GENETIC APPROACHES TO MOLECULAR AND CELLULAR COGNITION: A Focus on LTP and Learning and Memory

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Abstract  Long-term potentiation (LTP) is the predominant experimental model for the synaptic plasticity mechanisms thought to underlie learning and memory. This review is focused on the contributions of genetics to the understanding of the role of LTP in learning and memory. These studies have used a combination of genetics, molecular biology, neurophysiology, and psychology to demonstrate that molecular mechanisms of synaptic plasticity are critical for learning and memory. Because of the large scope of this literature, we focus primarily on genetic studies of hippocampal-dependent learning. Altogether, these findings not only demonstrate a role for plasticity in learning, they also lay down the foundations for the new field of molecular and cellular cognition.

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INTRODUCTION

Synaptic Mechanisms of Learning and Memory

Memory allows organisms to make adaptive changes that improve their fitness. For example, the ability to remember the territories of rival males allows a mouse to minimize possible life-threatening confrontations. There is now an overwhelming amount of data that suggest that synaptic changes play a key role in learning and memory. Thirty years before the discovery of long-term potentiation, Donald Hebb proposed that memory is stored in patterns of synaptic connections established during learning. The idea is that neurons that are repeatedly coactivated during learning, strengthen their synaptic connections, and that these changes in neuronal connectivity can be used to recreate the information acquired during learning. This simple idea is the basis of the most influential theory of learning and memory and accounts for a very large body of molecular, neurophysiological, and behavioral data in multiple systems and organisms.

While studying the basic electrophysiological properties of excitatory neurons in the dentate gyrus of the hippocampus, Lomo & Bliss discovered a phenomenon in which excitatory postsynaptic potentials (EPSPs) were stably enhanced after specific types of high-frequency stimulation [reviewed in (15)]. This electrophysiological finding was the first demonstration of long-term synaptic plasticity and a confirmatory hint of Hebbian ideas about learning and memory. The high-frequency stimulation protocols used by Lomo & Bliss were subsequently adapted to studying LTP in hippocampal slices. Additional studies have also discovered long-term depression (LTD) in which a stable decrease in baseline EPSPs occurs after low-frequency activation of the postsynaptic cell. Theoretical studies had predicted the existence of LTD, since without some mechanism to balance LTP, synapses in neuronal circuits would progressively become saturated and thus unable to store information.

Synaptic plasticity in the dentate gyrus, CA1, and CA3 neurons of the hippocampus is an attractive candidate mechanism for learning and memory, including declarative memory, a form of hippocampal-dependent memory for facts and events [reviewed in (34)]. Studies of HM, a patient who received medial temporal lobe lesions to alleviate his intractable epilepsy, revealed that the hippocampus plays a pivotal role in this form of memory. The studies with HM had an enormous impact because they demonstrated that memory is a phenomenon separate from other cognitive abilities, such as attention and general intelligence. The hippocampus also has a key role in place learning in mammalian species as diverse as mice and men. Therefore, spatial and contextual learning have had a prominent role in studies of hippocampal-dependent learning and memory. Pharmacological and genetic manipulations that interfere with LTP are often found to affect place learning and memory, although there are some interesting exceptions to this correlation. Although mechanisms that result in stable and long-lasting changes in synaptic function are ensconced as learning and memory mechanisms, many
questions remain unanswered. Among these are how synaptic inputs are modulated during learning, how cells integrate their inputs, and how that code is transferred, processed, and stored over multiple networks of neurons in the brain.

The Genetic Basis of Learning and Memory

The idea that there is a genetic basis for learning and memory originated nearly a century ago, even before the physical mode of inheritance was known. Since then, many studies have shown that learning and other cognitive abilities have a genetic basis [reviewed in (117)]. Current genetic approaches to learning and memory came from three main sources: forward genetic studies in fruit flies heralded the advent of the genetic dissection of behavior in model systems (32), mouse reverse genetic approaches to behavior started by asking how genes affect plasticity and learning and memory in mice (101, 103), and more recent studies ascertained the feasibility of performing mutagenic screens in mice (60, 115). The original forward genetic studies of learning and memory in Drosophila not only showed that there are discrete loci for learning and memory, but also identified key signaling pathways that continue to be intensively investigated [reviewed in (116)].

The genetic study of molecular and cellular cognition in mice benefited tremendously from both behavioral neuroscience and neurophysiology. A key contribution of reverse genetic studies of molecular and cellular cognition has been to bring psychology and neurophysiology closer together. The availability of mutants has allowed behavioral neuroscientists and neurophysiologists to study and analyze the same genetically modified mice. This has led to a more coherent integration of findings in both fields, to the development of a common language between these areas, and to the emergence of a new field, molecular and cellular cognition, where genetics, physiology, and behavior are used side by side in integrative explanations of cognition.

Important developments have also been made in forward genetic approaches. Quantitative trait loci (QTL) that account for small differences in behavior have been identified in many inbred mouse strains (86), including those associated with Pavlovian fear conditioning (114, 119). These and other findings indicated that genetic loci can account for the complex behavioral variance among inbred mouse strains. These studies have also highlighted the critical importance of controlling for genetic background when carrying out genetic studies of behavior (26, 102).

