrus (Lin et al., 2006) and thalamic and cortical inputs to the amygdala (Shin et al., 2006). Different STDP rules were found at cortical versus thalamic projections to the same postsynaptic neuron in the lateral amygdala (Humeau et al., 2005). Whereas thalamic afferents showed bidirectional STDP, the cortical afferents showed no changes in synaptic strength.

In the dorsal cochlear nucleus the coincident activation of parallel fibers and either fusiform principal neurons or cartwheel inhibitory interneurons results in different STDP curves. Whereas the fusiform principal neurons exhibit LTD for post-pre pairing and LTP for pre-post pairing, cartwheel interneurons show LTD for the latter sequence (Tzounopoulos et al., 2004). A 'reversed' STDP curve with LTP for post-pre pairing and LTD for pre-post pairing has been described for Purkinje-like cells in the electrosensory lobe of mormyrid electric fish (Bell et al., 1997) and at the corticostriatal synapse (Fino et al., 2005). Such STDP curves are

described as 'anti-Hebbian,' since Hebbian learning is considered to result in LTP with pre- before post-synaptic spike pairing. In the spinal cord, substantia gelatinosa neurons show Hebbian spike-timing-dependent LTP that can be switched to LTD by blocking group I metabotropic glutamate receptors (mGluRs) or by release of Ca^{2+} from internal stores (Jung et al., 2006). These results indicate that the expression of LTP or LTD by a spiking pattern depends on additional factors, like the presence of mGluRs or neuromodulators.

All the examples of STDP described above were related to changes in glutamatergic synaptic transmission. STDP of inhibitory postsynaptic potentials results from correlated spiking of a cortical layer 5 pyramidal neuron and an inhibitory interneuron (Holmgren and Zilberter, 2001). Inhibitory synaptic transmission of interneurons in entorhinal cortex exhibits an asymmetric STDP curve with maximal depression for post- before presynaptic AP at $\Delta t = -10$ ms and maximal potentiation for pre- before postsynaptic AP at $\Delta t = +10$ ms, without any change in synaptic strength for shorter time intervals (Haas et al., 2006).

The multitude of synapses which can be modified bidirectionally by the precise timing of pre- and postsynaptic activity suggests that STDP could be a widely occurring mechanism for the regulation of synaptic strength in an activity-dependent manner. The variety of time windows and effective induction protocols for LTP and LTD suggests, however, that the characteristics of STDP depend critically also on other factors.

4.39.4.2 Effects of Action Potentials and Calcium Transients at the Synapse

A backpropagating AP paired with synaptic activation by a presynaptic spike can result in LTP or LTD. Therefore the backpropagating AP can be regarded as a retrograde signal to the dendritic arbor that reports the output AP activity back to the sites of synaptic input.

4.39.4.2.1 Long-term potentiation

Long-term changes in synaptic efficacy are often dependent on increased dendritic Ca^{2+} influx through NMDA receptors and voltage-dependent Ca^{2+} channels (VDCCs), suggesting that backpropagating APs are part of a mechanism that controls the change in synaptic strength through metabolic cascades that are driven by a brief rise in intracellular Ca^{2+} concentration (Lisman, 1989; Lisman et al., 2002). A backpropagating AP depolarizes the dendritic tree, including the activated synapse, and results in Ca^{2+} influx in most parts of the dendritic tree through VDCCs. In addition, the backpropagating AP briefly relieves magnesium block of NMDARs, thereby further enhancing Ca^{2+} influx, specifically at activated synapses (**Figure 4**). The resulting Ca^{2+} transient in an active spine is larger than the sum of the Ca^{2+} transients evoked by backpropagating AP and synaptic stimulus alone (Yuste and Denk, 1995; Nevian and Sakmann, 2006). This supralinear Ca^{2+} transient occurs when a backpropagating AP follows an EPSP within a time window of 50 ms, similar to the time window for the induction of spike-timing-dependent LTP. Therefore the NMDAR detects coincidence of glutamate release and of membrane depolarization, evoked by pre-before-postsynaptic APs. The spine-restricted supralinear Ca^{2+} influx triggers input-specific modifications in synaptic strength. Supralinear increases in Ca^{2+} concentration evoked by coincident EPSPs and backpropagating APs mediated by the NMDAR have been demonstrated in hippocampal CA1 neurons (Yuste and Denk, 1995), cortical layer 2/3 and layer 5 pyramidal neurons (Köster and Sakmann, 1998; Nevian and Sakmann, 2006), spiny stellate neurons in neocortical layer 4 (Nevian and Sakmann, 2004), cerebellar Purkinje cells (Wang et al., 2000), striatal medium spiny neurons (Carter and Sabatini, 2004), and a variety of interneurons in neocortical layer 2/3 (Goldberg et al., 2003; Kaiser et al., 2004). In dendritic spines of layer 4 spiny stellate neurons the time course of the supralinear summation of Ca^{2+} signals matches the deactivation time course of NMDAR channels (Nevian and Sakmann, 2004). Therefore the NMDAR kinetics probably determines the LTP timing window.

Induction of STDP requires coincident occurrence of pre- and post-synaptic spiking. Neither pre- nor post-synaptic spiking alone is sufficient when repeated a similar number of times as during pairing protocols. Backpropagating APs alone, as well as EPSPs alone, cause a postsynaptic elevation in Ca^{2+} concentration, but with different time courses and amplitudes from Ca^{2+} transients during coincident activity (Sabatini et al., 2002). As a result of these differences, APs and EPSPs alone presumably do not result in the activation of the biochemical signaling cascades that eventually cause changes in synaptic strength (Rubin et al., 2005). A possible explanation is that the Ca^{2+} binding proteins triggering LTP are

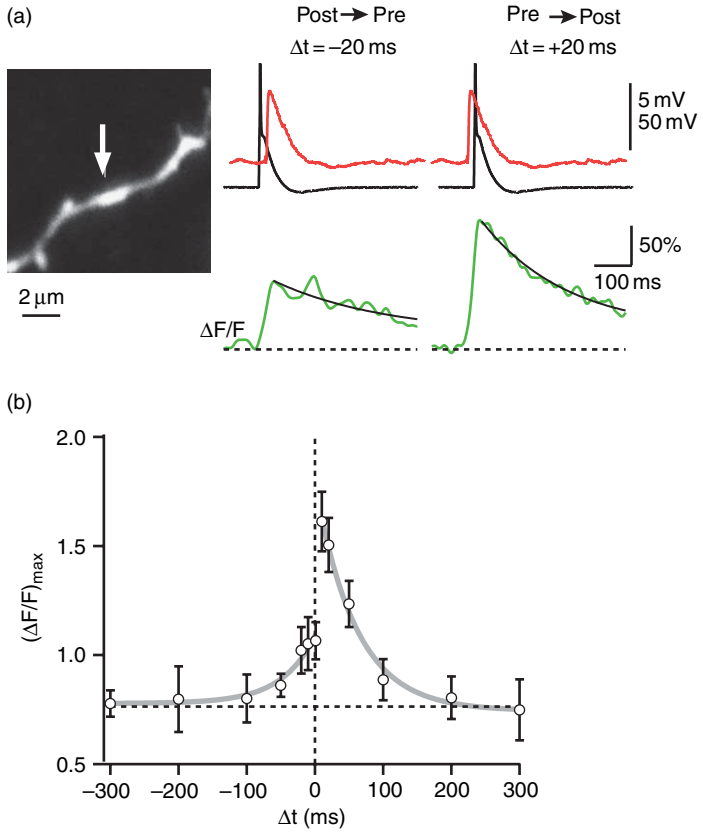


Figure 4 Dendritic spine Ca^{2+} signaling. (a) Two-photon image of a dendrite with spines. The arrow indicates the active synaptic contact in which the Ca^{2+} transients to the right were measured. The traces to the right illustrate the pairing protocols for the induction of STDP and the corresponding Ca^{2+} transients. The activity pattern of a backpropagating AP preceding an EPSP (post–pre, $\Delta t = -20$ ms) results in a smaller Ca^{2+} transient than the reverse order of a backpropagating AP following an EPSP (pre–post, $\Delta t = 20$ ms). (b) Spike-timing-dependent peak Ca^{2+} transient amplitude as a function of the timing interval between EPSP and backpropagating AP. Negative intervals correspond to the order post–pre and positive intervals to the order pre–post. The timing curve is asymmetric with larger calcium transients for positive time intervals. Adapted from Nevian T and Sakmann B (2004) Single spine Ca^{2+} signals evoked by coincident EPSPs and backpropagating action potentials in spiny stellate cells of layer 4 in the juvenile rat somatosensory barrel cortex. *J. Neurosci.* 24: 1689–1699.

located in close proximity to the NMDAR channel and sense the elevation of Ca^{2+} concentration only in the immediate proximity of the site of influx (Franks and Sejnowski, 2002). Such microdomains of Ca^{2+} elevation would result in a Ca^{2+} source-specific activation of biochemical cascades. However, this view was recently challenged for STDP-inducing protocols by the finding that the magnitude of both LTP and LTD was correlated the volume-averaged elevation of Ca^{2+} in a synaptic spine (Nevian and Sakmann, 2006).

4.39.4.2.2 Long-term depression

The LTP-part of the STDP-curve can be explained by activation of the NMDAR, but the mechanisms underlying the induction of LTD are probably more diverse. Two broad forms of spike-timing-dependent LTD have been observed: postsynaptically expressed LTD (Froemke et al., 2005) and presynaptically expressed LTD (Sjöström et al., 2003). Both types of LTD require an increase in postsynaptic Ca^{2+} concentration (Sjöström et al., 2003). LTD is induced at most synaptic contacts when the postsynaptic spike precedes the presynaptic spike, and the resulting postsynaptic Ca^{2+} signals either add linearly or sublinearly (Köster and Sakmann, 1998; Nevian and Sakmann, 2004). This result suggests that moderate elevations of intracellular Ca^{2+} are the trigger for the induction of LTD (Bear et al., 1987; Artola and Singer, 1993; Hansel et al., 1997). In agreement, the mechanism for the induction of postsynaptically expressed LTD depends on shunted Ca^{2+} influx

through the NMDAR. It has been suggested that backpropagating AP-evoked Ca^{2+} influx through VDCCs inhibits subsequent Ca^{2+} influx through the NMDAR (Froemke et al., 2005), which is prone to inactivation by intracellular Ca^{2+} (Legendre et al., 1993; Umemiya et al., 2001). The resulting expression of LTD is postsynaptic and due to modification of postsynaptic AMPA receptors (Lee et al., 2000). Thus, the waveform of the backpropagating AP can influence LTD induction, since it determines the size and time course of dendritic depolarization. In particular, a postspike after-depolarization could facilitate current flow through the NMDAR by relieving magnesium block while an after-hyperpolarization could have the reverse effect (Sjöström et al., 2001; Kampa et al., 2006).

By varying the number, frequency, and timing of a burst of APs preceding an EPSP, one can establish pairing protocols that induce LTD and have similar peak Ca^{2+} transient amplitudes to pairing protocols that induce LTP (Nevian and Sakmann, 2006). This result suggests that the peak amplitude of the Ca^{2+} transient alone is not sufficient to determine the direction of the change in synaptic efficacy. This finding hints to presynaptically expressed LTD, which depends on the release of retrograde messengers from the postsynaptic site that modify the presynaptic release probability (Egger et al., 1999; Sjöström et al., 2003, 2004). In cortical layer 5 and layer 2/3 pyramidal neurons the retrograde messenger is an endocannabinoid (Sjöström et al., 2003; Bender et al., 2006; Nevian and Sakmann, 2006). This form of LTD is independent of postsynaptic NMDARs but requires the activation of VDCCs, mGluRs, and presynaptic NMDARs (Anwyl, 2006; Duguid and Sjostrom, 2006). The results suggest that the sequence of post-before-presynaptic APs activates a putative coincidence detector other than the NMDAR (Karmarkar and Buonomano, 2002; Bender et al., 2006; Nevian and Sakmann, 2006). This coincidence detector senses Ca^{2+} influx through VDCCs before activation of an mGluR and a G-protein-coupled signaling cascade. It triggers the synthesis and release of endocannabinoids. Phospholipase C, which is involved in the Ca^{2+}-dependent synthesis of endocannabinoids (Hashimotodani et al., 2005; Maejima et al., 2005), might be this putative coincidence detector (Nevian and Sakmann, 2006). The sequence for the induction of LTD is qualitatively different from the sequence of activation for the induction of LTP. In the case of LTP, synaptic activation and thus mGluR activation precede (or are coincident with) Ca^{2+} influx, rendering the LTD pathway silent.

Modeling studies also indicate that the relationship between synaptic Ca^{2+} concentration increase and the magnitude and direction of the change in synaptic strength is more complex. Simulating the AP-evoked Ca^{2+} transients and correlating the concentration level of Ca^{2+} to a change in synaptic strength can predict the STDP curve for single spike pairs when a second coincidence detector is added to the model (Karmarkar and Buonomano, 2002) or if the stochastic opening of the NMDAR is taken into account (Shouval and Kalantzis, 2005). More complex spike patterns require differential binding kinetics of Ca^{2+} binding proteins to decode the Ca^{2+} transients (Rubin et al., 2005).

4.39.4.3 Additional Factors Affecting Spike-Timing-Dependent Plasticity

Synapses distributed throughout an elaborate dendritic tree are not equally influenced by backpropagating APs. This can have implications for the STDP learning rules at different synapses. Furthermore, the active dendritic properties of dendrites can result in the generation of local dendritic spikes, which change the timing dependence for STDP. Finally, modulation of dendritic channels and excitability can influence synaptic plasticity.

4.39.4.3.1 Synapse location

Since backpropagating APs attenuate as they invade the dendritic tree, the retrograde signal they provide depends on synapse location. For example, in the apical dendrites of layer 2/3 pyramidal neurons of the visual cortex, the time window for the induction of LTD depends on the distance of the synaptic input from the soma (Froemke et al., 2005). Similarly, in the apical dendrites of cortical layer 5 pyramidal neurons, synapse location determines the sign of the change in synaptic efficacy, with the same STDP induction protocol resulting in LTP at proximal and LTD at distal synapses (Letzkus et al., 2006; Sjöström and Häusser, 2006). Furthermore, when the distal dendrite is depolarized, by EPSPs for example, the amplitudes of backpropagating APs may be enhanced, converting distal LTD to LTP (Sjöström and Häusser, 2006). Hence the sign and magnitude of changes in synaptic efficacy depend upon the location of the synapse and the electrical state of the dendrite during the induction of changes in synaptic strength.

4.39.4.3.2 Local dendritic spikes

In the forms of STDP that we have described, axosomatically initiated APs, which backpropagate into the dendritic tree, are supported by Na^+ conductances and result in brief depolarizations of the dendritic membrane. Dendritic Ca^{2+} channels are activated, but contribute relatively little to the voltage waveform of the AP. This AP waveform interacts with synaptic conductances to evoke a large Ca^{2+} signal that is mainly restricted to dendritic spines. Local dendritic spikes, including sodium, calcium, and NMDA spikes (see the section titled 'Local dendritic action potentials'), have also been implicated in the induction of STDP (Holthoff et al., 2006). In the apical dendrites of hippocampal CA1 pyramidal neurons, for example, local dendritic spikes in the distal apical dendrite can substitute for backpropagating APs in the induction of LTP (Golding et al., 2002). This demonstrates that in these cells, at least, backpropagating APs are not necessary for the induction of LTP, since the depolarization required for Ca^{2+} influx can also be provided by local regenerative events.

In layer 5B neocortical pyramidal neurons a calcium spike can be triggered in the distal apical dendrite by strong synaptic activation (Schiller et al., 1997) or by pairing a backpropagating AP with a distal EPSP (Larkum et al., 1999b). Similarly, a train of backpropagating APs above a critical frequency (Larkum et al., 1999a) can result in the regenerative activation of voltage-dependent Ca^{2+} channels at a distal dendritic site. Pairing EPSPs with high-frequency bursts of APs above the critical frequency, which evokes a large dendritic depolarization and Ca^{2+} transient, reverses the timing rule for the induction of STDP at the distal apical dendrite in layer 5B pyramidal neurons. Here calcium spikes precede the EPSPs and result in LTP, whereas the reverse order results in LTD (Letzkus et al., 2006). A similar shift in the STDP curve occurs when pairing a presumed calcium spike in the basal dendrites of layer 5 pyramidal neurons with an EPSP (Kampa et al., 2006; Kampa and Stuart, 2006). The induction of LTP for this sequence of local dendritic spike followed by EPSP may result from the prolonged depolarization and maximal NMDAR activation, even for negative time intervals.

NMDA spikes were first described in the distal basal dendrites of layer 5 pyramidal neurons. Their initiation requires correlated and clustered synaptic input. They are generated by strong local synaptic stimulation (Schiller et al., 2000). Once the threshold for regenerative currents through NMDARs is passed, strong depolarization and large concomitant Ca^{2+} influx occur for several tens of micrometers along the dendritic branch. Interestingly, NMDA spikes paired with EPSPs do not induce LTP in distal basal dendrites. However, when NMDA spikes and EPSPs are paired with locally applied brain-derived neurotrophic factor (BDNF), LTP is induced at these locations within a time window of 200 ms between NMDA spike and EPSP and preferentially when the NMDA spike precedes the EPSP (Gordon et al., 2006). This is another example of an anti-Hebbian learning rule. This form of synaptic plasticity associates inputs that cause an NMDA spike with subsequent EPSPs arriving at synaptic sites in close spatial proximity.

Finally, dendritic spikes contribute to STDP mechanisms even in cell types that do not support the backpropagation of APs. For example in cerebellar Purkinje cells the coincident activation of parallel and climbing fibers results in a timing-dependent spineous and branchlet Ca^{2+} transients and parallel fiber LTD (Wang et al., 2000).

4.39.4.3.3 Neuromodulation

The mechanisms that result in changes in synaptic strength can be influenced by neuromodulators. For example, the induction of LTD can be converted to LTP by the presence of dopamine in the prefrontal cortex (Matsuda et al., 2006). In other instances dopamine can facilitate the induction of LTD (Otani et al., 2003). The mechanisms by which neuromodulators affect STDP are currently unknown. However, neuromodulators can influence AP backpropagation, including the shape of the AP waveform in the dendrite, and the shape and size of the synaptic potential, all of which can influence STDP. Furthermore, activation or inactivation of the biochemical signaling cascades that are required for synaptic modifications could alter the direction of plastic changes for the same pre- and postsynaptic spiking pattern. For example, blocking the activation of mGluRs can result in the spike-timing-dependent induction of LTP with a stimulation pattern that would otherwise result in LTD (Nevian and Sakmann, 2006). The influence of BDNF on the induction of LTP during an NMDA spike (Gordon et al., 2006) is another example of neuromodulatory effects on biochemical cascades in STDP. Therefore STDP at a given synapse might not be a static and fixed learning rule. It might rather be modifiable by the actual

state of the neuronal network, which is represented by the ambient concentration of neuromodulators.

Modifications in synaptic strength due to repeated correlated pre- and post-synaptic activity can also be accompanied by changes in dendritic excitability (Desai et al., 1999; Daoudal et al., 2002; Daoudal and Debanne, 2003; Frick et al., 2004). Induction of STDP that results in a bidirectional modification of synaptic strength was shown to also induce a bidirectional modification of the excitability in the presynaptic neuron (Li et al., 2004). Therefore STDP is a mechanism not only by which changes in synaptic strength are regulated but also by which also changes in dendritic excitability of both the pre- and postsynaptic neuron are controlled.

4.39.4.4 Spike-Timing-Dependent Plasticity *in vivo* and Circuit-Level Effects

STDP has been most extensively studied in brain slice preparations, but several groups have provided evidence that STDP is of importance *in vivo*. Convergent retinotectal synapses from developing *Xenopus* tectal neurons undergo LTP when synaptic inputs to the tectal neuron are stimulated 20 ms before spiking of the neurons, whereas the reverse order results in LTD (Zhang et al., 1998). The measured STDP curve for the retinotectal synapse was similar to recordings made *in vitro* (Bi and Poo, 1998). Nevertheless, the expression of long-lasting potentiation can be prevented by spontaneous spiking in the tectal neuron or random visual input (Zhou et al., 2003). Developing tectal neurons can be trained to become direction sensitive by repeated presentation of a moving bar to the retina (Engert et al., 2002). The asymmetric modification of the receptive field can be explained by spike-timing-dependent LTP and LTD (Mu and Poo, 2006). Subregions of receptive fields can be modified or even erased by STDP (Vislay-Meltzer et al., 2006). All these findings support the idea that STDP induces experience-driven refinement of developing neural circuits.

In the rat visual cortex visually evoked synaptic responses can be modified bidirectionally depending on the temporal order of synaptic responses evoked by visual stimulation and postsynaptic spikes (Meliza and Dan, 2006). Pairing of visual stimuli presented at two orientations results in a shift in the orientation tuning in the visual cortex of cats, and this shift depends on the temporal order of the visual stimulus pair (Yao and Dan, 2001). Asynchronous visual stimuli presented to two adjacent regions on the retina control the relative spiking of two groups of cortical neurons with high temporal precision (Fu et al., 2002). Repeated pairing of these asynchronous stimuli resulted in modifications of intracortical connections and shifts in receptive fields depending on the temporal order and timing interval between the pair of visual stimuli. Similarly, in kitten visual cortex, cortical map plasticity depends on the timing of visual stimuli and electrical cortical stimulation (Schuett et al., 2001). A shift in orientation preference toward the paired orientation occurs when the electrical stimulation follows the visual stimulus, consistent with STDP. Experience-dependent plasticity of somatosensory responses also depends on dendritic excitability (Komai et al., 2006), and whisker deprivation results in cortical map plasticity based on spike-timing-dependent mechanisms (Celikel et al., 2004).

Hence there is growing evidence that STDP is a fundamental synaptic mechanism by which experience forms memory traces and neural circuits are refined in an activity-dependent manner. This hypothesis could explain the remarkable capability of the nervous system to constantly adapt to changes in the environment and to modify its behavioral responses to sensory inputs. The notion that APs in the dendrites are at the heart of this mechanism highlights the importance of dendritic structure and ion channel distribution for our capability to learn and think.

4.39.5 Conclusions

We have provided an overview of the current understanding of dendritic excitability and its relationship to synaptic plasticity. While a number of key mechanisms have been identified, such as backpropagating APs, local dendritic spikes, and timing-based rules for changes in synaptic efficacy, we are still just beginning to understand their functional importance for signal processing in neural circuits in awake animals. Likewise we need to further understand the molecular bases for the marked variations in these mechanisms, i.e., the underlying distributions of ion channels and the activation of particular signaling pathways. Future research will doubtless focus on both the molecular basis of dendritic signaling and also the functional implications for circuit function. Although many mysteries about dendritic function remain, it is now evident that dendrites are sophisticated and powerful computational devices.

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4.40 Plasticity of Intrinsic Excitability as a Mechanism for Memory Storage

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4.40.1 Introduction

During the past several decades, the analysis of the cellular and molecular mechanisms underlying learning and memory revealed two major targets for learning-dependent neuronal modulation: synaptic efficacy and intrinsic excitability (for review see Byrne, 1987).

The efficacy of a synapse is highly plastic and can be modified by neuronal activity in different ways. Indeed, different patterns of neuronal activity can lead to distinct and enduring changes in synaptic strength associated with phenomena such as long-term potentiation (LTP) and long-term depression (LTD). In addition, several behavioral training tasks, capable of inducing nonassociative and associative forms of learning, alter synaptic efficacy, thus supporting a role for synaptic plasticity in the storage of memory (e.g., Martin and Morris, 2002). Because of the extremely large number of individual synaptic contacts that neurons can form with other neurons as well as because of some computational properties that synapses exhibit such as associativity and input specificity, a memory storage system based on changes in synaptic strength has a potentially massive storage capacity (Poirazi and Mel, 2001). Consequently, theories based on persistent experience-driven changes in synaptic function have been extensively used to explain the storage of information (e.g., Fusi et al., 2005).

Nevertheless, an accumulating body of work indicates that learning also leads to persistent changes in intrinsic neuronal excitability (e.g., Brons and Woody, 1980; Crow and Alkon, 1980; Moyer et al., 1996; Cleary et al., 1998; Antonov et al., 2001; Brembs et al., 2002; Lorenzetti et al., 2006). These changes are due to modifications of membrane conductances and can affect electrophysiological properties including the resting potential, the input resistance, the shape and the threshold of the action potential, and the discharge frequency (for reviews see Daoudal and Debanne, 2003; Debanne et al., 2003; Zhang and Linden, 2003). What is the functional relevance of changes in intrinsic neuronal excitability to memory storage?

In this chapter, we will review some of the earlier work illustrating plasticity of intrinsic excitability produced by experience together with some more recent findings. In addition, we will discuss the implications of these results and the extent to which changes in synaptic efficacy and changes in intrinsic excitability can both contribute to memory storage.

4.40.2 Changes in Intrinsic Excitability Produced by Learning and Experience

Research over the past three decades has discovered modifications in the intrinsic membrane properties occurring in invertebrates and vertebrates following different forms of nonassociative and associative learning.

4.40.2.1 Invertebrate Models

Crow and Alkon (1980) were the first to report that classical conditioning (i.e., the learned ability to

associate a predictive stimulus with a subsequent salient event) altered the excitability of the photoreceptors in the nudibranch mollusk *Hermissenda crassicornis*. The positive phototaxis that the animal normally exhibits in response to illumination was reduced when the animal was trained with multiple paired presentations of light (conditioned stimulus, CS) and rotation (unconditioned stimulus, US), which activates the vestibular system (Crow and Alkon, 1978). This form of learning was associative, and the suppression of phototaxis, the conditioned response, persisted for several days (Crow and Alkon, 1978). Classical conditioning produced changes in several membrane properties of the type B photoreceptors, which are the primary sensory neurons of the CS pathway. The type B photoreceptors are excited by light and also by synaptic drive from the statocyst cells, which are part of the vestibular system activated by rotation. The changes produced by classical conditioning included increases in input resistance, spontaneous firing and spike activity in response to light presentation or current injection, and generator potentials evoked by the CS (Crow and Alkon, 1980). Importantly, these changes persisted when the synaptic input to the photoreceptors was removed. Voltage-clamp analysis of the type B photoreceptors revealed that pairing-specific changes in several ionic currents were the biophysical substrate of the aforementioned altered membrane properties. Classical conditioning decreased two K^+ currents, a rapidly activating and inactivating K^+ current (I_A) and a Ca^{2+}-activated K^+ current ($I_{K,Ca}$) (Alkon et al., 1982, 1985; Farley, 1988), and also altered a voltage-dependent Ca^{2+} current (Collin et al., 1988). These biophysical changes were consistent with the increase in excitability observed in the type B photoreceptors following classical conditioning because a reduction of I_A and $I_{K,Ca}$ would tend to produce an enhanced depolarization and increased spike activity in the photoreceptors in response to the presentation of light. The pairing-specific increase in intrinsic excitability was observed for several days following conditioning. Also, the magnitude of the increase in excitability was positively correlated with the degree of phototactic suppression, thus suggesting a causal relationship between the biophysical changes in the type B photoreceptors and the expression of the conditioned response (Crow and Alkon, 1980).

Following the results observed in *Hermissenda*, other learning-related changes in intrinsic excitability were identified in other invertebrates including annelids and mollusks. In the leech *Hirudo medicinalis*, habituation and sensitization, two forms of nonassociative learning, altered in opposite directions the intrinsic excitability of an identified neuron. *Hirudo* exhibits a defensive withdrawal reflex that consists of whole-body shortening in response to light touch of the skin (Sahley, 1995). This reflex can be habituated, when it is repetitively evoked, or sensitized, when a strong, noxious stimulus is delivered (Sahley, 1995). Sensitization, which is mediated by serotonin (5-HT), requires the activity of a group of electrically coupled interneurons called the S-cells (Sahley et al., 1994). Sensitization increased the input resistance of the S-cells as well as their spike threshold and the number of action potentials evoked by intracellular depolarization (Burrell et al., 2001). These effects were mimicked by exogenous application of 5-HT (Burrell and Sahley, 2005). Conversely, habituation produced a decrease in intrinsic excitability (Burrell et al., 2001). These results indicate that in the leech bidirectional changes in intrinsic excitability represent cellular correlates of two distinct forms of nonassociative learning.

The marine mollusk *Aplysia californica* has been probably the most extensively used invertebrate model to study the cellular and molecular mechanisms of learning and memory. Several examples of experience-dependent changes in intrinsic excitability have been reported in *Aplysia* following both nonassociative and associative learning of simple defensive reflexes as well as associative learning of more complex behaviors such as feeding. Long-term (24-h) sensitization was associated with facilitation of the sensorimotor neuron synapse (Cleary et al., 1998) as well as changes in the biophysical properties of the tail sensory neurons (Cleary et al., 1998). The intrinsic changes included increases in the excitability and in the afterdepolarization following either a single spike or a burst of action potentials (Cleary et al., 1998). Voltage-clamp analysis revealed that long-term sensitization reduced the net outward current in the tail sensory neurons, which is consistent with the increased excitability observed in these neurons following learning (Scholz and Byrne, 1987). Interestingly, long-term sensitization training also altered the biophysical properties of tail motor neurons. These changes included a more hyperpolarized resting membrane potential and a decrease in the threshold for spike initiation (Cleary et al., 1998). The changes produced by long-term sensitization in the sensory neurons resembled those induced by short-term sensitization, thus suggesting that the phenotype of the expression mechanism for the

short-term memory is preserved for the long-term memory. However, this is not always the case. Indeed, a more robust sensitization training protocol, which produced morphological changes in the sensory neurons and long-term synaptic facilitation of the sensorimotor neuron synapse (Wainwright et al., 2002), did not change the intrinsic excitability of the sensory neurons (Wainwright et al., 2004), but instead it induced narrowing of their action potentials (Antzoulatos and Byrne, 2007).

In *Aplysia*, classical conditioning also produced changes in the intrinsic excitability of sensory neurons. In a simplified preparation of the *Aplysia* siphon-withdrawal reflex, classical conditioning led to an increased input resistance and excitability of the siphon sensory neurons (Antonov et al., 2001, 2003). The changes in intrinsic excitability, as well as the pairing-specific strengthening of the sensorimotor neuron synapses, were restricted to the sensory neurons that were activated by mechanical stimulation of the siphon (i.e., on-field neurons), thus providing CS specificity to the expression of the conditioned response (Antonov et al., 2001, 2003). The presence of biophysical as well as synaptic changes following both nonassociative and associative learning suggested the intriguing hypothesis that the same cellular phenotype underlying nonassociative learning may also serve as the physiological substrate for associative memory (Byrne, 1987; Lechner and Byrne, 1998).

In *Aplysia*, the cellular mechanisms of associative learning were also studied using a more complex behavior (for a review see Baxter and Byrne, 2006). Appetitive forms of classical and operant conditioning (i.e., the ability to learn the consequences of a behavior) both increased the frequency of feeding behavior after training (Lechner et al., 2000a,b; Brembs et al., 2002; Baxter and Byrne, 2006; Lorenzetti et al., 2006). Also, both operant and classical conditioning changed the biophysical properties of neuron B51, a key element of the feeding neural circuitry, but the changes were remarkably different. Operant conditioning induced changes in B51 membrane properties (i.e., increased input resistance and decreased burst threshold), suggesting an increase in intrinsic excitability, which can contribute to the increased activity in B51 observed after training (Nargeot et al., 1999a,b; Brembs et al., 2002; Mozzachiodi et al., 2006). In contrast to operant conditioning, classical conditioning did not alter the input resistance, but increased the burst threshold of B51, resulting in a decreased intrinsic excitability (Lorenzetti et al., 2006).

These findings provided a biophysical substrate for the hypothesis that at the cellular level operant and classical conditioning are mediated by fundamentally different mechanisms (for a review see Baxter and Byrne, 2006). The decreased excitability in B51 was counterintuitive because classical conditioning enhances feeding in response to the CS and also strengthens the CS-evoked excitatory drive to B51 (Lorenzetti et al., 2006). However, the analysis of the responsiveness of B51 to the CS after training revealed that classical conditioning indeed facilitated the recruitment of B51, indicating that the factors that enhanced the recruitment of B51 overpowered the diminished excitability (Lorenzetti et al., 2006). Although experimental evidence is needed, the pairing-specific decrease in the excitability of B51 might represent an adaptive mechanism to help shape the CS specificity produced by classical conditioning.

Although most of the effects of learning on the membrane properties involved changes in input resistance and/or intrinsic excitability, other forms of intrinsic modifications were described as a result of experience in mollusks. For example, as mentioned above, long-term sensitization in *Aplysia* was associated with an increase in the resting potential of the motor neurons (Cleary et al., 1998). In the pond snail *Lymnaea stagnalis*, long-term memory for appetitive classical conditioning of feeding, induced by repetitive paired presentations of mechanical stimulation of the lips (CS) and application of sucrose as a feeding stimulant (US; for a review see Benjamin et al., 2000), was associated with a persistent depolarization in the modulatory neuron CV1a involved in the initiation of feeding movements (Jones et al., 2003). The pairing-specific depolarization of CV1a was positively correlated with the fictive feeding response to the CS in reduced preparations from conditioned animals, but it was not associated with any change in other membrane properties such as input resistance, spike threshold, or spike frequency (Jones et al., 2003). The time course of the persistent depolarization also mirrored the duration of retention of long-term memory (Jones et al., 2003). This pairing-specific depolarization would render CV1a more likely to fire in response to the CS and allow the CS to more effectively generate feeding responses.

4.40.2.2 Vertebrate Models

The first evidence of experience-dependent changes in neuronal excitability in a vertebrate model system was reported by Brons and Woody in 1980.

These authors used a conditioning paradigm in the cat in which repetitive paired presentations of an auditory click (CS) and a glabella tap (US), which evoked an eyeblink response, led to the appearance of the conditioned response consisting of a combined eyeblink and nose twitch (Brons and Woody, 1980). Cellular analysis conducted in the sensorymotor cortical areas and in the facial nucleus of awake cats revealed that classical conditioning was associated with an increased excitability as measured by the minimum amount of injected current necessary to evoke an action potential (Brons and Woody, 1980). An increase in input resistance was also measured in neurons of the facial nucleus. The persistence of the increased excitability, once the conditioned response was extinguished, indicated that this mechanism was not directly implicated in the formation and retention of the conditioned response (Brons and Woody, 1980). A detailed analysis of the biophysical substrate of the increased excitability has not been pursued.

Several other examples of experience-dependent changes in intrinsic excitability were identified in vertebrate models. In the rabbit, delay eyelid conditioning, which is performed using a tone as CS and a co-terminating air puff as US, depends on the activation of both the cerebellar cortex and cerebellar deep nuclei (for reviews see Kim and Thompson, 1997; Christian and Thompson, 2003). Recordings from Purkinje cells revealed a reduced spike threshold and a reduced afterhyperpolarization (AHP) evoked by bursts of spikes in slices from conditioned animals when compared to control animals (Schreurs et al., 1998). The AHP that follows action potentials is an important determinant for the firing activity of neurons, and a pairing-specific reduction of its amplitude is consistent with an increased excitability observed following classical conditioning. Importantly, these changes were long-lasting (up to 30 days), restricted to defined microzones of the cerebellar lobule HVI and the degree of reduction in spike threshold positively correlated with the conditioned response in the paired animals (Schreurs et al., 1997, 1998). Another form of classical conditioning is trace eyelid conditioning, which requires the hippocampus and occurs when a trace interval is imposed between the CS offset and the US onset. Cellular analysis of trace eyelid conditioning revealed that hippocampal pyramidal neurons in the CA1 and CA3 areas from conditioned animals exhibited a greater number of spikes evoked by depolarizing current injections (Moyer et al., 1996; Thompson et al., 1996) and a decreased AHP due to a reduction in the Ca^{2+}-dependent K^+ current underlying the AHP as compared to control animals (Coulter et al., 1989; Moyer et al., 1996; Thompson et al., 1996). These changes were consistent with a pairing-specific increase in excitability. Although the increased excitability was similar to that found following delay conditioning, it was not restricted to a specific area, but it was instead widespread through the dorsal hippocampus and also could not be detected 7 days after training when the memory for trace eyelid conditioning was still retained (Moyer et al., 1996; Thompson et al., 1996). These findings indicate that it is unlikely that this transient increased excitability in hippocampal neurons constitutes a part of the memory trace itself, but it could represent a mechanism through which the hippocampal circuit is set in a more permissive state for input-specific synaptic modification to occur during memory formation. A similar learning-dependent reduction in AHP also occurred in CA1 pyramidal neurons in the dorsal hippocampus following a spatial learning task that depends on the hippocampus (Oh et al., 2003).

In rats, pairing-specific enhancements in both CS-evoked synaptic drive and neuronal excitability (i.e., increased input resistance and a reduced amount of injected current necessary to elicit an action potential) were measured *in vivo* in neurons from the lateral nucleus of the amygdala of anesthetized rats trained with a conditioning paradigm during which an odor (CS) was repetitively paired with a foot shock (US; Rosenkranz and Grace, 2002). The synaptic and the intrinsic changes were both prevented when dopamine signaling, which is relevant for amygdala-dependent forms of learning, was pharmacologically blocked in the lateral nucleus of the amygdala (Rosenkranz and Grace, 2002). These results are consistent with the aforementioned studies of *Aplysia* withdrawal reflexes in which both synaptic and intrinsic plasticity occur together during memory formation.

Changes in intrinsic excitability have also been found in vertebrates following operant conditioning tasks. In monkeys, operant conditioning of the spinal stretch reflex is associated with changes in the intrinsic properties of the motor neurons. The spinal stretch reflex is among the simplest vertebrate reflexes and is largely mediated by a monosynaptic pathway between Ia afferent neurons and alpha motor neurons (Wolpaw, 1997). Because this reflex is influenced by descending activity from supraspinal structures, it can be operantly conditioned and

animals can learn to gradually increase or decrease this reflex or its electrical analog, the H-reflex (Wolpaw, 1997). In particular, operantly conditioned decrease in the H-reflex in monkeys was associated with an increase in the depolarization needed for the spinal motor neuron to fire and a decrease in its conduction velocity (Carp and Wolpaw, 1994). These apparently intrinsic changes were consistent with the hypothesis that a positive shift in motor neuron firing threshold and a consequent increase in the depolarization needed to reach that threshold could contribute to weaken the magnitude of the H-reflex following operant conditioning. Modeling studies proposed that a positive shift in the activation of voltage dependence of Na^+ channels might represent the biophysical substrate for the operantly conditioned decrease in the H-reflex (Halter et al., 1995).

Changes in excitability were also reported in rats following an olfactory discrimination task (for a review see Saar and Barkai, 2003). Rats were trained to discriminate pairs of odors for a water reward. Several consecutive days of training were required for a rat to successfully discriminate between a first pair of odors, but once the rat reached good performance, its ability to learn to discriminate between a new pair of odors improved dramatically and only 1 day was needed to achieve good performance (Saar and Barkai, 2003). Cellular analysis revealed that both spike frequency adaptation and AHP amplitude were reduced in pyramidal neurons of the rat olfactory cortex following olfactory discrimination training when compared to naive or pseudo-trained animals (Saar et al., 1998, 2001). These changes were detected 1–3 days after training, but they decayed to baseline values by 5–7 days after training (Saar et al., 1998, 2001). It has been speculated that this example of increased excitability is not *per se* a mechanism of memory storage, but it might be involved in rule learning, setting the neural circuits in the piriform cortex in an excitable, more permissive state for activity-dependent synaptic modifications to occur (Saar and Barkai, 2003).

4.40.3 Activity-Dependent Modulation of Intrinsic Excitability

In addition to learning tasks *in vivo*, intrinsic excitability can be modulated by patterns of electrical stimulation of neurons and neural pathways. Depending on the protocol of stimulation, some of these patterns of neuronal activity may induce either an enhancement or a reduction of synaptic strength, or LTP (for a review see Lynch, 2004) and LTD (for a review see Ito, 2001), respectively. LTP, which is probably the most studied example of activity-dependent modulation of synaptic efficacy, can be induced in several areas of the vertebrate brain, including the hippocampus, the amygdala, the neocortex, and the cerebellar cortex, and is commonly considered to be a mechanism underlying aspects of learning and memory (for reviews see Bliss and Collingridge, 1993; Martin and Morris, 2002). Because several forms of LTP and LTD can be induced in isolated brain slices, the biochemical cascades underlying these forms of neuronal plasticity can be analyzed.

Although most of the work on LTP has focused on the mechanisms underlying the persistent augmentation of synaptic efficacy, several lines of evidence indicate that, at least in some cases, LTP is also accompanied by modifications in intrinsic excitability. Bliss and Lømo, who first described LTP in the dentate area of the rabbit hippocampus in 1973, also reported an increase in the population spike (i.e., the extracellularly recorded signal representing the summation of the evoked action potentials in postsynaptic neurons) accompanying LTP, which could not be entirely explained by the LTP-evoked increase in the population excitatory postsynaptic potential (EPSP). This nonsynaptic component of LTP was termed E-S potentiation, or E-S potentiation (Bliss and Lømo, 1973). The mechanisms underlying E-S potentiation are still subject to some debate. Two hypotheses have been developed to explain this phenomenon: an LTP-induced decrease in the ratio of inhibitory to excitatory drive (e.g., Abraham et al., 1987; Chavez-Noriega et al., 1989) and an LTP-induced increase in the intrinsic excitability of the postsynaptic neurons through modulation of voltage-dependent channels (e.g., Taube and Schwartzkroin, 1988; Bernard and Wheal, 1995). Several lines of evidence indicate that a reduction in the ratio of inhibitory to excitatory drive is not sufficient to account for the E-S potentiation, thus pointing to a role for LTP-induced intrinsic plasticity, the consequence of which would lead to a higher efficiency of E-S coupling (e.g., Wathey et al., 1992). One example, which suggests the modulation of voltage-dependent channels as a mechanism underlying the increased excitability accompanying synaptic LTP, has been reported in hippocampal CA1 pyramidal neurons. In these neurons, a pattern of synaptic inputs paired with postsynaptic spikes induced a long-term increase of

intrinsic excitability concurring with synaptic LTP (Xu et al., 2005). The increased excitability was manifested as a decrease in the action potential threshold that was attributable to a shift in the activation curve of voltage-gated Na^+ channels (Xu et al., 2005). This form of enduring increase of intrinsic excitability shared a similar signaling pathway with the late phase of synaptic LTP, which included the requirement of activation of glutamate receptors of the *N*-methyl-D-aspartate (NMDA) type, the influx of Ca^{2+}, the activity of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), and changes in protein synthesis (Xu et al., 2005).

The modulation of the E-S coupling appears to be bidirectional. In the CA1 region of the hippocampus, LTD, normally induced by low-frequency patterns of electrical stimulation, was accompanied by a reduction of the E-S coupling (Daoudal et al., 2002). This reduction of the E-S coupling was largely due to a decrease in the intrinsic excitability of hippocampal cells (Daoudal et al., 2002). Therefore, opposite modulation of the synaptic strength is associated with bidirectional changes in E-S coupling, which reflect bidirectional modifications of intrinsic excitability.

Other examples of activity-dependent changes in intrinsic excitability produced by synaptic activation have been reported in different brain areas. For example, in the rat entorhinal cortex, persistent graded increases in firing frequency were induced in pyramidal neurons by repetitive excitatory synaptic activation (Egorov et al., 2002). These sustained levels of firing could be either increased or decreased in an input-specific manner and relied on activity-dependent changes of Ca^{2+}-dependent cationic current (Egorov et al., 2002). This intrinsic ability of the neurons in the entorhinal cortex to generate graded persistent activity has been proposed as a cellular mechanism for working memory (Egorov et al., 2002). Another example of modulation of intrinsic excitability induced by synaptic activation has been reported in the cerebellar cortex. High-frequency stimulation of the mossy fiber-to-granule cells pathway led to LTP and was also accompanied by a persistent enhancement of intrinsic excitability of granule cells, which was associated with increased input resistance and decreased spike threshold (Armano et al., 2000). Neurons of the cerebellar deep nuclei also exhibited a rapid, synaptically driven, increase in their intrinsic excitability, which required Ca^{2+} influx through activation of NMDA-type glutamate receptors (Aizemann and Linden, 2000). However, in this case, the pattern of synaptic stimulation used to activate these neurons did not produce any facilitation of the synaptic input (Aizemann and Linden, 2000). This last example indicates that modulation of intrinsic excitability can be expressed without the occurrence of changes in synaptic function, thus providing a contribution to the information storage independent from synaptic plasticity.