Transgenic Approaches

Manipulations that alter gene function include functional deletions of genes, overexpression of wild-type or mutated genes, and gene replacements with mutant alleles. Collectively, these manipulations are referred to as transgenic manipulations, to distinguish them from transgenic approaches that refer specifically to studies done with mice derived by injections of constructs into the pro-nuclei of
Oocytes. Germline deletions of genes typically include the insertion of a neomycin cassette near the beginning of the open reading frame of a gene, causing truncation and instability of resulting transcripts. The neomycin cassette can sometimes disrupt nearby loci and thus confound the interpretation of the results. Therefore, targeted mutations in which the neomycin cassette is removed prior to blastocyst injection are being generated. Although this approach is the mainstay of reverse genetic studies of learning and memory, considerable effort has been placed into developing techniques that improve the temporal, regional, and biochemical specificity of the mutations studied. Proteins can have multiple biochemical roles, in different tissues and over time. Therefore, it is critical to develop methods that unravel the multiple roles of single genes in specific cells and brain regions. The development of the Cre/loxP system represents a significant step toward this goal. Cre recombinase deletes sequences flanked by its 34-bp recognition sites, loxP. Therefore, specific exons flanked by loxP (or floxed) can be deleted in tissues expressing this recombinase. The expression of the recombinase can be restricted with tissue-specific promoters. For example, an 8.5-kb segment of the αCaMKII promoter is sufficient to direct expression of a reporter gene to postnatal excitatory neurons of the forebrain (74). Furthermore, transcriptional regulatory elements in the genomic integration site can further modify the expression profile of the transgenic Cre recombinases. Thus, gene expression can be limited to specific regions of the brain, such as the CA1 pyramidal field of the hippocampus (112). Localized transgenic expression can also be obtained by region-specific injection of virally driven genes [e.g., (58)].

Temporal control over gene expression in the brain has also been achieved with both transcriptional and posttranscriptional methods. The tetracycline transactivator (tTA) system uses the bacterial tetracycline-sensitive activator to direct expression from the Tet operon promoter (tetO). In this system, administration of a tetracycline analog (doxycycline) turns off gene expression driven from the tetO promoter. In a mutated version of this system (the reverse tTA or rtTA system), administration of the tetracycline analog induces expression from the tetO promoter. In the LBD posttranscriptional system, a mutant ligand-binding domain (LBD) of the estrogen or progesterone receptor (12, 118) is fused with a protein of interest. The LBD fusion will be transcribed and translated, but will be inactive until the LBD binds its ligand. This system allows activation of proteins of interest within hours rather than days (62), as is the case with the tTA systems. Improvements in these inducible and tissue-restricted systems will continue to benefit studies of learning and memory.

Searching for Learning and Memory Genes in the Mouse

Taking a cue from learning and memory mutant screens in fruit flies, recent forward genetic experiments with traditional ENU mutagenesis are attempting to identify new mouse learning and memory mutations. Similarly, our laboratory has also developed phenotypic screens of previously derived knock-outs (KO). Although most
of these KO mutant mice were derived to study other biological problems, such as development, cancer, or immunology, many of the mutated genes are expressed in the brain. Therefore, those mutations could also have behavioral phenotypes. Indeed, a limited screen of randomly selected KO mice has already identified several new learning and memory genes (A. Matynia & A.J. Silva, unpublished data).

Analysis of genes expressed after behavioral training has also been used to identify potential new learning and memory genes. Numerous microarray and SAGE (serial analysis of gene expression) experiments have identified genes normally expressed in structures with a known role in certain forms of learning, such as the amygdala (127) and hippocampus (29, 82) as well as genes that are up- or down-regulated during associative learning (21; Y. Robles & S. Pena-de-Ortiz, personal communication). Data from these types of experiments show the diverse array of proteins required for learning and memory (21; Y. Robles & S. Pena-de-Ortiz, personal communication). Attempts have also been made to develop methods to visualize gene expression in the brains of behaving animals. Gene expression profiles may very likely represent functional signatures of different memory processes. In combination with genetics, these genomic approaches will be an important tool to dissect and uncover novel learning and memory mechanisms. Thus, transgenetic approaches should continue to play an important role in uncovering the mechanisms responsible for the acquisition, processing, storage, and recall of information.

Below, we highlight key findings and insights gained from the analysis of mutant mice in the field of molecular and cellular cognition. Because of the large scope of this literature, we focus our review on transgenetic studies of hippocampal-dependent learning and memory.