Recent findings indicate that changes in intrinsic excitability may not require synaptic activation. For example, a train of high-frequency intracellular depolarizations delivered to pyramidal neurons in layer V of the primary visual cortex produced an enduring increase in intrinsic excitability (Cudmore and Turrigiano, 2004). This change in excitability required Ca^{2+} entry during neuronal activity and did not affect the passive membrane properties, thus suggesting the involvement of voltage-dependent channels (Cudmore and Turrigiano, 2004). Another example of synaptic-independent modulation of intrinsic excitability has been reported in hippocampal CA1 pyramidal neurons (Fan et al., 2005). A pattern of suprathreshold repeated intracellular stimulation (i.e., theta-burst firing) produced a decrease in somatic excitability, which was due to an upregulation of the hyperpolarization-activated cationic current I_h (Fan et al., 2005). This decrease in excitability was prevented by Ca^{2+} chelators; by low concentrations of tetrodotoxin, which blocked back propagation of action potentials from the soma to the dendrites; and by NMDA receptor antagonists even in the absence of synaptic activation (Fan et al., 2005). These findings suggest that during theta-burst firing, back-propagating action potentials would lead to Ca^{2+} influx through the NMDA receptor, which, in turn, would increase the I_h via a mechanism mediated by CaM kinase II.

Recent findings indicate that the synaptic drive does not need to be excitatory to induce an increase in intrinsic excitability. In the medial vestibular nucleus, brief periods of inhibitory synaptic input, or direct membrane hyperpolarization, triggered a long-lasting increase in both the spontaneous firing rate and firing responses to intracellular depolarization (Nelson et al., 2003). This increase in excitability, termed firing rate potentiation, was due to a decrease in cytosolic Ca^{2+}, which reduced CaM kinase II activity and, in turn, downregulated BK-type $I_{K,Ca}$ (Nelson et al., 2005). This novel form of neuronal plasticity might contribute to motor learning in the vestibulo-ocular reflex.

The intrinsic changes produced by patterns of neuronal activity are not restricted to ion channels and can

include modulation of the activity of membrane transporters. For example, interneurons of the rat dentate gyrus exhibited a long-term depolarization of their resting membrane potential after high-frequency stimulation of the perforant path that was attributed to an activity-dependent change in the rate of the electrogenic Na^+ pump (Ross and Soltesz, 2001). This activity-dependent depolarization, which occurred in the absence of any potentiation of the excitatory synaptic input, required the rise of intracellular Ca^{2+} and the activation of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), but not NMDA receptors (Ross and Soltesz, 2001). As a result of the depolarization, the interneurons of the dentate gyrus responded with action potential discharge to previously subthreshold EPSPs even in the absence of synaptic potentiation, thus indicating an increase in the E-S coupling (Ross and Soltesz, 2001). Activity-dependent changes in the function of the electrogenic Na^+ pump have been found also in invertebrate neurons. In the leech, low-frequency repetitive stimulation of touch (T) sensory neurons led to a lasting increase in the amplitude of the AHP produced by the firing discharge, which is due to an increase in the activity of the electrogenic Na^+ pump (Scuri et al., 2002). The modulation of the Na^+ pump activity was regulated by both the influx of Ca^{2+} during neural activity and the release of Ca^{2+} from intracellular stores. In addition, phospholipase A2 and the downstream activation of arachidonic acid metabolites, derived from the 5-lipoxygenase pathway, were necessary for the increase of the AHP amplitude (Scuri et al., 2005). The increase of the AHP amplitude was associated with a persistent depression of the synaptic connection between T cells and their follower neurons (Scuri et al., 2002). This synaptic depression may be a cellular mechanism contributing to short-term habituation. Since 5-HT mediates sensitization in the leech (e.g., Catarsi et al., 1990; Sahley et al., 1994; Zaccardi et al., 2004), it has effects on the amplitude of the AHP in T neurons opposite those produced by repetitive stimulation. Indeed, 5-HT reduced the AHP amplitude by inhibiting the Na^+ pump (Catarsi and Brunelli, 1991) in a cyclic adenosine monophosphate (cAMP)-dependent manner (Catarsi et al., 1993). Injection of cAMP into a T neuron enhanced the synaptic connection between the T cell and its follower neurons (Scuri et al., 2007). Together these findings indicate that bidirectional changes in the AHP amplitude that alter synaptic efficacy may represent cellular mechanisms underlying habituation and sensitization, two simple forms of learning.

4.40.4 Plasticity of Intrinsic Excitability as a Mechanism for Memory Storage: Hypotheses and Lines of Evidence

The general motif that emerges from the examples above is that several different learning tasks, nonassociative and associative, as well as activation of neurons and neural pathways, induce changes in the biophysical properties of neurons in both invertebrates and vertebrates. These neurophysiological correlates have been identified at the level of sensory neurons, interneurons, and motor neurons.

What is the functional relevance of these changes? In some cases, a direct role for the intrinsic plasticity in memory storage appears straightforward. For example, in *Aplysia* and *Hermissenda*, because the loci of plasticity induced by classical conditioning were found at the level of the first central relay in the circuits controlling the responses, neuron-wide changes in intrinsic excitability in response to the conditioned stimuli appear to be an appropriate memory mechanism to express and retain the conditioned response. However, in other cases the changes in intrinsic excitability failed to correlate, or correlated poorly, with the learned behavioral changes, thus making it difficult to assess their contribution to the storage of memory (e.g., Cleary et al., 1998). Although additional results are required, one hypothesis is that some of the intrinsic changes may not function as part of the engram itself, but they may represent either adaptive mechanisms to shape the stimulus specificity of the learned response (as in the case of classical conditioning of feeding in *Aplysia* (Baxter and Byrne, 2006; Lorenzetti et al., 2006), or mechanisms through which a neural circuit is set to a permissive state to facilitate the occurrence of the synaptic modifications necessary for memory formation (as in the case of trace eyelid conditioning in rabbits (Moyer et al., 1996; Thompson et al., 1996) or olfactory operant conditioning in rats (Saar and Barkai, 2003).

Another issue that requires more investigation is the extent to which the changes in intrinsic excitability are complementary to experience-dependent modifications in synaptic efficacy. The majority of theoretical models of memory storage have been largely based on persistent experience-driven changes in synaptic function (Poirazi and Mel, 2001; Fusi et al., 2005). Certain unique properties of the synapses such as associativity and input specificity cause

systems of memory storage based on changes in synaptic strength to have a potentially massive storage capacity (Poirazi and Mel, 2001; Fusi et al., 2005). In contrast, global changes in intrinsic excitability would theoretically alter the throughput of all the synaptic inputs impinging on a given neuron. If this is the case, memory mechanisms based on neuron-wide altered intrinsic excitability would have a lower storage capacity and would be less versatile than systems of memory storage based on changes in synaptic strength.

Some recent results may help to reconcile differences between models of memory storage based on synaptic plasticity and models based on intrinsic plasticity. Patch-clamp techniques and simultaneous Ca^{2+} imaging that allow for recordings from individual dendrites (for a review see Magee and Johnston, 2005) have revealed that the induction of LTP was accompanied by a local increase in dendritic excitability that favored back propagation of action potentials into that dendritic region with a subsequent boost in the Ca^{2+} influx (Frick et al., 2004). A shift in the inactivation curve of a transient A-type K^+ current was found to account for the enhanced excitability (Frick et al., 2004). Importantly, the activity-dependent increase in dendritic excitability was localized at, or in the vicinity of, the synaptic site, which was potentiated in an input-specific manner following electrical stimulation (Frick et al., 2004). A local increase in dendritic excitability could facilitate enhanced propagation of synaptic potentials toward the site of action potential initiation (for a review see Magee and Johnston, 2005). Therefore, enhancement of local excitability could contribute to the higher efficiency of coupling between synaptic potentials and spike as observed following LTP induction.

Although validation in *in vivo* preparations is required, these findings indicate that changes in intrinsic excitability can be induced in restricted membrane compartments and can be co-expressed with changes in synaptic efficacy in a manner that affects signal integration locally and can preserve input specificity.

4.40.5 Summary

Data collected over the past decades in both vertebrate and invertebrate model systems indicate that learning and memory as well as patterns of electrical stimulation of neurons and neural pathways not only alter synaptic function but also produce changes in intrinsic excitability. These changes in intrinsic excitability can be neuron-wide or restricted to specific membrane compartments such as the dendrites, thus affecting neuronal function and signal integration either globally or locally. Although the functional relevance of certain changes in intrinsic excitability in the context of a given form of learning has not been fully elucidated, it is becoming clear that experience-dependent changes in intrinsic excitability may function as part of the engram itself, as adaptive mechanisms to shape the stimulus specificity of the learned response or also as mechanisms through which a neural circuit is set to a permissive state to facilitate the occurrence of the synaptic modifications necessary for memory formation and retrieval.

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4.41 Neurogenesis

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4.41.1 Introduction

During the development of the central nervous system (CNS), neural stem cells give rise to neurons, oligodendrocytes, and astrocytes, the three major lineages that constitute the brain. Although a fundamental dogma of neuroscience predicted that neurogenesis ceases with the end of development, as early as 40 years ago studies by Altman and Das showed that dividing cells persist throughout life in the mammalian

CNS (Altman and Das, 1965). Indeed, Altman and colleagues' data suggested that not only glial cells but also new neurons are continuously added into the adult brain circuitry. Despite confirming reports by other groups (Hinds, 1968; Kaplan and Hinds, 1977), the finding that neural stem cells persist and can give rise to new neurons even in the adult brain remained a subject of great controversy. Nevertheless, improved techniques to identify newborn cells and their respective neural phenotype within the adult tissue finally led to the acceptance of the fact that (1) neural stem cells persist in the adult brain and (2) new neurons are continuously generated in the adult CNS in two restricted areas: the hippocampal dentate gyrus (DG) and the subventricular zone (SVZ) of the lateral ventricle (Ming and Song, 2005). In the meantime, adult neurogenesis was found to exist in all mammals, including humans (Eriksson et al., 1998). In this chapter, we mainly focus on adult neurogenesis in the hippocampal dentate gyrus and present recent advances in the understanding of the biology of adult neural stem cells, the maturation of new neurons into the preexisting circuitry, and the potential significance of adult neurogenesis in hippocampal function.

4.41.2 Stem Cells in the Adult Brain

4.41.2.1 Neural Stem Cells *in vitro*

Despite the early reports by Altman and colleagues in the 1960s, it took almost another three decades to successfully grow neural stem cells that were isolated from the adult brain in the culture dish (Reynolds and Weiss, 1992). The *in vitro* characterization of proliferative cells that were isolated from adult brain tissue was crucial to identifying a subpopulation of them as stem cells. Even though the consensus definition of a stem cell has been modified several times (Smith, 2006), two characteristics are generally required for a cell to be identified as a true stem cell: (1) the capacity for theoretically unlimited self-renewal and (2) the ability to generate cells different from themselves through asymmetric cell division. In general, stem cells can be divided into several levels of potency: (1) the 'totipotent' zygote capable of generating a complete organism when implanted into the uterus of a living animal; (2) the 'pluripotent' embryonic stem cell, which can give rise to all body tissues except the trophoblasts of the placenta and, perhaps, the gonads; and (3) the 'multipotent' stem cell that self-renews but gives rise to only tissue-specific cell types. Even though there is recent evidence that neural stem cells (NSCs) can give rise to tissue types other than CNS tissue under *in vitro* conditions (Wurmser et al., 2004), they are still mainly classified as multipotent stem cells (Gage, 2000).

4.41.2.1.1 Culturing of neural stem cells

The neurosphere assay is the most commonly used technique to analyze the stem cell capacity of isolated brain cells [**Figure 1(a)**] (Reynolds and Weiss, 1992; Lie et al., 2004). In this preparation, the brain area of interest is dissected and plated as a single-cell suspension that is propagated as floating aggregates. The addition of

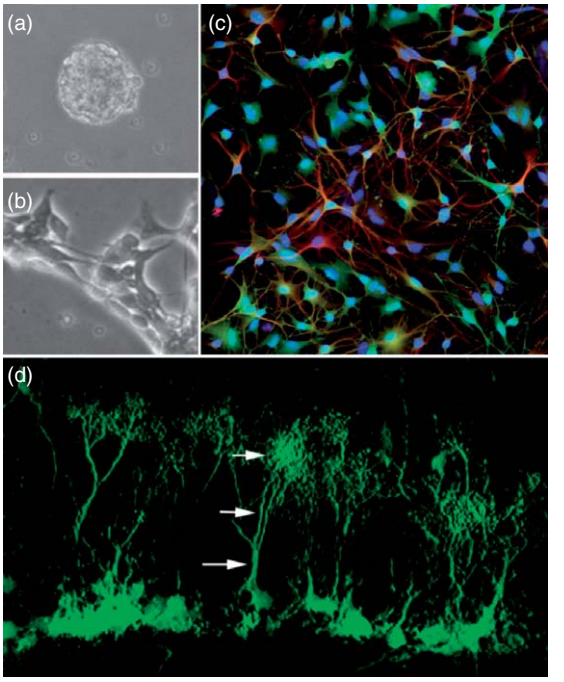


Figure 1 Neural stem cells (NSCs) *in vitro* and *in vivo*. NSCs can be grown as free-floating cell aggregates, so called neurospheres (a), or under adherent culture conditions forming monolayers (b). In both conditions strong mitogens such as EGF and/or FGF-2 are added (for details see Ray and Gage, 2006). *In vitro* propagated stem cells retain the capacity to differentiate into all three neural lineages. (c) Rat NSCs (expressing green fluorescent protein, green) that were differentiated into neuronal cells expressing MAP2ab (red) with the addition of retinoic acid and forskolin in the culture medium for 4 days. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). (d) A population of NSCs residing in the adult brain is expressing nestin. Shown is the dentate area of an adult transgenic mouse expressing green fluorescent protein under the control of the nestin promoter. Note the typical morphology of type 1 nestin cells with a tree-like process that branches in the inner molecular layer (arrow). In contrast to this morphology, type 2 cells are small, rounded cells that show a higher proliferative activity *in vivo* than type 1 cells (for details see Kempermann et al., 2004).

strong mitogens such as epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF-2) allows the propagation of endogenous proliferative cells (the putative NSCs). The forming spheres, referred to as neurospheres, can be propagated over many passages and have the potential to differentiate into all three neural lineages after the withdrawal of mitogens and/or addition of differentiating factors. Even though it appears that neurospheres do not grow clonally (Fessberger et al., 2006; Singec et al., 2006), self-renewal can be tested by the formation of secondary or tertiary spheres; a single cell is grown in a miniwell until the sphere reaches a certain size, after which the sphere is again dissociated into single cells that can give rise to a new, and thus secondary, multipotent neurosphere. An alternative method to grow adult NSCs is the so-called monolayer assay, where multipotent cells grow adherent to the dish surface [**Figure 1(b)**] (Ray et al., 1993; Gage et al., 1995; Palmer et al., 1995). Using these culturing techniques, NSCs can be isolated and propagated from many species, including humans (Palmer et al., 2001). Using the neurosphere assay, several laboratories have tried to identify differences in the potency of NSCs derived from different regions of the adult brain. Due to conflicting *in vitro* results, which might be due to differences in dissection and culturing methods, there is an ongoing controversy about whether the hippocampus contains true stem cells with theoreticallyunlimited self-renewal, or whether hippocampus-derived neurosphere cultures have only limited proliferative and differentiating capacities (Seaberg and van der Kooy, 2002).

4.41.2.1.2 Proliferation and differentiation of NSCs

The *in vitro* propagation of adult NSCs allowed the extensive biochemical and molecular characterization of fate choice, proliferative capacity, and cellular potency. *In vitro* studies identified a variety of factors and mechanisms that regulate cell proliferation and instruct NSCs to adopt a neuronal or glial fate [**Figure 1(c)**] (Lie et al., 2004; Ming and Song, 2005). Subsequent studies showed an *in vivo* role for most of those factors as well, thus confirming the *in vitro* NSC model system. New mechanisms that affect proliferation and/or differentiation of NSCs are constantly being discovered; the regulation of proliferation and differentiation clearly occurs on multiple molecular levels. We and others have shown that epigenetic mechanisms, growth factors such as brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF), sonic hedgehog signaling, WNT signaling, small double-stranded RNAs, retrotransposition, transcription factor expression such as E2F, cyclin-D2 expression, and cell-cycle inhibitors such as p27kip1 (Lie et al., 2004; Ming and Song, 2005; Cao et al., 2006) are among important regulators of proliferation and/or neuronal differentiation. Considerably less is known regarding differentiation toward glial cells, though several important signaling pathways have been identified that induce NSCs to differentiate into oligodendrocytes, such as insulin-like growth factor (IGF) signaling (Hsieh et al., 2004), or astrocytes, such as bone morphogenetic protein (BMP) and leukemia inhibitory factor (LIF) signaling (Bonaguidi et al., 2005).

Despite the great utility of *in vitro* assays in the biochemical and molecular characterization of NSCs, there are several important caveats about using them to study NSCs. Isolated cells must be exposed to high levels of mitogens that potentially induce cellular changes different from those seen in an *in situ* stem cell. Furthermore, NSCs in culture are 'naked' and may not receive the same factors as those that are embedded in their respective cellular niche. Importantly, cells with stem cell capacity can be easily isolated from almost all areas of the adult brain and the spinal cord, even though only two areas in the adult brain are capable of generating new neuronal cells.

4.41.2.2 Neural Stem Cells *in vivo*

The adult DG continuously produces new neuronal cells. Neurons born in the adult hippocampus are glutamatergic granule cells, the principal cell type of the DG. Granule cells reside in the densely packed granule cell layer (GCL), receive their main excitatory input from layer II of the entorhinal cortex (EC), and largely project toward area 3 of the cornu ammonis (CA3) via the mossy fiber tract. The DG is a very densely packed structure: a single mouse DG consists of approximately 300,000 granule cells, rat DG about 1 million, and human DG between 15 and 20 million (Simic et al., 1997). These cell numbers are very large relative to the DG's input (EC) and output (CA3) structures, both of which have an order of magnitude fewer neurons.

4.41.2.2.1 The hippocampal neurogenic niche

Proliferative activity within the dentate area occurs largely in the zone just below the GCL and the hilus. This area, called the subgranular zone (SGZ), does not have strict boundaries and is commonly defined as an intermediate region about two cell layers deep into the hilus below the GCL (corresponding to

approximately 20–30 μm). Significant research has been devoted to determining how to identify dividing cells in the SGZ morphologically and whether they can be classified using differential gene expression. Acutely dividing cells can be visualized by the detection of cell cycle–associated genes such as Ki-67, PCNA, and phospho-histone H3.

Whether or not those proliferating cells in the DG are true NSCs *in vivo* is still unknown. This categorization requires the observation of self-renewal and multipotency, and neither has been definitively shown due to the technical difficulties in following single cells and their lineages over time *in vivo*. Several labeling techniques have been used to label proliferating populations of cells in the DG and track their progeny, including bromo-deoxyuridine (BrdU) and tritiated (^{3}H)-thymidine (nucleotide analogues) and marker-expressing retroviruses [**Figure 2(a)**].

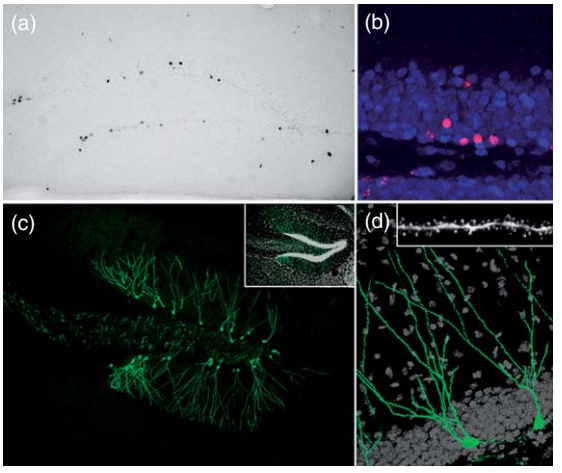


Figure 2 Labeling of newborn cells *in vivo*. The thymidine analogue bromo-deoxyuridine (BrdU) is integrated into the DNA of proliferating cells during S-phase. (a) BrdU-labeled cells can be visualized in the adult dentate gyrus with specific antibodies. The example shows a section of an adult mouse that received five consecutive BrdU injections and was sacrificed 4 weeks after the last injection. (b) The phenotype of the BrdU-positive cells (red) can be reliably analyzed by confocal microscopy using, for example, neuronal markers such as NeuN (blue), again 4 weeks after BrdU injection. Dividing cells and their progeny can also be visualized with retroviruses expressing green fluorescent protein (or any other label that can be later visualized), allowing the direct detection of newborn cells without the need for post hoc detection methods. (c) A 40-μm section of an adult mouse that was injected 4 weeks earlier with a retrovirus expressing green fluorescent protein under the control of the CAG promoter. (d) Retroviral labeling visualizes the whole cell (including dendritic and axonal processes) and even allows the analysis of neuronal fine structures such as dendritic spines (inset in (d); for details see Zhao et al., 2006).

The most commonly used label is BrdU, a thymidine analogue that becomes integrated into the DNA during the S-phase of the cell cycle. BrdU-labeled cells can be visualized with a specific antibody and then analyzed histologically to determine their neural phenotype [**Figure 2(b)**]. The major advantage of BrdU labeling is the easy and reliable quantification of new cells. There are several drawbacks to BrdU, however. Because BrdU incorporates into newly formed DNA, several successive rounds of DNA replication after BrdU administration will dilute the signal. Furthermore, the BrdU signal is restricted to the nucleus and thus does not show the morphology of newborn cells. Given these limitations, the use of retroviral vectors expressing green fluorescent (GFP) or red fluorescent protein (RFP) to visualize the whole cell morphology opened up a completely new level of analysis [**Figures 2(c) and 2(d)**] (van Praag et al., 2002). Because retroviruses require the breakdown of the nuclear membrane for genomic integration, only dividing cells are transduced. Retrovirus infection and delivery are not as ubiquitous as BrdU uptake, limiting their value as quantification tools; however, the transduction of progenitors results in a stable and robust expression of a marker protein that is detectable in both fixed and live tissue.

Using these markers, it has been shown that proliferating cells that can generate all three lineages exist in the DG (Kempermann et al., 2004). As it is unclear that these cells are individually multipotent and self-renewing, they are referred to as neural progenitor cells (NPCs). Accumulating evidence suggests that there are actually several types of dividing cells within the adult hippocampus, and their relationship to one another remains somewhat elusive (Kempermann et al., 2004). One type of NSC within the hippocampal DG appears to be very similar to a mature astrocyte [**Figure 1(d)**]. These cells express glial fibrillary acidic protein (GFAP) (Seri et al., 2001), have the electrophysiological properties of astrocytes, and possess astrocytic vascular end feet (Filippov et al., 2003). Depending on the nomenclature used, these cells are referred to as type 1 or B-cells. Type 1 cells also express the intermediate filament protein nestin, and a subpopulation of type 1 cells also colabels with the HMG-transcription factor Sox-2. The low proliferative activity of type 1 cells may indicate that these cells represent a largely quiescent, true stem cell within the hippocampus, but this hypothesis remains to be proven. In contrast to the slowly dividing type 1 cells, type 2 cells exhibit a much higher proliferative activity. Type

2 cells are negative for GFAP but still express nestin and Sox-2, and a fraction of type 2 cells expresses early neuronal markers such as doublecortin (DCX) and the bHLH transcription factor Prox-1. One theory is that the expression of these neuronal markers in a subset of dividing cells indicates that type 2 cells (and type 3 cells that divide but are negative for nestin and Sox-2 and positive for DCX) represent committed neuroblasts that have lost their multipotentiality (Kempermann et al., 2004).

The rate at which neurons are born in the adult hippocampus is not fixed but is instead dynamically regulated by a variety of factors. Despite increasing knowledge about how progenitor cells 'translate' signals such as local network activity into altered cell division or differentiation, the exact mechanisms remain unclear. Recent reports have shown that depolarizing events trigger NSCs to initiate neuronal differentiation (Deisseroth et al., 2004; Tozuka et al., 2005), providing an explanation for how network activity within the dentate gyrus may shape the cellular composition of the area itself. Furthermore, elegant *in vivo* studies have shown the synaptic integration of nestin-expressing cells in the SGZ and their functional GABAergic input (Tozuka et al., 2005). Those reports have led to the hypothesis that dentate NSCs may 'sense' the activity of surrounding mature, neuronal networks and respond with a distinct action, such as increased or decreased cell proliferation and/or differentiation. The regulation of adult neurogenesis on a systems level will be extensively discussed in the section titled "Systems regulation of adult neurogenesis."

As stated earlier, NSCs can be isolated and grown *in vitro* from almost all brain areas. Furthermore, Sox-2, nestin, and dividing GFAP-positive cells are scattered throughout the adult brain. One major question is what allows the DG and SVZ to be unique in permitting the generation of new neurons in the adult. Most likely, there is something in the microenvironmental niche that surrounds NSC *in vivo* that permits and/or supports neuronal differentiation and maturation. A detailed study by Seri and colleagues (2004) showed a close association of hippocampal NSCs with the vasculature, a proximity that might be a critical factor in the distribution of trophic support for not only NSCs but also immature neurons (Palmer et al., 2000). Importantly, hippocampal astrocytes also play a pivotal role in making the DG a neurogenic area (Song et al., 2002). While astrocytes from nonneurogenic regions such as the spinal cord appear to inhibit neurogenesis, hippocampal astrocytes appear to be the source of important differentiation and/or stem cell maintenance factors, such as WNT proteins (Lie et al., 2005).

4.41.2.3 Neurogenesis in Nonneurogenic Areas Following Manipulation

Although the DG and SVZ are the only brain regions that undergo continual neurogenesis in the healthy adult, it appears that endogenous NSCs throughout the nervous system maintain the capability to become neurons. When grown *in vitro,* NSCs from nonneurogenic areas can be induced to differentiate into neurons, and when transplanted into a neurogenic area they will differentiate into neurons. The exciting possibility also exists that nonneurogenic brain areas can be manipulated by traumatic insults or other means to induce the production of new neurons. A recent report showed that the production of new neurons in the hypothalamus could be induced by injection of ciliary neurotrophic factor (CTNF), which is critically involved in feeding behavior (Kokoeva et al., 2005). Thus, new neurons in the hypothalamus feeding centers may be involved in regulating the energy balance in rodents. The potential to produce new neurons apparently also holds true for the striatum and cornu ammonis area 1 (CA1) following ischemic stroke and for the cortex following targeted cellular ablation (Abrous et al., 2005). However, the numbers of new neurons produced following strokes, for example, are extremely low, and it is not clear what their functional impact may be. Nevertheless the potential for endogenous repair has sparked high levels of excitement, and future studies will have to determine the feasibility of neuronal repair from endogenous NSCs following brain injury.

4.41.3 Maturation of Adult-Born Granule Cells

4.41.3.1 Molecular Maturation and Identification of Adult-Bom Granule Cells

As described earlier, the availability of robust and reliable methods to label cells and/or their progeny enabled the identification of genes expressed during the maturation of adult-born granule cells (AGCs). Importantly, and in striking contrast to neuronal maturation during embryonic development, all stages of maturation coexist in the adult DG at any given time point. Therefore, every observation has to focus

on a single cell and is a mere snapshot of neuronal development in the adult brain. This heterogeneity of adult neurogenesis has complicated large-scale approaches to identifying specific gene expression patterns of AGCs at a certain stage of maturation. Moreover, the development of new neurons is accompanied by gliogenesis (Kempermann et al., 2004), and if the current assumption of a common precursor for all three neural lineages holds true, the decisive branching point between neuronal and glial development has not yet been identified. Finally, only a subset of the AGCs that are born and are clearly classifiable as neuronal cells eventually becomes stably integrated into the dentate circuitry (Kempermann et al., 2004). However, the work of several laboratories has generated an astoundingly clear picture of the molecular maturation of AGCs (Brandt et al., 2003; Fukuda et al., 2003; Kempermann et al., 2004; Gleiberman et al., 2005). Much of this information has been generated by characterizing the molecular makeup of BrdU-positive cells at different time points after the injection of the thymidine analogue.

The molecular characteristics of adult progenitors were described in the earlier section covering NSCs. **Figure 3** shows examples of several immature neuron–specific markers. The first gene considered to be neuronal is expressed in dividing cells: DCX. DCX is a microtubule-associated protein that is critically involved in neuronal migration during development; mutations in the DCX gene cause lissencephalic malformations in humans. Even though DCX is expressed in acutely dividing cells, expression persists for approximately 3 weeks after BrdU injection. Thus, the DCX-expressing cell population consists of a very heterogeneous population of newborn cells. The functional importance of DCX in AGC maturation remains unclear. Comparable to the timing of DCX expression is the expression of the polysialylated form of the neural cell adhesion molecule (PSA-NCAM) – a protein that appears to be involved in cell migration, axonal fasciculation, and neurite outgrowth. The proneural basic helix-loop-helix transcription factor NeuroD1 is expressed early after cell division and tapers off approximately 7 days after BrdU. NeuroD1 has been found to be critically involved in neuronal fate instruction, particularly for granule cells, as *NeuroD1* −/− mice fail to ever develop a DG. Expression of Prox-1, the mammalian homologue of the transcription factor prospero that is critically involved in cell cycle regulation, appears together with DCX and PSA-NCAM in newborn

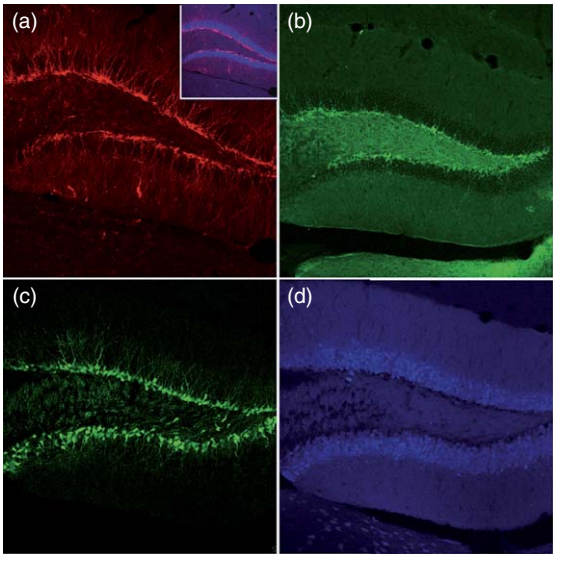


Figure 3 Endogenous and transgenic markers of adult neurogenesis. There are numerous endogenous proteins that are expressed during specific stages of neuronal maturation in the adult dentate gyrus. (a) Doublecortin (DCX) labeling of immature neurons lining the inner granule cell layer (visualized by prox-1 staining, blue in inset). (b) polysialylated neural cell adhesion molecule labeling of immature neurons, which includes staining of axons. (c) The dentate gyrus from a *POMC*-driven green fluorescent protein transgenic mouse (green) that was generated by Overstreet-Wadiche and colleagues. (4) Calbindin, the mature granule cell Ca^{2+}-binding protein, is only expressed in granule cells after 3–4 weeks of maturation.

cells. In contrast to DCX and PSA-NCAM, prox-1 remains expressed throughout the life of a granule cell. This expression pattern is similar to the pan-neuronal protein NeuN, which can be detected as early as 3 days after BrdU injection but remains expressed later on. The expression of the Ca^{2+}-binding protein Calretinin marks the definite exit from cell cycle. Calretinin is replaced approximately 3 weeks later by calbindin, the Ca^{2+}-binding protein of mature granule cells (Kempermann et al., 2004). As described later, the regulation of the survival and integration of AGCs seemingly occurs during many, if not all, stages of neuronal maturation.

4.41.3.2 Electrophysiology of Maturing AGCs

Critical to our understanding of the role adult neurogenesis has in cognition is the functional maturation of these neurons. Increasingly, it appears that these new neurons become functional parts of the DG circuit, passing through several maturation

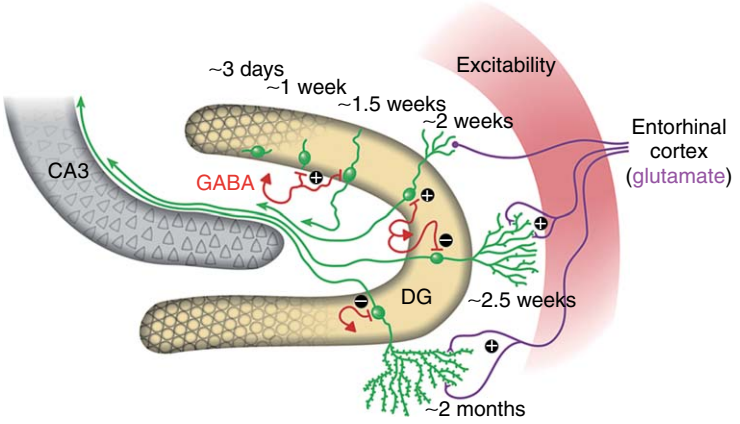


Figure 4 Maturation of newborn neurons. Adult-born granule cells progress through several states before they reach full maturity. Early in maturation, the neurons have limited processes and receive only GABA inputs from local interneurons. By 2 weeks, the neuron's dendrite protrudes into the molecular layer. Soon thereafter, spine formation begins, indicating the onset of glutamatergic input. After several months of maturation, the newborn neuron becomes indistinguishable from other granule cells. The bar labeled "excitability" indicates a period in which immature neurons may be more responsive to the network due to depolarizing GABA, depolarized resting potentials, and increased LTP. Figure from Aimone JB, Wiles J, and Gage FH (2006) Potential role for adult neurogenesis in the encoding of time in new memories. *Nat. Neurosci.* 9: 723–727, with permission.

stages and ultimately developing a cellular physiology indistinguishable from that of embryonic-born neurons (**Figure 4**). This section reviews the maturation process of newborn granule cells.

4.41.3.2.1 Techniques used in characterizing maturation stages of AGCs

Following the characterization of the critical steps leading to the molecular maturation of AGCs, several laboratories have made significant strides in characterizing the physiological maturation of AGCs using several different approaches to labeling and recording from specific ages of new neurons. One approach has been to segregate cells based on location and passive membrane properties (i.e., membrane resistance) and to use post hoc histological and morphological observations to confirm maturation state. While this approach has proven effective at distinguishing between mature and immature neurons, it is difficult to *a priori* identify neurons of a specific age or maturation state. Because early cell division markers such as ^{3}H-Thy and BrdU only label nuclei, studies investigating the maturation of AGCs were limited until the development of more sophisticated labeling techniques using fluorescent genetic markers. Two approaches in particular have boosted research in this area: GFP retrovirus and GFP driven by immature granule cell–specific promoters. By using a retroviral vector that only incorporates itself into dividing neurons to deliver GFP, researchers can identify and observe the morphology of cells that divide at a known time. This process allows the determination of the actual 'age' of the neuron. On the other hand, GFP driven by promoters that specifically label immature neurons or NPCs, such as *Pomc,* allows the identification of a group of cells at a similar maturation state (Overstreet et al., 2004). Importantly, since not all AGCs mature at the same rate, the retrovirus-GFP and cell-specific GFP labeling approaches are not immediately comparable, and care must be taken when interpreting data across experiments.

4.41.3.2.2 Depolarizing GABA input

Using retrovirus labeling, neurons – as defined by the ability to fire an action potential – can be observed as 1 day postinjection (dpi) in slice (Esposito et al., 2005). These early neurons have little distinguishing morphology beyond possibly a few small nonoriented projections. These young neurons lack synaptic inputs, though it appears that even at this young age there is a tonic GABA input, suggesting the presence of nonsynaptic somatic receptors that are sensitive to levels of local interneuron activity.

By around 1 week of age, these neurons begin to take on a neuronal morphology. Although at this age most neurons still lack a robust dendritic arborization, oriented processes extending to the molecular layer can be seen. It is at this stage in development that these

young neurons begin to receive synaptic GABAergic inputs from the local interneuron population. The fact that GABA-releasing axons are the first to contact the young neurons is important for several reasons. First, having GABA synapses preceding glutamate synapses is similar to the order seen during embryonic development. Like in development, this early GABA has a depolarizing impact on the neuron. Second, this depolarizing GABA appears to have long-lasting implications for the neurons' development, suggesting the onset of activity-dependent maturation.

By 2 weeks postinjection, there appears to be a substantial amount of depolarizing GABA input. A similar group of neurons has also been identified by Overstreet-Wadiche and colleagues (2005) by using GFP driven by the *Pomc* promoter. These GABA-only neurons have identifiable dendrites, but they barely extend into the molecular layer with limited branching. Despite not receiving glutamatergic inputs, they can be depolarized by perforant path stimulation, presumably via polysynaptic activation of GABAergic basket cells.

Although 1- to 2-week-old neurons receive depolarizing synaptic inputs, it is not clear at what point they begin to communicate with other neurons. While these GABA-excited neurons are capable of exhibiting action potentials, their spiking is not typical of mature granule cells' bursting patterns. The extent to which GABA can induce action potentials *in vivo* remains to be seen. GFP-labeled axonal processes do not appear in the CA3 until about 11 or 12 dpi and do not reach the CA3/CA2 boundary until 16 dpi.

4.41.3.2.3 Spine formation and the onset of glutamatergic inputs

According to several studies (Esposito et al., 2005; Zhao et al., 2006), spine formation begins just after 2 weeks and continues beyond 1 month of age. This time frame is consistent with electrophysiology studies, which show the onset of weak glutamatergic inputs at around 2 weeks of age (Esposito et al., 2005; Ge et al., 2006). The earliest stages of glutamate inputs are characterized by a high *N*-methyl-D-aspartate (NMDA) dependence; the blockade of NR1 receptors has been shown to kill most immature neurons between 2 and 3 weeks of age (Tashiro et al., 2006). Spine formation is rapid, increasing from about 0.43 spines/μm to 1.95 spines/μm between 21 and 28 dpi.

It is at these early glutamate stages that synaptic plasticity has been best characterized. Numerous studies have observed a difference in long-term potentiation (LTP) between immature and mature DG granule cells. Schmidt-Hieber and colleagues (2004) showed that high-resistance, PSA-NCAM+ neurons (corresponding to between 1 and 3 weeks of age) experienced associative LTP more readily than mature neurons (Schmidt-Hieber et al., 2004). Similarly, Wang et al. (2000) showed, using several paradigms, that medial perforant path stimulation did not induce LTP in mature neurons unless GABA was blocked with bicucilline, whereas immature neurons had robust LTP regardless of GABA blockade (Wang et al., 2000). Consistent with this increased propensity for LTP, Zhao et al. (2006) showed that immature neurons had higher levels of spine motility and a lower proportion of large mushroom spines.

By around 1 month of age, the neurons are similar to fully mature neurons, but not yet identical. Electrophysiologically, there are few significant differences at this stage, though it appears that some differences in plasticity remain when compared to fully mature neurons (van Praag et al., 2002). Morphologically, the dendritic arborizations appear roughly similar, but there are still morphological differences. Overall spine density continues to increase until about 2 months of age, after which the density remains relatively constant. Likewise, spine motility at 28 dpi is still higher than that seen in fully mature neurons. Furthermore, Toni et al. (in press) show that spines on new neurons preferentially form connections on already existing synaptic sites.

4.41.3.2.4 Timeline of projections to CA3

All indications suggest that AGCs project axons to the same neurons to which embryonic and postnatal granule cells project, specifically the CA3 and interneurons in the hilus, although these studies are limited. Markakis and Gage (1999) and Hastings and Gould (1999) both showed that fluorescent retrograde tracers injected into the CA3 colocalized with BrdU cells in the DG (Hastings and Gould, 1999; Markakis and Gage, 1999). Colabeling can be seen in some cells as early as 10 days after BrdU was first administered. This finding is consistent with observations using GFP retrovirus. Zhao et al. (2006) showed that GFP driven by the CAG promoter is present in the axons of immature neurons (Zhao et al., 2006). At 10 dpi, axons are restricted to the hilus, but they reach the CA3/CA2 boundary by 16 dpi.

Although the inputs to AGCs have been well characterized electrophysiologically, the physiology of newborn axonal projections to the CA3 has not

been studied. The mossy fiber projection of dentate granule cells has several unique characteristics, including its sparse topography – one granule cell connects to only about a dozen CA3 pyramidal neurons – and the ability of a single active mossy terminal to fire its downstream target. It will be interesting to see whether the unusual properties of DG mossy fibers are also found in adult-born neurons.

4.41.3.2.5 Regulation of maturation process

Although the phases of AGC maturation mimic those of the embryonic and early postnatal granule cells, the network into which they are maturing is unique in that it is a real-time functioning network. Not surprisingly, the maturation of AGCs is a tightly regulated process at each stage. Increasingly more is being learned about what regulates the way in which these new neurons integrate into the network.

Experience-dependent regulation of maturation appears to begin very soon after differentiation. Ge and colleagues (2006) have demonstrated that 3-dpi neurons receive tonic GABA depolarization several days before the appearance of synapses, and the depolarizing nature of this input is important for further development (Ge et al., 2006). Their data suggest that the depolarizing effects of GABA in very immature neurons are regulated by a tight balance between NKCCl and KCC2 transporters, and the disruption of this balance results in dramatically underdeveloped dendritic arborizations by 2 weeks.

After this GABA-dependent phase of maturation, the neuron appears to enter an NMDA-dependent maturation phase. A study by Tashiro et al. (2006) demonstrated that immature neurons deficient in the NR1 subunit of the NMDA receptor develop normally for the first 2 weeks, but by 3 weeks most are dead. Furthermore, this required NMDA activation is relative to the local network; if NMDA is globally blocked using AP5, the *NR1*−/− neurons survive more successfully.

In summary, the maturation of new neurons appears to take a course similar to that seen during development, but over a more extended time scale. Importantly, at each stage of development, the efficacy of integration appears to be critical for further maturation and ultimate survival. Consistent with this dependence on activity, these neurons appear to be much more responsive to the network, in terms of both synaptic plasticity (spinogenesis, LTP) and basic physiological states (more depolarized resting potentials, higher input resistances, longer membrane time constant, higher E_{GABA}). Their excitable state stands out in contrast to mature granule cells, which are characterized by very low activity due to high levels of tonic inhibition.

4.41.4 Systems Regulation of Adult Neurogenesis

4.41.4.1 Physiological Regulators of Adult Neurogenesis

The amount of progenitor cell division and the subsequent numbers of new neurons that are born in the adult DG are dynamically regulated by a variety of both physiological and pathological factors. The finding that adult neurogenesis is a highly dynamic feature of adult brain plasticity challenged our understanding of how the mature brain responds to its environment and showed that the cellular composition in the adult dentate area is subject to constant change. How adult neurogenesis is regulated and what factors influence the number of adult-generated neurons have been important questions in the field.

The number of new granule cells can be changed by two mechanisms: (1) an increase in progenitor cell proliferation and (2) an increase in newborn neuron survival. Regulators of adult neurogenesis often have an effect on both proliferation and survival, and currently there is no easy way to completely separate these two mechanisms. However, some regulators act more strongly on proliferation than on survival and vice versa. The regulators discussed here and in the next section represent only the major factors that influence adult hippocampal neurogenesis, and the list of manipulations that enhance or abate adult neurogenesis is growing constantly (Ming and Song, 2005). These results are summarized in **Table 1**.

4.41.4.1.1 Natural variation in adult neurogenesis

Without a doubt there is a very strong genetic impact on the number of adult-born neurons. Inbred mice strains can differ 10-fold or more in the number of new granule cells, either by dramatically decreased/elevated levels of cell proliferation or by low/high levels of cell survival, respectively. Using quantitative trait loci analyses of inbred mice strains, several genetic loci have been identified that appear to be critically involved in the regulation of adult neurogenesis (Kempermann et al., 2006). Further studies are designed to narrow the identified loci down to single genes.

Table 1 Regulation of adult neurogenesis

Regulator	*Proliferation*	*Survival*	*Neuronal differentiation*
Physiological Regulators of Adult Neurogenesis			
Genetic background	+/−	+/−	+/−
Enriched environment	?	+	+
Physical exercise	+	no change	+
Learning	no change	+	?
Aging	−	?	−
Dietary restriction	no change	+	?
Neurotransmitters	see text		
Pathological Regulators of Adult Neurogenesis			
Stress	−	no change	?
Seizure activity	+	+ (?)	+
Ischemia	+	+ (?)	+
Irradiation	−	−	−
Neurodegenerative Diseases			
Alzheimer's disease	+/−	?	?/−
Huntington's disease	−	no change	no change
Parkinson's disease	−	?	?
Drugs			
Opiates	−	?	?
Antidepressants	+	+	+
Ethanol	no change/−	−	?

4.41.4.1.2 Environmental enrichment

Genetic background is not alone in having a powerful influence on adult hippocampal neurogenesis. It has been known for a long time that housing laboratory animals in an enriched environment exerts positive effects on the animals' behavior (van Praag et al., 2000), even though the structural correlate of this improvement remained unclear. Kempermann and colleagues found that housing adult mice for several weeks together in a large cage with toys and a changing environment had strong effects on their performance in hippocampus-dependent learning tasks (Kempermann et al., 1997). In addition to improving learning, environmental enrichment doubled the number of new neurons in the DG. This dramatic effect on hippocampal neurogenesis appeared to be mainly due to an increased survival of the new neurons, preventing the apoptotic death of immature neurons. Later studies showed that the effects of environmental enrichment on adult neurogenesis are mediated by VEGF signaling in the dentate area (Cao et al., 2004). Recent work has shown that 2- to 3-week-old neurons appear to be the most affected by an enriched environment, as cells labeled that far ahead of enrichment are substantially more likely to survive and respond to that environment at a later date (Tashiro et al., 2007).

4.41.4.1.3 Physical exercise

After the robust effects of environmental enrichment on adult neurogenesis were discovered, it remained unclear which of the multimodal effects of enrichment on the animals' life was responsible for the enrichment-induced increase in neurogenesis: larger cages may result in more physical activity, and bigger housing groups increase social interaction (to prevent the strong formation of social dominance, all initial experiments were performed using female mice). van Praag and colleagues tried to dissect out those factors and found that the physical activity itself – in contrast to the survival-promoting effect of enrichment – had a very potent effect on progenitor cell division, subsequently leading to an increased net number of newborn neurons (van Praag et al., 1999b). The exercise-induced increase in newborn neurons in the dentate area also correlated with improved performance in learning tasks and facilitated the induction of LTP on perforant path/granule cell layer synapses (van Praag et al., 1999a).

4.41.4.1.4 Learning

Hippocampus-dependent learning itself can increase the number of surviving neurons, even though the survival-increasing effect of learning on immature neurons appears to be restricted to a limited stage of neuronal development (Gould et al., 1999).

Supporting the finding of learning-enhanced neurogenesis are reports that the *in vivo* induction of LTP elevates the number of newborn neurons (Bruel-Jungerman et al., 2006). Recent experiments suggest that this effect on the hippocampal structure may also occur in humans (Draganski et al., 2006). However, it remains unclear whether the observed changes in hippocampal volume are indeed causally related to increased neurogenesis.

4.41.4.1.5 Aging

The age of the animal also has a dramatic influence on the number of adult-generated neurons. According to van Praag et al. (2005), the number of AGCs surviving for a month is estimated to be between 500 and 1000 per week in young adult mice (between 2 and 6 months of age) but drops to about 100 per week in aged animals. The reasons for this strong decrease are both low levels of progenitor cell proliferation and low levels of neuronal differentiation compared to young animals. An elegant study by Cameron and colleagues indicated that corticosteroids play a pivotal role in the age-related decline of adult neurogenesis (Cameron and McKay, 1999). However, it is important to notice that even in old age, both environmental enrichment and physical activity are still powerful tools to increase the number of newborn neurons (Kempermann et al., 2002; van Praag et al., 2005). To date it is still unclear whether decreased neurogenesis in aged animals is caused by an intrinsic progenitor cell failure or by changes in the neurogenic environment, but studies are under way that will address this critical question.

4.41.4.1.6 Neurotransmitters

Most neurotransmitters that have been studied appear to have an influence on the number of new neurons formed, although in most cases it has been technically very difficult to distinguish whether their effects on NPCs are direct or occur through the transmitters' effects on the surrounding network. For example, the direct roles of glutamate and GABA are still unclear. The principal input to the DG is glutamate from the EC. Disruption of the perforant path in the rat results in an increase in cell proliferation, suggesting that excitatory input might inhibit neurogenesis. Similarly, systemic NMDA receptor blockade enhances proliferative activity (Nacher and McEwen, 2006). However, as discussed in the following text, the activation of kainic acid glutamate receptors dramatically increases cell proliferation, and depolarization of progenitor cells appears to facilitate neurogenesis. Given these findings, the simple assumption that glutamatergic excitation generally downregulates neurogenesis might be too simple, especially considering that glutamate is rarely released in the DG without simultaneous release of GABA from interneurons. GABA signaling is not only involved in maturation – as outlined in earlier sections – but is also critically involved in proliferation (Tozuka et al., 2005; Ge et al., 2006). Therefore, it is probable that a delicate balance between excitation and inhibition is responsible for modulating the levels of neurogenesis (Deisseroth et al., 2004).

In addition to the glutamatergic EC input and internal circuitry, the DG receives inputs from a variety of different brain regions carrying a wide range of neurotransmitters. Cholinergic (as well as GABAergic and glutamatergic) fibers from the septum and nucleus basalis terminate throughout the DG, and particularly at the SGZ. Selective lesions of cholinergic neurons in the forebrain revealed a survival-promoting effect of acetylcholine, as lesions increased apoptotic cells' deaths and lowered neurogenesis levels (Cooper-Kuhn et al., 2004). Likewise, there is a strong serotonergic innervation of the SGZ from the raphe nuclei, and gain- and loss-of-function studies showed that release of serotonin strongly increased the numbers of newborn neurons (Gould, 1999). It has also been shown that drugs increasing serotonin levels such as fluoxetine upregulate adult neurogenesis (see following section for more details).

The DG also receives norepinephrine from the locus coereleus and dopamine from the ventral tegmental area. Less is known regarding the overall role of these neurotransmitters in the adult DG, but there are indications that both transmitters influence the levels of adult neurogenesis (Abrous et al., 2005). Furthermore, a wide range of neuropeptides and other molecules are released by axons in the DG, and several of these, including endocannabinoids, NPY, and endogenous opiates, appear to have effects on the proliferation of NPCs.

4.41.4.1.7 Additional regulators of adult neurogenesis

Another strong modulator of adult neurogenesis is dietary restriction, which increases the number of newborn granule cells by increased proliferation (Prolla and Mattson, 2001). Dietary intake may strongly influence the hormonal state, and several hormones have been shown to have a strong effect on the number of newborn neurons in the dentate area. Both positive (e.g., dehydroepiandrosterone,

DHEA) and negative (e.g., corticosterone) hormonal regulators could be identified (Abrous et al., 2005). Another modulator of adult neurogenesis is sleep deprivation. Interestingly, a single night of sleep deprivation appears to increase adult neurogenesis, whereas more chronic forms of sleep restriction had the opposite effect (Guzman-Marin et al., 2005; Grassi Zucconi et al., 2006). It is important to note that the number of physiological modulators is constantly growing, and the list described here is not complete (Abrous et al., 2005).

4.41.4.2 Pathological Regulators of Adult Neurogenesis

The previous section indicated the enormous degree of regulation that adult neurogenesis undergoes under normal conditions. Thus, it is not surprising that a long list of pathological conditions also have an effect on the number of newborn neurons in the DG.

4.41.4.2.1 Stress and depression

One very prominent negative regulator of adult neurogenesis is stress. Pioneering work by Gould and colleagues showed that psychosocial stress leads to a strong decrease in progenitor cell proliferation and new neuron numbers (Abrous et al., 2005). Mechanistically, the negative effects of stress on neurogenesis appear to be largely mediated by corticosteroids; adrenalectomy has been shown to prevent the stress-induced decline in new neuron production in rodents. Importantly, very similar results were obtained using primates (Abrous et al., 2005). The finding that stress has a strong, negative effect on adult neurogenesis lead to the hypothesis that hippocampal neurogenesis may also be critically involved in the disease process of depressive disorders. Interestingly, many human patients suffering from major depression have elevated serum levels of corticosteroids as well as reduced hippocampal volume, as measured by MRI (Sapolsky, 2000). Furthermore, it has been shown that several antidepressive drugs, such as fluoxetine, which belongs to the class of selective serotonin reuptake inhibitors (SSRIs), have a positive impact on adult neurogenesis. In addition, electroconvulsive seizures, which are a powerful clinical tool used to treat certain forms of major depression, increase the number of newborn neurons (Warner-Schmidt and Duman, 2006; and see following). The delay in the effectiveness of a variety of antidepressants for several weeks nicely fits with the idea that new neurons may be partially responsible for the drug's efficacy. In fact, Santarelli and colleagues showed that the inhibition of adult neurogenesis abolished the behavioral effects of fluoxetine on certain aspects of anxiety and stress (Santarelli et al., 2003). The link between adult neurogenesis and emotions will be discussed in more detail in the next section.

4.41.4.2.2 Seizures

Compared to stress and depression, seizure activity has the opposite effect on adult neurogenesis within the hippocampal circuitry. Epileptic discharges lead to a massive increase in cell proliferation and subsequent integration of new neurons into the dentate area (Parent, 2002). The exact mechanisms of how seizures result in increased numbers of new neurons is not fully understood, but it seems that cell death following seizure activity is not a critical factor. Even though the number of new neurons is dramatically upregulated within the first several days following seizures, recent data suggest that, after the initial insult, numbers drop below controls (Hattiangady et al., 2004). Whether this decrease is due to an 'exhaustion' of the progenitor population or to environmental changes in the neurogenic niche remains unclear.

Notably, a significant fraction of seizure-generated neurons either has aberrant basal dendrites (Shapiro and Ribak, 2006) or is ectopically located at the hilar/CA3 border (Parent et al., 1997; Scharfman et al., 2000). These basal dendrites reach deep into the hilus and, as observed with DCX analyses, have immature synapses that appear to integrate into the circuitry. However, little is known about the stability of these aberrant connections and what their functional impact on synaptic transmission through the dentate circuitry might be. Besides having abnormal dendritic processes, many seizure-generated granule cells ectopically migrate into the hilus toward area CA3. Studies by Scharfman provided evidence that these aberrant granule cells show synchronous firing patterns with CA3 pyramidal cells and may initiate recurrent excitation onto granule cells (Scharfman et al., 2000). These abnormal features of seizure-generated granule cells have led to the hypothesis that seizure-induced neurogenesis might be responsible for certain aspects of the epileptogenic disease process (Parent, 2002). However, it may also be true that seizure-induced neurogenesis is instead an attempt by the injured brain to replace lost cells and to rebuild damaged hippocampal structure (Bjorklund and Lindvall, 2000).

4.41.4.2.3 Ischemia

Similar to seizure activity, though less dramatic, ischemic insults to the adult brain increase the number of new neurons (Zhang et al., 2005). Unlike ischemia-induced neurogenesis in the SVZ, where newborn cells appear to migrate toward the lesion and possibly participate in repair function, the cause or value for increased neurogenesis within the hippocampus following ischemia remains unclear. Importantly, short ischemic episodes that do not result in measurable cell death still affect neurogenesis (Zhang et al., 2005). There are certain types of ischemic stroke induction, such as the four-vessel occlusion, that specifically target the hippocampal area in rodents. Experiments from the Nakafuku laboratory showed that poststroke infusion of the growth factors EGF and FGF-2 led to a robust repopulation of the damaged CA1 area with functional pyramidal neurons (Nakatomi et al., 2002), suggesting that endogenous progenitors are capable of repopulating substantial lesions in the adult brain. Further studies will have to determine the feasibility of this approach and what the actual benefit of endogenous neuronal replacement in injured subjects is.

4.41.4.2.4 Irradiation and inflammation

Brain irradiation is often used clinically to control tumorous growth within the CNS, especially in children. Palmer and colleagues showed that brain irradiation almost completely abolished cell proliferation within the SGZ, resulting in a lasting decrease in neurogenesis (Monje et al., 2002). Interestingly, the depletion of neurogenesis following irradiation was not entirely due to the radiation effect alone. Rather, the inflammatory response that accompanies irradiation strongly decreases the number of new neurons, and anti-inflammatory treatment augmented neurogenesis in radiated animals (Monje et al., 2003). The detrimental role of inflammation regarding neurogenesis was confirmed by studies showing that the decrease in neurogenesis following endotoxin-induced neuroinflammation was blocked by anti-inflammatory treatment (Hagberg and Mallard, 2005).

4.41.4.2.5 Neurodegenerative diseases and drugs

Clearly, the above-mentioned pathological regulators are not a complete list of disease states that influence the number of newborn neurons. If anything, people have sought unsuccessfully for a disease or pathological state that does not affect adult neurogenesis. There are reports that neurogenesis appears to be disturbed in animal models of Alzheimer's disease and in specimens from human Alzheimer's patients (Jin et al., 2004a,b). However, these findings remain controversial (Donovan et al., 2006). In addition, several other neurodegenerative disorders, such as Huntington's and Parkinson's diseases, have been shown to affect the number of newborn neurons in the dentate area (Abrous et al., 2005). The number of new neurons is also greatly influenced by abuse of substances such as ethanol, opioids, cannabionoids, barbiturates, nicotine, and benzodiazepines (Abrous et al., 2005).

Adult hippocampal neurogenesis is highly responsive and vulnerable to a variety of pathologies affecting the CNS. In most cases, however, the underlying cause or consequence of altered neurogenesis levels in the disease process of many neurological disorders remains unclear and may simply be epiphenomenological. Nevertheless, the finding that neurogenesis in the adult hippocampus is misregulated in many diseases has opened up several new approaches to understanding and ultimately curing human neurological disease.

4.41.5 Function of Neurogenesis

Two theories of hippocampal function have developed in parallel over the past 50 years, and both have been considered in the search for possible roles for neurogenesis. The first, originating in the 1950s from human amnesia studies, is that the hippocampus plays a critical role in the formation of episodic memories. Medial temporal lobe patients – the most famous being H.M. – display a robust, temporally graded retrograde amnesia (Milner et al., 1998). Hippocampal lesion patients, as well as monkeys and rodents, display difficulties in the recall of declarative memories (Squire et al., 2004). The second theory of hippocampal function originated in the 1970s from studies showing that hippocampal neurons in rats have strong place fields (O'Keefe and Conway, 1978; Jung and McNaughton, 1993; Best et al., 2001). This spatial map theory has been very well supported by the use of *in vivo* recordings of hippocampal and entorhinal cortex neurons. Despite these distinct theories, there is increasingly a consensus that the hippocampus has roles in both types of processing.

Although the precise function of granule cell neurogenesis in cognition is still not clear, computational and behavioral studies have led to more sophisticated ideas of what the addition of new neurons would mean to this well-studied circuit. This section describes the progress of both computational approaches and knock-down behavioral experiments.

4.41.5.1 Hippocampal Circuit Function and the Role of the DG

One reason that the hippocampus has been one of the more well-studied regions of the mammalian brain over the past century has been its seemingly simple neural circuitry. Although an oversimplification, the idea of a trisynaptic excitatory circuit (DG → CA3 → CA1) has proven very accessible to both electrophysiology and theoretical work. In particular, the recurrent connectivity of the CA3 region and the presence of strong place fields in CA1 have led to numerous theoretical ideas about the different hippocampal subregions.

Despite the lack of a consensus on the function of the hippocampus as a whole and for its subregions, a general agreement exists that the DG is most probably responsible for separating inputs presented to the hippocampus. This agreement has been based on the anatomy of the DG (Patton and McNaughton, 1995) and on *in vivo* electrophysiology studies (Jung and McNaughton, 1993), behavioral lesion studies (Kesner et al., 2004), and computational models (Rolls and Kesner, 2006). The region receives a very divergent projection from the EC (~200,000 cells project to >1 million granule cells in the rat) while having a very sparse projection onto the CA3. Within the network, granule cells receive strong tonic inhibition from local interneurons, and they are rarely active during behavior. However, when they are active, they have a potent effect on downstream CA3 neurons (Henze et al., 2002). These properties were used, and in some cases even predicted, by computational models describing the computational need for the DG in the production of sparse codes to facilitate memory formation (Rolls and Kesner, 2006).

4.41.5.2 Theoretical Functions of Adult Neurogenesis

Like most theories of hippocampal computation, most neurogenesis theories and network models have focused on a role in memory formation. However, as mentioned before, the DG's presumed role in pattern separation is similar in both mnemonic and spatial processing, suggesting that several of these conclusions are relevant for both general hippocampal functions.

Early theoretical and computational considerations of adult neurogenesis focused on the idea that neurogenesis might be a real-time regulator of memory capacity (Schinder and Gage, 2004). Indeed, several neural network studies demonstrated exactly this. Chambers et al. (2004) and Deisseroth et al. (2004) showed that a simple, three-layer neural network experiencing cell death and neurogenesis is capable of learning more information than a fixed-layer network. Although these models were only conceptual models of hippocampal circuitry and did not explicitly investigate pattern separation, their conclusions are still relevant to this theory of DG function. By replacing neurons in an input layer, they showed that the number of possible states of the output layer increases considerably, indicating increased memory capacity regardless of where the storage occurs.

Becker (2005) was one of the first to consider the effects of neurogenesis in a full hippocampal model and to limit the role of the DG to the encoding phase of learning, a hypothesis that has been the developing consensus in the hippocampal literature (Becker, 2005). The key result of this model was that DG size directly affects the storage capacity of the hippocampus, supporting the idea that neurogenesis facilitates the formation of new memories by permitting more network states. However, contrary to earlier models that involved the DG in the retrieval phase, Becker's model suggests that neural turnover actually reduces possible interference between memory traces in the CA3. The idea that neurogenesis keeps old and new memories separated was examined in detail by the network model of Wiskott et al. (2006). The Wiskott model suggests that interference between memories in the hippocampus is reduced by only permitting new neurons to adapt to new memories, consistent with physiological data showing increased LTP in immature neurons (Wiskott et al., 2006).

Most of these computational theories of neurogenesis have sought a role for new neurons in the framework of functions already considered for the hippocampus. Another view proposes that neurogenesis may have a function that is distinct from those traditionally assigned to the DG. The rationale behind this view is that most current hippocampal theories were derived without regard for adult neurogenesis.

For example, Aimone et al. (2006) described how increased excitability in immature neurons may contribute to the formation of temporal linkages between memories, a function that had not previously been attributed to the DG.

It is likely that any additional functions that neurogenesis contributes (beyond simply improving the DG's function) would be hippocampus dependent as well, just as linking memories in time would still be categorized as a type of declarative memory. However, without the benefit of the existing paradigms used to test memory, such novel functions will likely be difficult to investigate and will present additional challenges to behavioral scientists seeking evidence of a neurogenesis function in rodents. Despite the potential difficulties, it would not be surprising for neurogenesis to have several effects on cognition, as it increasingly appears that the hippocampus serves several functions in the brain.

4.41.5.3 Experimental Evidence for Functional Significance of Adult Neurogenesis

As outlined above, the hippocampus is critically involved in a variety of processes underlying certain forms of learning and memory. New neurons in the dentate area represent only a very small part of the hippocampal structure. As a result, a conclusive answer regarding the function of adult-born neurons is not easy to obtain experimentally. The difficulty in analyzing the functional significance of adult neurogenesis begins in choosing the right test. As mentioned above, the exact function of new neurons might be best appreciated in tests specifically designed to challenge new neurons. Furthermore, there might be species-dependent differences in the use for neurogenesis. Nevertheless, it appears to be mandatory to characterize a potential contribution of new neurons in standard hippocampus-dependent tasks to reach the goal of designing adult neurogenesis–specific tests.

The gold standard of testing hippocampal function remains the Morris water maze (MWM), which is highly sensitive to hippocampal lesions. Many of the behavioral data discussed below are indeed derived from using this test. Recent studies have provided evidence that newborn neurons functionally respond to the acquisition and recall phase during water maze testing (Jessberger and Kempermann, 2003; Kee et al., 2007). However, it is crucial to keep in mind that even the very standardized MWM tasks have an endless list of variations that may utilize newborn neurons differently. The following section will discuss correlational evidence and emerging causal evidence for a role for adult neurogenesis in hippocampal function.

4.41.5.3.1 Correlational evidence

Because the number of neurons born in the adult DG is highly dynamic, an extensive number of studies have been conducted correlating adult neurogenesis with hippocampus-dependent behavior. Kempermann and colleagues used a large set of recombinant inbred (RI) mice and found that the number of new neurons correlates with certain aspects of the MWM (Kempermann and Gage, 2002). Even though the findings using RI mice are still correlational, they are especially valuable because the number of strains analyzed was very large. Reduced latencies for the RI mice to reach the hidden platform in the water maze compared to standard housed control animals were also found when the net number of newborn granule cells was increased with environmental enrichment or physical exercise in animals with identical genetic backgrounds (Kempermann et al., 1997; van Praag et al., 1999a). The same was true when aged animals that show an age-dependent decline in adult neurogenesis were housed in the enriched environment or had access to a running wheel: both numbers of newborn neurons and performance in the Morris water maze were enhanced (Kempermann et al., 2002; van Praag et al., 2005). However, it is obvious that alterations induced by enrichment or physical exercise may not be exclusive to adult neurogenesis. Thus, additional changes within the hippocampal formation that are different from increased neurogenesis may also be partially responsible for the observed behavioral effects.

In addition to these positive correlations, there are also several studies that showed a relationship between low levels of neurogenesis and poor performance in hippocampus-dependent learning tasks. The low levels of neurogenesis in aged animals showing an age-related memory decline correlated well with the performance in the MWM (Zyzak et al., 1995). Furthermore, the age-dependent decline in neurogenesis was also associated with high levels of serum corticosteroids, confirming a potential mechanism explaining how aging may affect neurogenesis (Montaron et al., 2006).

Correlations between neurogenesis and performance in hippocampus-dependent memory tasks were also found in a variety of transgenic mice. The genetic deletion of methyl-CpG binding protein results in low levels of neurogenesis and is also associated with impaired MWM performance (Zhao

et al., 2003). The same is true for NT-3 mutant mice and neuropeptide Y mutant mice, both showing reduced numbers of new neurons in the adult hippocampus and impaired learning and memory (Howell et al., 2005). Using a conditional knock-out of the *presenelin* gene, Tsien and his laboratory found decreased numbers of newborn neurons, whereas the acquisition of hippocampus-dependent learning was normal (Feng et al., 2001). However, the mutant animals showed apparent deficits in the clearance of older memories, suggesting that new neurons might be important not only for the acquisition but also for the clearance of memory traces (Feng et al., 2001).

4.41.5.3.2 'Causal' evidence

The correlative nature of the above-mentioned studies implies several caveats regarding the specificity of the manipulation of neurogenesis or genetic mutations. Therefore, current endeavors in the field aim to specifically knock down hippocampal neurogenesis without affecting other neural structures. To date, there is no widely accepted technical approach that is devoid of any unwanted side effects. This section discusses current strategies as well as the advantages and potential disadvantages of three experimental approaches: cytostatic drugs, irradiation, and molecular knock-downs.

An early study that was designed to reduce neurogenesis and analyze its impact on hippocampus-dependent learning was a report by Shors and colleagues (Shors et al., 2001). In this study, the cytostatic drug toxin methylazoxymethanol acetate (MAM) was used to block cell division and subsequent neurogenesis in the adult rat DG. Rats treated with MAM were found to be impaired in a trace eyeblink-conditioning paradigm that was associated with a decrease in dentate synaptic plasticity. When the treatment was discontinued and neurogenesis was allowed to recover, performance in the trace eyeblink-conditioning was normal. Similar results were obtained when testing the animals in a trace fear-conditioning test, whereas other hippocampal memory tasks, such as the MWM and contextual fear conditioning, appeared to be unaffected by MAM treatment (Shors et al., 2002). MAM was also found to block the beneficial effects of environmental enrichment in an object recognition task (Bruel-Jungerman et al., 2005). Notably, in each of these experiments MAM was injected systemically and potentially resulted in secondary effects on the animals' health that might account for some of the observed behavioral deficits (Dupret et al., 2005). Furthermore, MAM has been shown to interfere with protein synthesis, a crucial requirement for long-term memory (Grab et al., 1979).

Another approach to knock-down cell proliferation has been to use X-ray irradiation, which results in a robust and lasting reduction of neurogenesis in the adult brain (Parent et al., 1999; Monje et al., 2002). Whole-brain irradiation of adult rats left the acquisition phase of the MWM unaffected but impaired the long-term retention (over 14 days after the last training trial) of the platform location, which is suggestive of a functional role of new neurons in spatial memory (Snyder et al., 2005). Furthermore, the performance in a non-matching-to-sample (NMTS) task was impaired when the delays between sample and test trials were relatively long (Winocur et al., 2006). In contrast to MAM-treated animals (Shors et al., 2002), irradiation also led to memory deficits in contextual fear conditioning (Winocur et al., 2006). Studies by the Hen laboratory have selectively targeted the hippocampus instead of using whole-brain irradiation to determine the contribution of newborn neurons in cognitive improvement following environmental enrichment. Irradiated mice showed no impairment in the water maze, and enriched housing was effective at improving the acquisition of the hidden platform location, even in the absence of new neurons (Meshi et al., 2006). These findings indicate that certain aspects of the beneficial effects of enrichment may not depend on adult neurogenesis.

As outlined in an earlier section, adult neurogenesis might be also associated with anxiety and depression, and targeted irradiation of the hippocampus was used to address the relationship between new neurons and antidepressant treatment. Indeed, intact hippocampal neurogenesis appears to be required for the effectiveness of the antidepressant fluoxetine (Santarelli et al., 2003). However, it seems that neurogenesis is not a critical component *per se* in emotional control, because the anxiolytic effects of environmental enrichment were completely unaffected by the ablation of new neurons (Meshi et al., 2006). Despite the robust and complete ablation of new neurons in the adult hippocampus following irradiation, this approach also implies some unwanted side effects, such as ablation of all dividing cells (including glial progenitors outside the dentate gyrus), inflammation, and potential radiation-induced damage of mature neurons.

The existing causal evidence regarding a functional role for new neurons in hippocampal function allows one to speculate that newborn neurons are

required for certain aspects of learning and memory and might also be involved in emotional behavior. However, the available data remain inconclusive. The hope for identifying the functional significance of adult neurogenesis unambiguously lies in the development of specific behavioral tests and new strategies to knock down neurogenesis as specifically and gently as possible. These new strategies will likely include the use of neural progenitor and immature neuron-specific promoters to drive the expression of suicide genes and the virus-mediated inhibition (or enhancement) of adult neurogenesis.

4.41.6 Conclusions

The discovery of ongoing neurogenesis throughout adulthood has undoubtedly challenged our understanding of neuronal development and adult hippocampal function. Even though our understanding of fate instruction, neuronal maturation, and integration is quickly growing, several key questions remain unanswered. From a cellular and molecular standpoint, it will be very important to understand the *in vivo* potency of NSCs and why neurogenesis only occurs in two restricted areas of the adult brain under normal conditions. Furthermore, little is known about which signaling pathways are involved in the extension and pathfinding of axonal and dendritic processes arising from newborn neurons.

Finally, the ultimate challenge will be to truly decode the functional role of adult neurogenesis. Defining that role might not only fundamentally change current concepts regarding hippocampal function but could also help us to understand and eventually improve treatment of human neurological disease.

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4.42 Epigenetics – Chromatin Structure and Rett Syndrome

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4.42.1 Introduction

Much scientific effort over the last three decades has focused on understanding the molecular basis of memory formation. A great deal of progress has been made, and we have a very comprehensive understanding of the molecules and signaling pathways engaged during the early stages of memory formation. Despite the wealth of knowledge that exists about the molecular basis for memory formation, we have an incipient understanding of how a memory persists in the brain for the lifetime of an organism. For instance, what molecules are used to actually store the memory of your first kiss, or your first car accident?

A major question that will drive the next three decades of research into the molecular mechanisms of information storage in the brain is what are the molecular substrates for ‘lifetime’ memory storage? This question is particularly intriguing when one considers that nearly every molecule in the brain has a lifetime that is measured in minutes to hours. It has been estimated that the molecular composition of the brain is renewed every 2 months. How can information persist in the face of constant molecular renewal?

The answer to the question of how information is stored in the nervous system for the lifetime of an organism might not lie in the classic mechanisms identified as important for information storage in the brain thus far. Every cell in a metazoan must ‘remember’ at least one complex piece of information for the lifetime of the organism: cellular phenotype.

Early in development, stem cells are induced to differentiate into phenotypically distinct tissues. Once differentiated, the memory for cellular phenotype must endure in the face of molecular turnover and even mitosis. Is it possible that the nervous system has co-opted this ancient and evolutionarily conserved form of cellular memory for use in lifelong storage of memory?

Cell phenotype is due, in large part, to epigenetic marking of the genome. Recent studies have demonstrated that epigenetic gene regulation has a critical role in neuronal cell fate specification, synaptic development, and synaptic function (reviewed in Hsieh and Gage, 2004, 2005). These epigenetic marks induce lifelong changes in gene expression that reside in the cell and determine its phenotype. Epigenetics and its associated terminology have several different connotations, and specific terms need to be defined before we can discuss them in detail. We define the genome as a complete set of haploid DNA and the functional units that it encodes. In the nucleus, DNA exists as a highly compressed structure known as chromatin. The epigenome is the sum of both chromatin structure and the pattern of DNA methylation, which is the result of an interaction between the genome and the environment. There are three commonly used definitions for the term 'epigenetic' in the literature currently.

The broadest definition of epigenetics includes the transmission and perpetuation of information through meiosis or mitosis that is not based on the sequence of DNA. This process is not restricted to DNA-based transmission and can also be protein-based. This definition is broadly used in the yeast literature, wherein phenotypes that can be inherited by daughter cells are perpetuated past cell division using protein-based mechanisms (Si et al., 2003a,b; Pray, 2004). A second definition of epigenetics has arisen from the field of developmental biology that posits some meiotically and mitotically heritable changes in *gene expression* are not coded in the DNA sequence itself. The altered patterns of gene expression can occur through several mechanisms that are based on DNA, RNA, or proteins (see below) (Egger et al., 2004). A third definition of epigenetics is the mechanism for stable maintenance of gene expression that involves physically 'marking' DNA or its associated proteins, which allow genotypically identical cells to be phenotypically distinct. The molecular and physical basis for this type of change in DNA or chromatin structure is the focus of this review (Rakyan et al., 2001). By this definition, the regulation of chromatin structure is equivalent to epigenetics.

Epigenetics is an attractive candidate molecular mechanism for lifetime storage of memory. DNA and chromatin are relatively constant molecular substrates in neurons and most other cell types. DNA and chromatin are not continually degraded and resynthesized as all other molecular constituents of a neuron are. Thus, the half-life of epigenetic marks is not affected by the lifetime of the molecule. Therefore, epigenetic marks are a more direct reflection of the ongoing state of the neuron, or any other cell (**Figure 1**(**a**)). This positions chromatin as an ideal molecular substrate for long-term signal integration and information storage (**Figure 1**(**a**)).

4.42.2 Mechanisms of Epigenetic Marking

There are several mechanisms whereby epigenetic marks can be placed on chromatin. Each mark has been associated with distinct effects on transcription and chromatin structure. We will briefly review several of the most commonly studied epigenetic marks.

4.42.2.1 Epigenetic Marking of Histones

Histones are highly basic proteins that comprise the major protein component of chromatin. Histones are some of the most evolutionarily conserved proteins but among the most variable with respect to posttranslational modifications, which include acetylation, phosphorylation, methylation, ubiquitination, and sumoylation (reviewed in Thiagalingam et al., 2003). One function of histones is structural; DNA is tightly packaged into chromatin through interactions with histone octamers, referred to as nucleosomes. Interaction between histones and DNA is mediated in part by the N-terminal tail of histone proteins. The primary interface between histone and DNA is believed to be an electrostatic interaction between positively charged lysine residues within the N-terminal tails of histones and the negatively charged sugar–phosphate backbone of DNA. Interestingly, structural studies indicate that the N-terminal tails of histones protrude beyond the chromosomes (Luger et al., 1997), where they could potentially serve as signal integration platforms. Posttranslational modification of

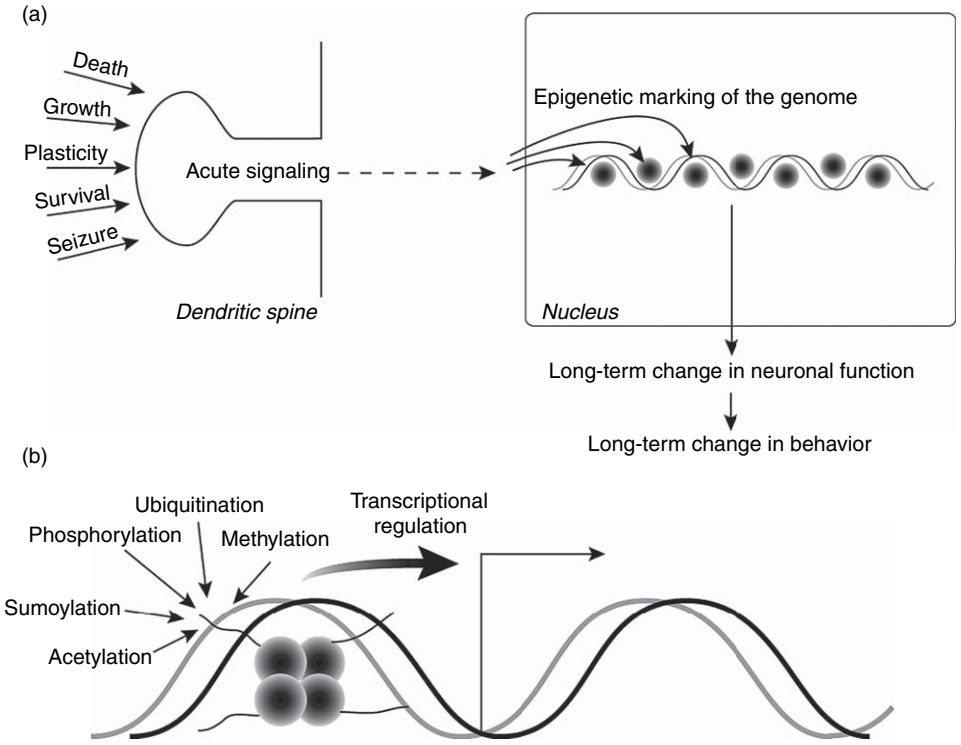


Figure 1 Chromatin as a substrate for signal integration. We propose that epigenetic marking of the genome represents a form of cellular memory utilized by neurons to subserve information storage. (a) Dendritic spines receive several distinct extracellular signals. These signals activate a variety of intracellular signaling cascades that ultimately lead to epigenetic marking of the genome. The half-life of these epigenetic marks is much longer than traditional posttranslational modifications, and so their effects on gene expression persist for much longer periods of time. Therefore, as a consequence of epigenetically modifying the genome, neuronal function and ultimately behavior of the animal are modified for very long periods of time. (b) Histone tails and cytosine residues of DNA can receive a variety of distinct epigenetic marks. Each mark has a different effect on transcription. The functional consequence of epigenetically marking the genome is ultimately precise modulation of transcription.

histones, either on their N-terminal tail or within the globular domain (van Leeuwen et al., 2002), represents one mechanism for epigenetic marking of the genome. Current hypotheses posit that post-translational modifications along the N-terminal tails are combined in a 'histone code' that ultimately directs the activity of numerous transcription factors, cofactors, and the transcriptional machinery in general (Strahl and Allis, 2000). The histone code is the specific pattern of posttranslational modifications of a given histone octamer in chromatin (**Figure 1(b)**). This code, or pattern, is read out as an influence on the specific level of expression of the associated gene(s). We have chosen to focus on N-terminal–directed posttranslation modifications because of the rapidly growing literature describing these different modifications, their associated effects on transcription, and their interdependence.

4.42.2.2 Histone Acetylation

Several specific sites of posttranslational modification exist within the N-terminal tails of histone proteins, and modifications of these sites can modulate the overall structure of chromatin. Currently, five post-translational modifications of histone tails have been characterized: acetylation, methylation, ubiquitination, sumoylation, and phosphorylation. Acetylation is the best characterized of the posttranslational modifications on histones to date. Acetylation of histones occurs on lysine residues, effectively neutralizing their positive charge and interfering with the electrostatic interaction between histone and nucleotide. Acetylation is catalyzed by histone acetyltransferases (HATs), which transfer an acetyl group from acetyl-CoA to the ε-NH^+ group of a lysine residue within a histone (Tanner et al., 1999, 2000a,b; Lau et al., 2000). Histone acetylation is reversed via the action of histone deacetylases (HDACs).

4.42.2.3 Histone Methylation

Histone methylation is another histone-directed epigenetic tag. Like acetylation, methylation of histones occurs on ε-NH^+ groups of lysine residues, and it is mediated by histone methyltransferases (HMTs). Unlike acetylation, lysines can accept up to three methyl groups, and methylation does not affect the positive charge of lysine residues. Thus, methylation of histones on lysine residues does not interfere with the electrostatic histone–DNA interaction. Methylation of histones can also occur on arginine residues, catalyzed by protein arginine methyltransferases (PRMTs). Arginines can be either mono- or dimethylated on their guanidine nitrogen.

4.42.2.4 Histone Ubiquitination

Ubiquitination of histones has only recently begun to be characterized in detail. Ubiquitin, a protein with 76 amino acids that is named for its ubiquitous distribution in all cell types and high degree of conservation across species, is a posttranslational modification usually used as a signal for degradation by the proteasome when substrates are polyubiquitinated (Pickart, 2001). Interestingly, most histones appear to be monoubiquitinated (reviewed in Hicke, 2001). Monoubiquitination has been shown to be involved in a range of cellular functions distinct from proteasome-dependent proteolysis (reviewed in Pickart, 2001; Murphey and Godenschwege, 2002). Histones, like other proteins, are ubiquitinated through attachment of a ubiquitin to the ε-NH^+ group of a lysine (Nickel and Davie, 1989). Ubiquitination of histones H2A, H2B, H3, and H1 has been observed (Goldknopf et al., 1975; West and Bonner, 1980; Chen et al., 1998; Pham and Sauer, 2000). Although sites of ubiquitination have been identified on histones, the function of histone ubiquitination remains unclear.

4.42.2.5 Histone Sumoylation

There are several ubiquitin-like posttranslational modifications that proteins can undergo. One example is the small ubiquitin-related modifier (SUMO) (Melchior, 2000; Hay, 2001; Johnson and Gupta, 2001). SUMO modifications are attached to proteins in a manner similar to the mechanisms involved in ubiquitination. Although SUMO is related to ubiquitination, sumoylation of proteins does not result in their degradation, suggesting that sumoylation of proteins has relevance in other signaling cascades. Initial studies suggested that histone H4 can be sumoylated, and that sumoylation of histone H4 is associated with transcriptional repression (Shiio and Eisenman, 2003). In yeast, the SUMO-conjugating enzyme Hus5 is highly enriched in heterochromatic regions, and knockout of SUMO results in loss of heterochromatic silencing, providing direct evidence that, at least in simple eukaryotes, SUMO modification of chromatin results in transcriptional silencing (Shin et al., 2005).

4.42.2.6 Histone Phosphorylation

Phosphorylation of histones H1 and H3 was first discovered in the context of chromosome condensation during mitosis (Bradbury et al., 1973; Gurley et al., 1974). Phosphorylation of histone H3 has since been associated with activation of mitogenic signaling pathways (Mahadevan et al., 1991). Phosphorylation of serine 10 on H3 is mediated by Rsk2, Msk1, and the aurora kinase family member Ipl1 (Sassone-Corsi et al., 1999; Thomson et al., 1999; Hsu et al., 2000; Di Agostino et al., 2002). Recent evidence also implicates aurora kinases in the phosphorylation of serine 28 in histone H3 (Goto et al., 2002). As with other proteins, phosphatases are responsible for catalyzing the removal of phosphate groups from histones (Mahadevan et al., 1991; Ajiro et al., 1996). To date, the phosphatases PP1 and PP2A have been shown to regulate levels of phosphorylation on H3 (Hsu et al., 2000; Nowak et al., 2003).

4.42.2.7 Other Histone Modifications

While a great deal of attention has been given to the N-termini of the histones, there is increasing evidence suggesting that other histone regions are targeted for modulation. For example, dot1p has been shown to methylate histone H3 on lysine 79, a residue that lies within the globular domain (van Leeuwen et al., 2002). Additionally, higher-order folding of chromatin is also undoubtedly involved in the regulation of gene expression. In this regard, there is increasing evidence that the linker histone H1 plays a role in modulation of chromatin structure (Brown, 2003).

4.42.2.8 DNA (Cytosine-5) Methylation

In addition to posttranslational modification of histones, DNA itself can be directly modified via methylation. Methylation of DNA is catalyzed by a

class of enzymes known as DNA (cytosine-5) methyltransferases (DNMTs) (Bestor et al., 1988; Yen et al., 1992; Okano et al., 1998; Lyko et al., 1999; Okano et al., 1999). DNMTs transfer methyl groups to cytosine (C) residues within a continuous stretch of DNA, specifically at the 5-position of the pyrimidine ring (Chen et al., 1991). Not all cytosines can be methylated; usually cytosines must be immediately followed by a guanine (G) to be methylated (Bird, 1978; Cedar et al., 1979). These 'CpG' dinucleotide sequences are highly underrepresented in the genome relative to what would be predicted by random chance; however, about 70% of the existing CpG dinucleotides are methylated (Cooper and Krawczak, 1989). The rest of the normally unmethylated CpG dinucleotides occur in small clusters, known as 'CpG islands' (Bird, 1986).

DNA methylation leads to marked changes in the structure of chromatin that ultimately result in significant downregulation of transcription. It can directly interfere with the ability of transcription factors to bind to regulatory elements. The transcription factor Ets-1 and the boundary element CCCTC binding factor (CTCF) can efficiently bind to nonmethylated, but not methylated DNA (Bell and Felsenfeld, 2000; Maier et al., 2003). Moreover, several proteins recognize and bind to methylated CpG residues independent of DNA sequence. The five proteins that are known to bind to methylated CpGs are MeCP2, MBD1, MBD2, MBD4, and Kaiso (Hendrich and Bird, 1998; Prokhortchouk et al., 2001). These proteins mediate transcriptional repression by recruiting chromatin remodeling enzymes. For example, MeCP2 directly associates with the transcriptional corepressor Sin3A and HDAC (Jones et al., 1998; Nan et al., 1998).

4.42.2.9 Epigenetic Modulation of Transcription

Several hypotheses have been put forth to explain how the many modifications made on histone proteins are interpreted to extend the information potential of the genetic (DNA) code. The most popular of these hypotheses is the histone code initially described by B. M. Turner (1993) and more recently elaborated and popularized by C. D. Allis and colleagues (Strahl and Allis, 2000; Jenuwein and Allis, 2001). Distinct histone posttranslational modifications can generate synergistic or antagonistic interaction sites for transcription factors and associated chromatin remodeling enzyme complexes. The combinatorial nature of these histone modifications may be thought of as a histone code that extends the information potential of the genetic code (Jenuwein and Allis, 2001). However, a very different hypothesis suggests that it is not combinatorial complexity, but rather a simple net charge effect. Genetic experiments, microarray analysis, and hierarchical cluster analysis were used to demonstrate that the interchangeability of acetylation sites on histone H4 provides support for a charge neutralization model (Dion et al., 2005; reviewed in Henikoff, 2005). Lastly, histone modifications have been related to signal-transducing modifications, and histone modifications provide switch-like properties and ensure robustness of the signal (Schreiber and Bernstein, 2002). This idea has been termed the signaling network model. These three hypotheses for how histone modifications direct transcriptional activation are not mutually exclusive, and thus, it will be interesting to see how future studies into chromatin regulation and information storage regulate cell fate, cellular memory, and as we propose below, long-term memory storage at the behavioral level.

4.42.3 Epigenetic Mechanisms in Synaptic Plasticity

Synaptic plasticity, defined as the activity-dependent change in synaptic strength, is currently viewed as a widely accepted hypothesis to explain the underlying cellular mechanisms of memory processes. The discovery of two forms of synaptic plasticity, long-term facilitation in *Aplysia,* where brief exposure to the neuromodulator serotonin induces a persistent enhancement of neurotransmitter release (Kandel and Tauc, 1964; Brunelli et al., 1976), and long-term potentiation (LTP), a form of synaptic plasticity whereby high-frequency stimulation of a neuronal pathway induces long-lasting increases in synaptic efficacy (*See* Chapter 4.16) (Bliss and Lomo, 1973), sparked decades of research into the role of synaptic plasticity in learning and memory. A recent review by Martin and Morris (2002) elegantly discusses the validity of synaptic plasticity as a cellular mechanism for memory formation. In the previous section of this article, we introduced the intriguing idea that epigenetic regulation of transcription may provide a mechanism for cellular memory in a neuron. This cellular memory may be involved in maintaining the long-lasting neuronal changes required for long-term synaptic plasticity changes. In this next section, we

discuss the recent evidence that epigenetic regulation of transcription may be required for certain forms of long-lasting synaptic plasticity.

4.42.3.1 Transcription and Chromatin Structure

Transcriptional activation is required for certain long-lasting forms of synaptic plasticity and memory storage (Nguyen et al., 1994; Korzus, 2003). Activation of transcription factors in neurons can occur via several mechanisms. In relation to activity-dependent synaptic plasticity and memory formation, it is generally accepted that increased neuronal activity engages a wide variety of intracellular signaling cascades including PKC, PKA, Ca^{++}, PI3-K, and ERK (*See* Chapters 4.23, 4.25) (Adams and Sweatt, 2002). Once engaged, these signaling pathways activate several distinct transcription factors, ultimately leading to the expression of gene products required for maintenance of synaptic plasticity and consolidation of long-term memory. For many actively transcribed genes, it is necessary to overcome the repressive chromatin structure surrounding the gene, especially in the promoter region. Genomic DNA is tightly packaged into chromatin, the major protein component of which is composed of nucleosomes that are octamers of core histone proteins. DNA is wrapped around the nucleosomes (~146 bp) and is then subjected to higher-order folding, resulting in a chromatin structure that is highly inhibitory to transcription. As described earlier, histone acetylation has been shown to alter chromatin structure (Norton et al., 1989), which can result in increased accessibility for transcriptional regulatory proteins to chromatin templates (Vettese-Dadey et al., 1996). The identification of transcriptional cofactors with HAT and HDAC activity has strengthened the relationship between histone acetylation and gene expression. Recently, several studies examining the role of HAT and HDAC enzymes in long-term synaptic plasticity demonstrated that there is a correlation between changes in synaptic plasticity and corresponding modifications of chromatin structure.

4.42.3.2 Chromatin Remodeling Enzymes and Synaptic Plasticity

One of the first demonstrations that chromatin remodeling enzymes are involved in synaptic plasticity came from a study in *Aplysia*. Several different forms of transcription-dependent synaptic plasticity have been identified in *Aplysia*, including long-term facilitation (LTF, characterized by enhanced synaptic transmission) and long-term depression (LTD, characterized by decreased synaptic transmission) (Montarolo et al., 1986, 1988; Hammer et al., 1989; Dale and Kandel, 1990). To understand the changes in chromatin structure that affect transcription required for LTF and LTD, Guan et al. (2002) examined the chromatin surrounding the promoter of ApC/EBP. ApC/EBP is an immediate early response gene critical for induction of LTF (Alberini et al., 1994) and is a downstream target of the transcription factor ApCREB1. Guan et al. (2002) showed that induction of LTF by treatment with serotonin activates ApCREB1, which recruits the transcriptional coactivator CREB binding protein (CBP, a potent HAT) to the ApC/EBP promoter (i.e., **Figure 2(a)**). Recruitment of cAMP response element binding protein (CREB) and CBP correlates with increased histone H4 acetylation and ApC/EBP expression (Guan et al., 2002). In contrast, induction of LTD by treatment with neuropeptide FMRFamide resulted in the recruitment of ApCREB2, an inhibitory form ApCREB that lacks a transcription activation domain, and an HDAC5-like molecule to the ApC/EBP promoter decreasing histone H4 acetylation and ApC/EBP expression (Guan et al., 2002). These two results suggest that, in *Aplysia* sensory neurons, the genome serves as a substrate for signal integration upon which long-term neuronal state is encoded. In support of this hypothesis, increasing levels of histone acetylation with the HDAC inhibitor trichostatin A (TSA) and mimicking the epigenetic state of the genome normally seen after induction of LTF transforms short-term synaptic facilitation into LTF (Guan et al., 2002). These results demonstrate that enzymes such as HATs and HDACs are actively involved in regulating chromatin structure at specific gene promoters relevant for induction of long-term synaptic plasticity.

Recently, several studies have demonstrated that CBP is involved in specific forms of hippocampal synaptic plasticity and hippocampus-dependent long-term memory formation (**Figure 2(a)**) (Oike et al., 1999; Bourtchouladze et al., 2003; Alarcon et al., 2004; Korzus et al., 2004; reviewed in Josselyn, 2005; Wood et al., 2005, 2006). These six studies utilized five different *cbp* genetically modified mice in which the activity of CBP was specifically impaired to investigate the role of CBP in synaptic plasticity and memory storage. With respect to synaptic

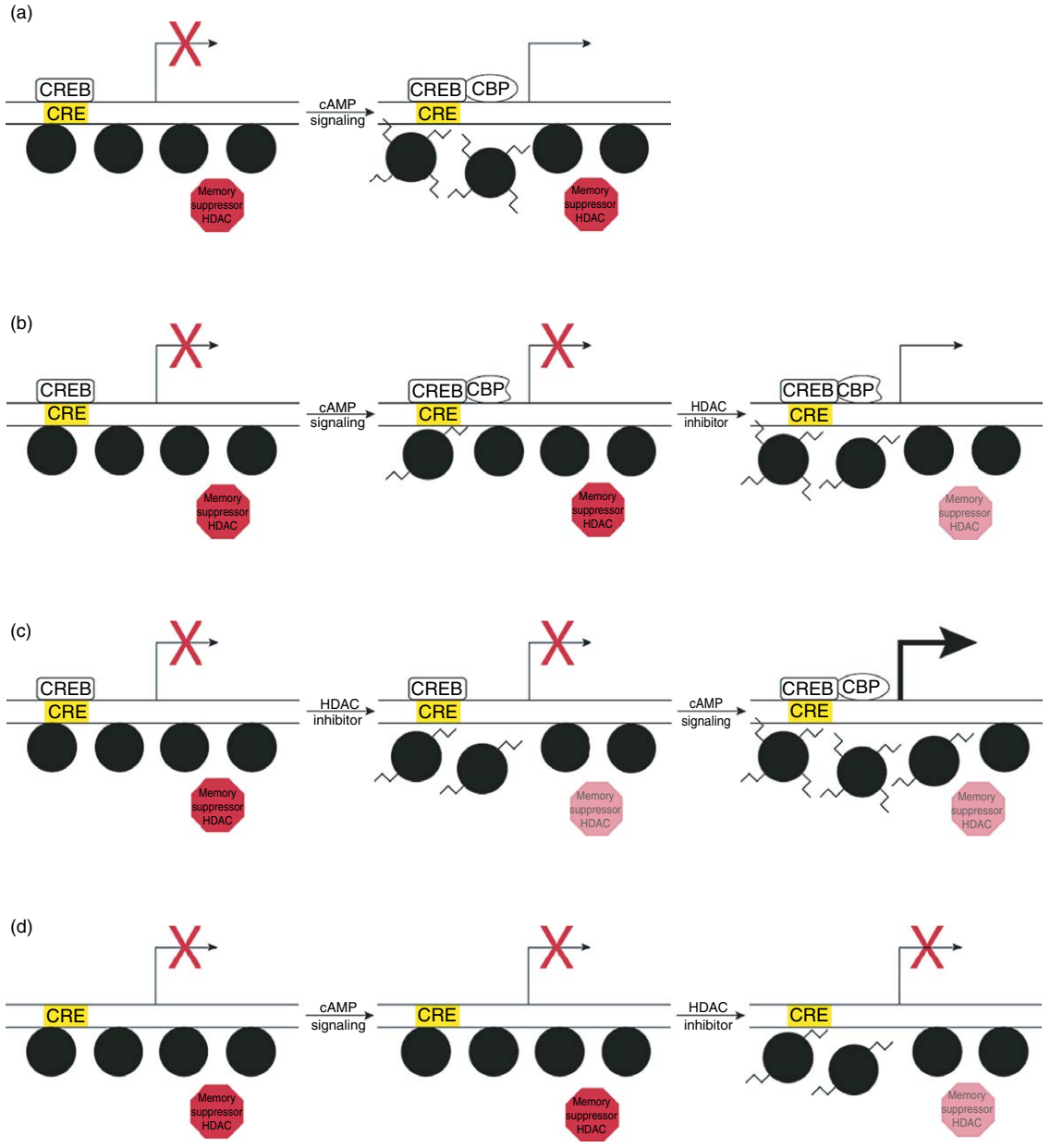


Figure 2 Epigenetics in synaptic plasticity and memory formation. Long-term forms of synaptic plasticity and memory require transcription for consolidation and maintenance. The diagrams indicate many aspects where epigenetics are involved in synaptic plasticity and long-term memory formation. (a) Induction of synaptic plasticity or formation of long-term memory requires the transcription factor CREB and the coactivator CBP. CBP is a HAT that acetylates histones and facilitates transcription of consolidation-associated genes. (b) Loss of CBP function, either through truncation mutations that eliminate the HAT domain or through haploinsufficiency, prevents CREB-mediated transcription of consolidation-associated genes. Addition of an HDAC inhibitor ameliorates the inability to induce long-term synaptic plasticity or form long-term memory. (c) Administration of HDAC inhibitors alone does not affect the expression of memory-associated genes. However, HDAC inhibitor preadministration facilitates the induction of synaptic plasticity and long-term memory. (d) Loss of CREB function, such as in CREB $\alpha\Delta$ mice, prevents induction of synaptic plasticity or formation of long-term memory. Administration of HDAC inhibitors does not restore plasticity or memory formation in these mice.

plasticity, $cbp^{+/-}$ heterozygous mice that lack one allele of *cbp* were found to have impairments in long-lasting LTP (L-LTP, a form of synaptic plasticity requiring transcription and translation), but normal early-phase LTP (E-LTP, a form of synaptic plasticity that is independent of transcription; *See* Chapter 4.16) (Alarcon et al., 2004). Similarly, transgenic mice expressing an inhibitory truncation mutant of CBP in forebrain neurons also exhibited deficits in L-LTP, with normal E-LTP (**Figure 2(b)**) (Wood et al., 2005). To overcome the deficits in histone acetylation and synaptic plasticity, mutant *cbp* mice were treated with HDAC inhibitors to induce a hyperacetylated genomic state. HDAC inhibition by suberoylanilide hydroxamic acid (SAHA) ameliorated impairments in hippocampal LTP in $cbp^{+/-}$ heterozygous mice (**Figure 2(b)**) (Alarcon et al., 2004). These results demonstrate that synaptic plasticity deficits in *cbp* mutant mice may be overcome by increasing histone acetylation via HDAC inhibition. However, the effect of modifying chromatin structure on synaptic plasticity and memory storage was not specifically addressed by these studies.

4.42.3.3 Histone Acetylation and Synaptic Plasticity

To directly examine the effect of increasing histone acetylation on synaptic plasticity, a recent study showed that the HDAC inhibitors TSA and sodium butyrate enhance LTP at Schaffer-collateral synapses in area CA1 of the hippocampus (**Figure 2(c)**) (Levenson et al., 2004b). Further, the authors demonstrated that the HDAC inhibitor–enhanced LTP is dependent on transcription. A similar result was observed in a study examining the effects of HDAC inhibition on E-LTP in the CA1 region of hippocampal slices from C57BL/6 mice. In this study, synaptic efficacy at both the collateral and commissural pathways was monitored simultaneously. Both pathways received baseline stimulation, but LTP was only induced in the collateral pathway, with the commissural fibers serving as a control. TSA had no effect on baseline responses in the commissural pathway as compared to vehicle, similar to other observations in TSA-treated slices (Alarcon et al., 2004; Levenson et al., 2004b). However, in the collateral pathways where E-LTP was induced, treatment with TSA converted transcription-independent E-LTP to L-LTP (Vecsey et al., in press). Other studies in the amygdala have shown that TSA can enhance forskolin-induced LTP at the sensory input synapses (**Figure 2(c)**) (Yeh et al., 2004). Together, these studies demonstrate that increasing histone acetylation via HDAC inhibitors can enhance synaptic plasticity in the hippocampus and amygdala.

What is the molecular mechanism whereby HDAC inhibitors enhance synaptic plasticity? A clue to answering this question came from examining the molecular mechanism underlying TSA-dependent enhancement of E-LTP. This form of E-LTP is independent of transcription, translation, protein kinase A (PKA), and CREB (reviewed in Nguyen and Woo, 2003; Pittenger and Kandel, 2003). This allowed Vecsey et al. (in press) to specifically examine whether the TSA-dependent enhancements in E-LTP were dependent on CREB function. CREB is thought to activate transcription through the coactivator CBP and its associated HAT activity. In addition, the significant phenotypic overlap between memory and synaptic plasticity deficits exhibited by transgenic mice expressing dominant negative inhibitors of CREB or CBP suggests that they function together during memory processes (Pittenger et al., 2002; Wood et al., 2005). Mice with targeted deletions of two *creb* isoforms (α and Δ; CREB $\alpha\Delta$ mice) on a defined F1 hybrid genetic background of C57BL/6 and 129/SvEvTac strains were used to investigate the role of CREB in TSA-dependent enhancement of E-LTP (Walters and Blendy, 2001; Graves et al., 2002). Hippocampal slices from CREB $\alpha\Delta$ mice did not exhibit the TSA-dependent enhancement of E-LTP observed in wild-type littermates, suggesting that a CREB-dependent process is involved in regulating the transcription of genes sensitive to histone hyperacetylation required for TSA-dependent enhancement of E-LTP. Further, genetically modified *cbp* mutant mice carrying a triple point mutation in the CREB-binding (KIX) domain of CBP ($CBP^{KIX/KIX}$ knockin mice) failed to exhibit TSA-dependent enhancement of E-LTP as well, specifically implicating the CREB:CBP complex in enhancement of LTP by HDAC inhibitors (Vecsey et al., in press). Together with the observations made concerning the effects of HDAC inhibitors on CBP-deficient and normal animals (Korzus et al., 2004; Levenson et al., 2004b; Yeh et al., 2004; Vecsey et al., in press), these results also suggest that HDAC inhibitors reduce the signaling threshold required for engagement of CREB-dependent maintenance of plasticity, but that these treatments by themselves do not induce expression of CRE-containing genes.

4.42.3.4 Histone Acetylation and Seizure

As reviewed at the beginning of this section, different patterns of synaptic activity can lead to differential

regulation of gene expression and ultimately a change in synaptic efficacy. A particularly dramatic example is seizure, which is a widespread burst of abnormal excitatory synaptic activity in the central nervous system (CNS). Seizure induces many changes in gene expression in the nervous system, which can lead to the development of chronic epilepsy and/or neurodegeneration. Early studies revealed that transcription of the immediate early gene *c-Fos* was upregulated in many regions of the brain after seizure (Morgan et al., 1987). Electron microscopy studies showed that c-Fos protein is preferentially localized to the euchromatic regions of chromosomes, which suggests that part of the transcriptional response to seizure involves changes in chromatin structure (Mugnaini et al., 1989).

Other studies have shown that expression of the glutamate receptor 2 (GluR2) alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor subunit and brain-derived neurotrophic factor (BDNF) are also regulated by seizure (Ernfors et al., 1991; Isackson et al., 1991; Timmusk et al., 1993; Kokaia et al., 1995; Nibuya et al., 1995; Binder et al., 1999; Grooms et al., 2000; Sanchez et al., 2001). Expression of GluR2 mRNA is downregulated by seizure, whereas expression of BDNF mRNA is upregulated (Ernfors et al., 1991; Timmusk et al., 1993; Nibuya et al., 1995; Grooms et al., 2000). If seizure-induced changes in gene expression are due to changes in chromatin structure, then changes in chromatin structure should occur around the genes that are regulated by seizure. Recent studies have used the chromatin immunoprecipitation (ChIP) approach to monitor posttranslational modifications of histones that are located in the promoters of the BDNF and GluR2 genes. The first study to characterize the epigenetic regulation of GluR2 and BDNF demonstrated that pilocarpine-induced seizure significantly decreased acetylation of histone H4 in the GluR2 promoter, whereas acetylation of H4 in the P2 promoter of BDNF was significantly increased (Huang et al., 2002). The GluR2 promoter contains a repressor element-1 (RE1)-like silencer element in its promoter, and a recent study demonstrated that treatment of cultured neurons with kainic acid, an *in vitro* model for induction of seizures, resulted in increased binding of the transcriptional corepressor REST (RE1 silencer transcription factor), suggesting one possible mechanism for the downregulation of histone H4 acetylation (Jia et al., 2006). In another line of experiments, Tsankova et al. (2004) investigated changes in chromatin structure after electroconvulsive seizures (ECS), a form of human antidepressant therapy. ECS increased the acetylation of H4 at the P2 promoter of BDNF, and acetylation and phosphoacetylation of H3 were regulated within the P2 and P3 promoters of BDNF (Tsankova et al., 2004). These results indicate that ECS induces complex regulation of the epigenetic state of the BDNF promoters. ECS also had significant effects on the acetylation of H4 and phosphoacetylation of H3 within the *c-Fos* promoter and on the acetylation of H3 and H4 in the CREB promoter (Tsankova et al., 2004). Together, these data indicate that the synaptic activity and/or action potential firing that occurs during seizure results in complex regulation of the epigenetic state of chromatin.

4.42.3.5 Epigenetics in Plasticity and Seizure – Conclusions

Observations made regarding the role of epigenetics in plasticity and seizure strongly support a role for chromatin remodeling enzymes and histone acetylation in activity-dependent changes in synaptic and neuronal function. Moreover, all of these results support the hypothesis that epigenetic changes in chromatin structure are the basis of a cellular form of memory that influences neuronal state. Further examination of additional chromatin remodeling enzymes, as well as the analysis of additional histone modifications and their coregulation, will undoubtedly expand our understanding of the transcriptional profiles regulated by chromatin structure that are involved in long-term memory processes.

4.42.4 Epigenetics in Memory Formation

There are numerous studies indicating that many of the molecules necessary for induction of LTP are also required for normal long-term memory storage (reviewed by Malenka, 2003; Lynch, 2004). In light of the relationship between LTP and long-term memory formation, we next review the evidence associating epigenetic changes in chromatin structure with long-term memory storage. We will focus on the chromatin remodeling enzymes that appear to be involved, the specific chromatin modifications that correlate with memory storage, and what chromatin remodeling may provide in terms of mechanistic advantage for long-term memory storage processes.

4.42.4.1 Chromatin Remodeling Enzymes and Memory Storage

Rubinstein-Taybi syndrome (RTS), a human disease characterized by developmental abnormalities and mental retardation, is caused by translocations, deletions, and point mutations of the *cbp* gene (Petrij et al., 1995; Coupry et al., 2004). As discussed above, CREB and CBP are critical for successful induction of synaptic plasticity in species ranging from flies and slugs to rodents. Considering the role of CREB in learning, memory, and synaptic plasticity that has emerged from studies in *Aplysia, Drosophila,* and mice (Silva et al., 1998), it was reasonable to hypothesize that the cognitive impairments observed in RTS patients may be due directly to alterations in CBP activity in the brain. In a screen designed to identify genes involved in developmental regulation in mice, Oike et al. (1999) isolated a truncation mutation of CBP that lacks the HAT domain. These mice, which have one wild-type allele of *cbp* and one truncated allele, phenocopied many aspects of RTS quite well, with mice even exhibiting memory impairments (Oike et al., 1999). More recently, these animals were used in a study by Bourtchouladze et al. (2003) in which mice possessing the truncated CBP exhibited long-term memory deficits but normal learning and short-term memory in an object-recognition task (Bourtchouladze et al., 2003). Interestingly, inhibitors of phosphodiesterase 4, which enhance CREB-dependent gene expression, ameliorate memory deficits for object recognition in a dose-dependent manner in the mice containing the truncated form of CBP (Bourtchouladze et al., 2003). This suggests that pharmacologically enhancing cAMP-mediated signaling can compensate for the disruptions caused by the inhibitory truncated-CBP mutant expressed in these mice. Together, these results suggest that CBP, a potent HAT and chromatin remodeling enzyme, is involved in formation of long-term memory. However, this interpretation must be viewed with the caveat that the mice generated by Oike et al. (1999) have severe growth and developmental abnormalities, suggesting the alternative hypothesis that the memory impairments in these CBP-deficient mice are secondary to a neurodevelopmental abnormality.

To more directly study the effect of altering CBP activity on memory storage, two recent studies generated mice carrying spatially and temporally restricted expression of CBP mutants. Korzus et al. (2004) generated conditional transgenic mice expressing a HAT-deficient CBP transgene. The CaMKIIα promoter was used to spatially restrict transgene expression to forebrain neurons (Mayford et al., 1996) and the tetracycline system to temporally restrict transgene expression. These mice exhibited impaired memory for spatial and object recognition tasks that were ameliorated by intraperitoneal administration of the HDAC inhibitor TSA (Korzus et al., 2004). Thus, inducing a hyperacetylated histone state was able to compensate for the lack of HAT activity in these HAT-deficient CBP transgenic mice (i.e., **Figure 2**(**b**)). In a second study, Wood et al. (2005) generated transgenic mice expressing an inhibitory truncation mutant of CBP that contains the CREB-binding domain but lacks the HAT domain. The CaMKIIα promoter was used to spatially restrict transgene expression to forebrain neurons (Mayford et al., 1996), and this promoter does not activate transcription until 10–21 days postpartum (Kojima et al., 1997). These mice exhibited long-term memory deficits when assessed with hippocampus-dependent tasks such as contextual fear conditioning and the spatial water maze, but not in amygdala-dependent cued-fear conditioning (Wood et al., 2005). Together, these results demonstrate that more spatially restricted and temporally restricted alterations of CBP activity affect long-term memory storage (**Figure 2**(**b**)). Further, Korzus et al. (2004) were able to ameliorate long-term memory deficits by increasing histone acetylation using HDAC inhibitors. Similarly, HDAC inhibitors ameliorate long-term memory deficits for contextual fear in CBP heterozygous mice (non-regionally restricted deletion of one endogenous *cbp* allele) that exhibit long-term memory deficits for contextual fear and object recognition (Alarcon et al., 2004). Together, these studies demonstrate that brain-specific reduction of CBP activity results in long-term memory impairments and that these impairments may be ameliorated by increasing histone acetylation in general via administration of HDAC inhibitors (**Figure 2**(**b**)).

4.42.4.2 Histone Acetylation and Memory Storage

If a reduction of CBP activity results in memory deficits, and increasing histone acetylation via HDAC inhibitors can overcome these memory deficits, then an intriguing hypothesis arises suggesting that increasing histone acetylation might be a mechanism to enhance cognitive ability in general. Even more fundamental is the question: how do

specific forms of memory formation affect histone acetylation? And lastly, what signaling cascades are involved in the transduction of information from the synapse to chromatin? Levenson et al. (2004b) addressed these questions in a set of experiments that were the first to demonstrate that chromatin structure modifications are involved in long-term memory formation. First, the authors examined histone acetylation in hippocampal area CA1 of rats subjected to contextual fear conditioning or latent inhibition (when an animal is preexposed to a novel context before receiving the shock in that context). They found that contextual fear conditioning correlated with increased acetylation of histone H3, whereas latent inhibition correlated with increased acetylation of histone H4 (Levenson et al., 2004b). These results demonstrate that differential regulation of hippocampal histone acetylation may be induced by specific forms of memory formation. Second, after demonstrating that fear conditioning correlates with increases in histone acetylation, the authors continued by examining the signaling cascades involved in histone acetylation changes. Using acute hippocampal slice preps, they found that *N*-methyl-D-aspartate receptor (NMDAR)-dependent synaptic transmission and signaling via the ERK-MAPK pathway were essential for acetylation of histone H3, but not acetylation of histone H4 (Levenson et al., 2004b). These results suggest that signaling pathways involved in the acetylation of H3 are different from those required for the acetylation of H4. Third, the authors showed that rats treated with HDAC inhibitors via intraperitoneal injection exhibited enhanced long-term memory for contextual fear conditioning when tested 24 h postconditioning, but normal short-term memory when tested 1 h postconditioning (**Figure 2**(**c**)) (Levenson et al., 2004b). Importantly, control experiments demonstrated that the HDAC inhibitors did not have an indirect effect on performance (Levenson et al., 2004b). Together, the study by Levenson et al. (2004b) demonstrated that changes in chromatin structure correlated with long-term memory formation and activation of second messenger signaling pathways that are known to be involved in memory processes.

In a similar study, Vecsey et al. (in press) found that delivering HDAC inhibitors directly to the hippocampus in mice via intrahippocampal cannulae enhanced long-term memory for contextual fear conditioning (**Figure 2**(**c**)). Interestingly, the authors found that delivery of the HDAC inhibitors during memory consolidation, but not during retrieval, enhanced memory. These results suggest that increasing histone acetylation during the consolidation stage of memory is involved in memory enhancement, a stage of memory formation that has been shown to be dependent on transcription (Abel and Lattal, 2001). The authors investigated the underlying molecular mechanism for HDAC inhibitor–dependent enhanced memory formation by examining the effect of HDAC inhibitors in CREB $\alpha\Delta$ knockout mice. These mice carry a targeted deletion of the α and Δ isoforms of CREB, the two most abundant isoforms of CREB (Hummler et al., 1994). CREB $\alpha\Delta$ knockout mice failed to exhibit enhanced memory for contextual fear after intrahippocampal administration of an HDAC inhibitor. These results suggest that the TSA-enhanced memory for contextual fear conditioning requires the transcription factor CREB (**Figure 2**(**d**)). As mentioned earlier in the synaptic plasticity section, the authors also demonstrated that HDAC inhibitor–dependent enhancement of LTP required CREB as well. Finally, the authors showed using quantitative real-time reverse transcriptase polymerase chain reaction that only a subset of CREB-target genes is affected by HDAC inhibition following contextual fear conditioning. Thus, CREB:CBP-mediated transcription appears to be essential for HDAC inhibitor enhanced memory and synaptic plasticity, which seems reasonable considering that CREB is a transcription factor that recruits CBP, a potent HAT, for transcriptional activation of memory-associated target genes.

4.42.4.3 Factor Acetylation and Memory Storage

Acetylation is a posttranslational modification that can occur on lysine residues in general. Therefore, acetylation can occur on non-histone (factor) proteins. For example, the histone acetyltransferase CBP acetylates several factors including the transcription factors p53, CREB, and NF-κB (Gu and Roeder, 1997; Furia et al., 2002; Kiernan et al., 2003; Yeh et al., 2004; Hassa et al., 2005). Thus, although HDAC inhibitor–dependent enhancement of memory and synaptic plasticity most likely occurs through histone acetylation that directly facilitates transcriptional activation, it is possible that this process may also be regulated by factor acetylation. For example, p65 (an NF-κB subunit also known as Rel-A) acetylation increases in the amygdala of rats subject to a fear-potentiated startle paradigm (Yeh et al., 2004). Increases in p65 acetylation correlate with increased

CBP interaction, whereas decreases in p65 acetylation correlate with increased HDAC3 interaction (Yeh et al., 2004). Interestingly, the HDAC inhibitor TSA blocks the interaction between HDAC3 and p65, suggesting that TSA may be able to increase NF-κB-mediated transcription in a manner that does not involve histone acetylation. Finally, the authors demonstrated that TSA injections directly to the amygdala enhanced fear-potentiated startle (Yeh et al., 2004). These results open the possibility that factor acetylation may also be involved in the mechanism by which HDAC inhibitors enhance memory storage. This idea is further supported by studies showing that the HDAC inhibitor TSA disrupts interactions between CREB and HDAC1/PP1, and Akt and HDAC1&6/PP1 (PP1 is a protein phosphatase that regulates CREB and other factors), thus increasing phosphorylation of CREB and Akt, directly regulating their function (Canettieri et al., 2003; Brush et al., 2004; Chen et al., 2005). An important distinction to make here is that acetylation of a factor will be transient. In contrast, histone acetylation establishes a pattern of histone modifications that may be much more stable, resulting in a long-term transcriptional profile required to maintain a cellular memory for memory storage. This idea is discussed in detail in the first section of this article.

4.42.4.4 DNA Methylation and 'Lifetime' Memory Storage

All of the studies discussed thus far have investigated the role of epigenetic marking of the genome in an early and acute phase of memory formation. A recent study published from the Meaney laboratory (Weaver et al., 2004) suggests that DNA methylation patterns of specific genes in the brain are used as part of the mechanisms for storing early childhood experiences. Mother rats exhibit strong nurturing behaviors toward their pups, most notably in the form of licking and grooming their offspring. Patterns of DNA methylation in the glucocorticoid receptor gene are directly correlated with the quality of maternal care, and these patterns of DNA methylation persist into adulthood (Weaver et al., 2004). Moreover, these changes in DNA methylation patterns result in decreased anxiety and a strong maternal nurturing instinct in the adult offspring (Weaver et al., 2004). Therefore, alterations in DNA methylation result in a 'learned' and persistent change in adult behavior. Interestingly, maternally induced patterns of DNA methylation can be altered by central infusion of either an HDAC inhibitor or L-methionine, a precursor in the synthesis of the methyl donor S-adenosyl-methionine, suggesting that learning-induced changes in patterns of DNA methylation are dynamic and can exhibit plasticity during the lifetime of the animal (Meaney and Szyf, 2005; Szyf et al., 2005; Weaver et al., 2005). It is important to note that disruption of the epigenetic state of neurons did not result in a loss of neuronal phenotype, as might be expected if the same kind of cellular memory were used to store long-term memory of phenotype and childhood experience. Moreover, persistence of neonatally acquired patterns of DNA methylation in the mature CNS is consistent with our hypothesis that epigenetic mechanisms contribute to persistent changes in neural function. Interestingly, this study suggests a unique mechanism whereby the status of at least one component of the epigenome is transmitted across generations using the basic mechanisms in place for information storage in the nervous system.

4.42.4.5 Epigenetics in Memory Formation – Conclusions

Formation of several different types of memory appears to be dependent upon some of the molecular machinery involved in chromatin structure remodeling; specifically HATs. Formation of some types of long-term memory is correlated with epigenetic tagging of the genome. Finally, treatment with HDAC inhibitors can compensate for loss of HAT function and even enhance normal long-term memory formation. All of these observations indicate that the processes involved in induction of long-term memory formation utilize epigenetic mechanisms. Future experiments should begin to investigate the role of epigenetics in other phases of long-term memory formation, including consolidation and lifetime or remote (see Frankland et al., 2004) storage.

4.42.5 Epigenetics in Cognition: Rett Syndrome

As reviewed, there is considerable evidence implicating epigenetic mechanisms in neural function and memory formation. Moreover, even though epigenetic regulation of chromatin structure is a vital step in cellular differentiation, the studies published

to date indicate an active and ongoing role for epigenetic regulation of chromatin structure in the nervous system as it relates to plasticity and memory formation. Thus, postdevelopmental regulation of chromatin structure in the nervous system appears to be an important component of cognition. This suggests that derangement of the mechanisms responsible for regulation of chromatin structure would lead to severe cognitive impairment. Rett syndrome (RS) represents one disease of cognition where a specific molecule important for regulation of chromatin structure is mutated, resulting in severe cognitive impairments.

RS, first described by Austrian pediatrician Andreas Rett (1966), is an inherited, X-linked disease that afflicts about 1 in 15 000 females by 2 to 18 years of age and is estimated to be the second leading cause of mental retardation in women (Ellaway and Christodoulou, 2001). Development during the first 3 to 6 months of life is normal in RS patients; symptoms of RS first appear between 3 months and 3 years of age. The trademark of RS is a display of continuous, stereotypical hand movements, such as wringing, washing, clapping, and/or patting, which appear after the loss of purposeful hand movement. Other signs of RS include decreased growth (including microcephaly), abnormal respiration, gait ataxia, autism, seizures, and other neurological dysfunctions. Mapping studies identified a putative RS locus at Xq28 (Sirianni et al., 1998). Recent studies indicate that, in a percentage of patients, a mutation of the methyl CpG binding protein 2 (MeCP2) located in Xq28 is correlated with Rett syndrome (Sirianni et al., 1998; Amir et al., 1999; Ellaway and Christodoulou, 2001).

MeCP2 is a member of a family of methyl CpG binding proteins that function to link DNA (cytosine-5) methylation with gene silencing. As discussed earlier, DNMTs catalyze the methylation of cytosines at the 5-position of the pyrimidine ring. Once methylated, 5mCpG is bound by MeCP2, which then recruits a complex of proteins including histone deacetylases and transcriptional corepressors such as Sin3A (**Figure 3**(**a**)) (Roopra et al., 2000). The histones associated with the 5mCpG become hypoacetylated, promoting tight association between DNA and histones, ultimately resulting in formation of a transcriptionally repressive heterochromatin complex (**Figure 3**(**a**)).

The role that MeCP2 might play in the memory deficits observed in RS is still unclear. DNA methylation is thought to be involved in genomic imprinting and dosage compensation. Therefore, MeCP2 could play a prominent role during development. Indeed, MeCP2 appears to be critically involved in neuronal maturation, and not surprisingly, early attempts to create mouse models lacking MeCP2 resulted in embryonic lethality (Tate et al., 1996; Cohen et al., 2003; Matarazzo et al., 2004; Matarazzo and Ronnett, 2004; Fukuda et al., 2005). However, RS is a progressive disease that does not result in symptoms until early childhood. More recent attempts to create MeCP2-deficient mice have succeeded (Chen et al., 2001; Guy et al., 2001). These mice display several of the characteristics of human RS; however, no studies of cognitive performance in these mouse models have been published to date (Chen et al., 2001; Guy et al., 2001). To more closely approximate the mutations commonly found in RS patients, another strain of mouse was developed where the last one-third of MeCP2 was removed ($MeCP2^{308/y}$) (Shahbazian et al., 2002). $MeCP2^{308/y}$ mice share phenotypic similarities with human RS, including stereotypy, spontaneous seizures, increased anxiety, altered diurnal activity levels, and abnormal social interaction (Shahbazian et al., 2002; Moretti et al., 2005). Initial studies of $MeCP2^{308/y}$ animals suggested that long-term memory formation was normal (Shahbazian et al., 2002); however, further studies of these mice have revealed significant deficits in formation of hippocampus-dependent long-term memory and induction of synaptic plasticity in the sensory-motor cortex and hippocampus (**Figure 3**(**b**)) (Moretti et al., 2006). The derangements observed in $MeCP2^{308/y}$ animals do not appear to be due to aberrant development, as mice where MeCP2 was removed from forebrain neurons postnatally display many of the same social and memory impairments (Gemelli et al., 2006).

Several recent studies have begun to explore the function of MeCP2 in neurons and have identified a few mechanisms by which loss of MeCP2 could result in the cognitive derangements observed in RS model mice and human patients. One study demonstrated that a truncated form of MeCP2 protein, which mimics the most common mutation observed in human RS patients, tightly associates with methylated DNA in *Xenopus* embryos, suggesting that mutated MeCP2 protein can have profound effects on early developmental processes and possibly also interfere with normal regulation of chromatin structure and gene expression in the adult (Stancheva et al., 2003). Morphological studies have revealed that MeCP2 is expressed in excitatory cortical

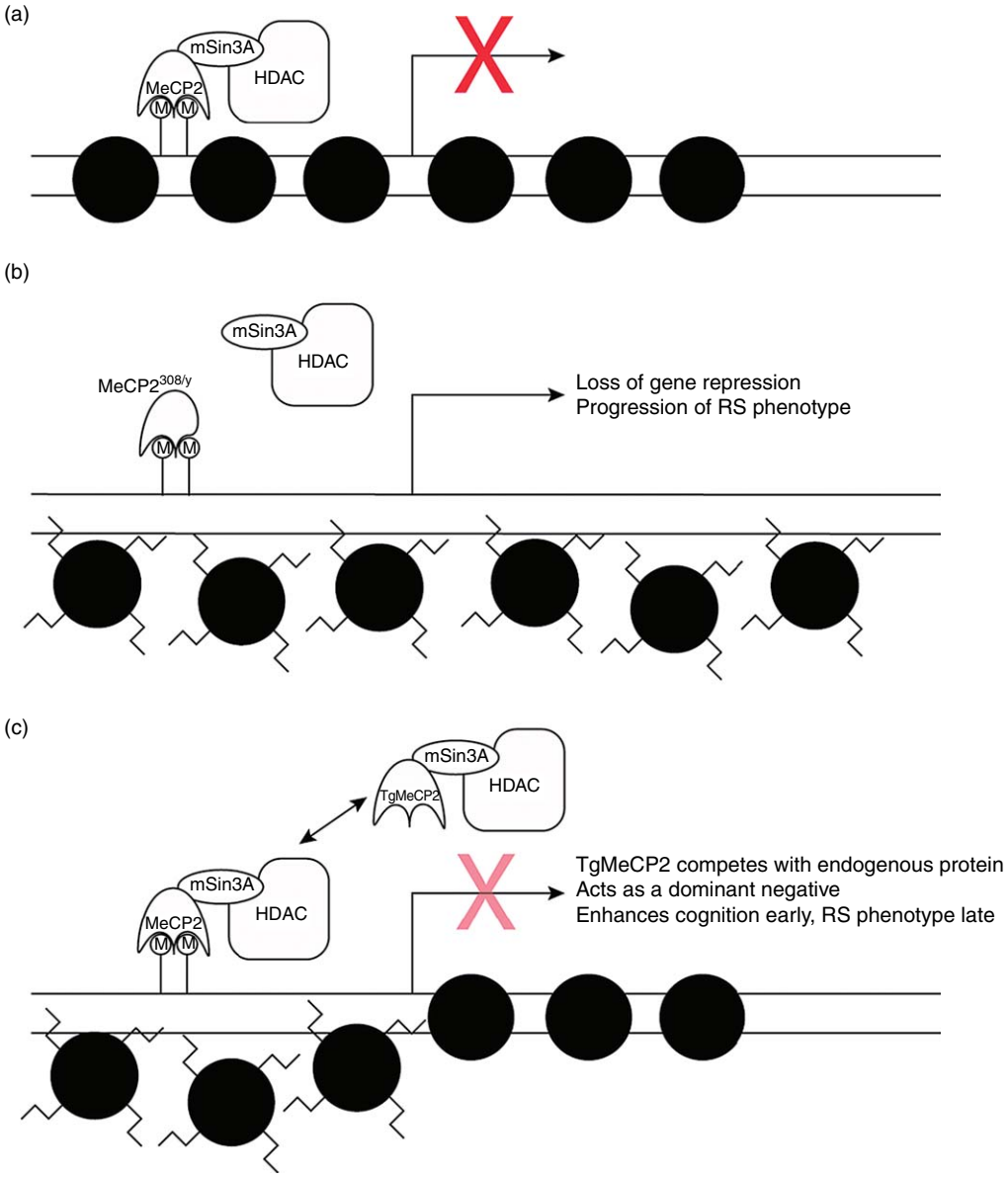


Figure 3 Role of MeCP2 in Rett syndrome, synaptic plasticity, and memory formation. MeCP2 is a member of the family of methyl CpG binding proteins. (a) MeCP2 binds to methylated cytosines and recruits the adapter protein mSin3A and HDAC to repress gene expression. (b) Loss of MeCP2 function through truncation mutations is thought to underlie RS. Truncation of MeCP2 results in loss of gene silencing, aberrant gene expression, and progression of the RS phenotype. (c) Overexpression of MeCP2 represents one possible therapeutic approach for treatment of RS. MeCP2 overexpression appears to have a dominant negative effect on normal MeCP2 function as determined through measures of synaptic plasticity and cognitive performance. Excess MeCP2 may sequester mSin3A and HDAC, limiting the ability of MeCP2 to repress gene expression. The early effects of MeCP2 overexpression lead to enhancement of synaptic plasticity and cognition. Continuous overexpression of MeCP2 eventually results in progression of a RS-like phenotype and death.

neurons and GABAergic interneurons, with little to no expression in glial cells (Akbarian et al., 2001; LaSalle et al., 2001; Adachi et al., 2005; Pelka et al., 2005). Restriction of MeCP2 expression to excitatory and inhibitory neurons in the cortex is consistent with the observations that expression of *dlx5*, a gene responsible for the regulation of GABA-synthesizing enzymes, is increased due to a loss of MeCP2-mediated imprinting in human fibroblasts (Horike et al., 2005), and cortical inhibitory tone is enhanced

in one strain of MeCP2-null mice (Dani et al., 2005). Investigating possible causes of the aberrant social behavior, several studies indicate that expression of stress-related genes is increased and levels of norepinephrine, dopamine, and serotonin were decreased in MeCP2-null mice (Ide et al., 2005; Nuber et al., 2005). Additionally, loss of MeCP2 leads to aberrations in chromatin structure around the Prader Willi and Angelman loci, two regions of the genome implicated in autism (Makedonski et al., 2005; Samaco et al., 2005). It is interesting to note that all of these aberrations observed in the context of loss of MeCP2 function are due to dysfunction of chromatin structure and gene expression (Tudor et al., 2002).

As detailed earlier, several studies indicate that loss of MeCP2 function leads to severe alterations in chromatin structure, gene expression, and neuronal function. Several labs have begun to explore the efficacy of overexpression of normal MeCP2 as a potential therapy to treat RS patients. In one study, overexpression of MeCP2 was shown to enhance long-term memory formation and the induction of hippocampal LTP in young mice (**Figure 3**(**c**)) (Collins et al., 2004). However, as the mice aged, overexpression of MeCP2 led to impairments in motor function and a decrease in longevity (Collins et al., 2004; Luikenhuis et al., 2004). Interestingly, overexpression of MeCP2 in mice that lack MeCP2 rescues the RS phenotype, suggesting that genetic replacement of MeCP2 expression is sufficient to rescue some of the RS-associated phenotypes (Luikenhuis et al., 2004). Moreover, these results suggest that levels of MeCP2 are coupled to cognitive performance, and suggest the broader implication that methyl-DNA binding protein function, and possibly DNA (cytosine-5) methylation itself, plays a significant role in induction of plasticity and memory formation in the adult CNS.

In support of an active role for DNA (cytosine-5) methylation in plasticity, a recent study by Levenson et al. (2006) demonstrated that inhibition of DNMT activity blocks induction of LTP in the hippocampus. Moreover, activation of PKC through the use of phorbol esters significantly increased expression of DNMT3A, suggesting that DNMT expression and activity is regulated by a signaling pathway that is crucial for the induction of synaptic plasticity and memory formation (*See* Chapter 4.25) (Lovinger et al., 1987; Abeliovich et al., 1993; Weeber et al., 2000). Collectively, these results are consistent with the observations made in MeCP2 model mice and suggest an active role for DNMT activity and DNA (cytosine-5) methylation in synaptic plasticity and long-term memory formation.

4.42.6 Conclusions

Epigenetic cellular memory is an ancient and evolutionarily conserved form of lifetime memory. Every cell in a metazoan relies on cellular memory to exist and function normally in its environment. A common theme emerging in the field of learning and memory is that the nervous system has co-opted several evolutionarily conserved processes to subserve long-term information storage. Some examples include molecules relevant for immune system function, such as class I major histocompatibility complex proteins and the NF-κB family of transcription factors (Meberg et al., 1996; Corriveau et al., 1998; Albensi and Mattson, 2000; Huh et al., 2000; Kassed et al., 2002; Yeh et al., 2002; Meffert et al., 2003; Levenson et al., 2004a; Oliveira et al., 2004) and signaling pathways involved in early development, such as the ras-MEK-ERK MAPK signaling pathway (English and Sweatt, 1996, 1997; Atkins et al., 1998; Silva et al., 1998; Selcher et al., 1999; Schafe et al., 2000; Giese et al., 2001). The realization that the processes involved in forming epigenetic cellular memory have been co-opted by the nervous system for induction of long-term memory and storage of some forms of lifetime memory has brought the field of neuroscience to an exciting juncture. For example, therapies based on modulation of epigenetic states could be used to treat a host of neurological conditions potentially including Huntington's disease, Alzheimer's disease, schizophrenia, and Rubinstein-Taybi syndrome (Kimberly et al., 2001; Steffan et al., 2001; Ferrante et al., 2003; Hockly et al., 2003; Kim et al., 2004; Numachi et al., 2004; Rouaux et al., 2004; Von Rotz et al., 2004; Grayson et al., 2005; Tremolizzo et al., 2005). Perhaps most relevant for the general population is the exciting possibility that, as we gain a deeper understanding of how epigenetics factors into cognition, novel drugs could be developed to enhance memory formation in otherwise normal individuals (Levenson et al., 2004b).

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