MUTATIONS AND INSIGHTS

NMDAR: A Molecular Coincidence Detector

The NMDA receptor is a glutamate-gated receptor composed of four to five subunits with at least one ζ and one ε subunit. The ζ1, also called the NR1 subunit, is ubiquitously expressed during development. Deletion of this subunit causes neonatal lethality (65). NR2B (ε2) and NR2D (ε4) are expressed embryonically, whereas all four subunits are expressed after birth. Postnatally, NR2A is expressed throughout the brain, NR2B is restricted to the forebrain, NR2C is expressed mainly in the cerebellum, and NR2D has limited expression in the brain stem. Activation of the NMDARs requires both binding of glutamate by any of the four NR2 subunits and release from a voltage-dependent magnesium block. Thus, sodium or calcium influx (e.g., through local depolarization or back-propagating spikes) alters the membrane potential, thereby alleviating the block and allowing activation. Since these two events are required simultaneously for activation, NMDARs have been called molecular coincidence detectors. After activation, calcium then enters through this channel and induces LTP. Both NR2A and NR2B bind
the alpha isoform of calcium/calmodulin-dependent kinase II (αCaMKII) as well as PostSynaptic Density-95 (PSD-95) via their carboxy-terminal tails, presumably linking channel activation with intracellular signaling pathways.

The role of NMDA receptors (NMDAR) in LTP and memory has been long established. Treatment with NMDAR blockers severely impaired performance in the spatial version of the Morris water maze. In this task, mice have to learn to find a submerged platform in a large pool of water using distal guiding cues (see Figure 1A) (83). In addition to their clear impact on learning in the Morris water maze, a task very sensitive to hippocampal lesions, NMDAR blockers also disrupted LTP in the hippocampus, suggesting that NMDAR-dependent induction of LTP in the hippocampus is critical for spatial learning. This study was one of the first to provide compelling evidence for a link between LTP and learning. However, follow-up studies showed that NMDAR blockers have many behavioral effects, other than blocking spatial learning (8, 93). With genetic approaches it has been possible to manipulate specific subunits and properties of these receptors. Individual subunits can not only be deleted, but also deleted in specific regions of the brain, thereby obviating the need to consider functional effects from regions other than the one of interest. This is particularly important when studying the NMDA receptor since deletion of the NR1, NR2A, or NR2B subunits results in neonatal lethality (38, 65, 66).

NR1 The properties of the NR1 subunit determine not only affinities for the channel modulators glycine and glutamate, but also Ca^{++} permeability, gating properties, potentiation, and the voltage-dependent Mg^{++} block. A key amino acid for function of this subunit is N598 that lies in the pore-forming region of the protein. To determine the role of the NR1 subunit in both LTP and learning, mice with a substitution of N598 for glutamine (conserving the charge) or arginine (altering the charge) were studied. Surviving only as heterozygotes, the N598Q mice exhibited normal LTP but increased mortality, whereas the N598R mice had decreased NMDA currents, decreased calcium permeability and died within 2 days (104). Given the essential nature of this gene, very careful manipulations must be made in the NR1 subunit to molecularly dissect the specific contributions of this subunit to LTP and learning and memory.

To investigate the role of glycine binding to the NR1 subunit and its phenotypic consequences, Kew et al, used the knock-in approach to make point mutations that disrupt glycine binding. Mice with a D481N mutation, which moderately decreases the affinity of the subunit for glycine, were viable. In acutely dissociated neurons, glutamate binding to NMDA receptors was normal and the decreased binding of glycine was confirmed. Hippocampal LTP could not be induced using theta-burst stimulation. Consistent with a relationship between LTP and spatial learning, these mice were also deficient in the hidden-platform version of the water maze (61).

To circumvent the lethality of NR1 deletions, this subunit was deleted specifically in the CA1 pyramidal field of the hippocampus, a region with a well-demonstrated role in learning and memory. This was done with the Cre/LoxP...
system under the regulation of the αCaMKII promoter (75). This promoter normally directs gene expression to postnatal excitatory neurons of the forebrain. However, interactions with putative transcriptional elements in the genomic site of transgene insertion result in specific expression patterns. For example, a specific insertion site restricted deletions of NR1 to the CA1 region (112). The loss of NR1 from CA1 cells resulted in a selective loss of NMDAR function without affecting other glutamate receptors (e.g., AMPA receptors). Neither NMDA-dependent LTP nor LTD could be induced in the CA1 region, although LTP and LTD was not affected in other regions studied, confirming the neuroanatomical specificity of this genetic manipulation. This CA1-specific deletion of NR1 also resulted in spatial learning deficits in the water maze. The CA1 NR1−/− mice were unable to learn to locate the hidden platform, even though they could learn to escape the water by swimming towards and climbing to a visible-platform in the water maze, suggesting that their spatial learning deficit was not due to abnormalities in motor coordination, motivation to escape the water or vision required for spatial navigation (113).

Consistent with the idea that abnormal CA1 function accounts for the spatial learning deficits of the CA1 NR1−/− mutant mice, single unit in vivo electrophysiological recordings of CA1 cells revealed interesting spatial deficits. The activity of these cells reflects the position of the animal in its environment. The firing pattern of different CA1 cells, as the animal travels through a given environment, can be used to reconstruct its trajectory. This and other results suggested that place cell maps have a role in spatial learning [reviewed in (96)]. Multi-electrode recordings in the CA1 region of CA1 NR1−/− mice showed that place-related firing is not affected, but that the average size of the place where CA1 cells fire (place fields) was larger in the mutant mice. Additionally, the coordinated firing of pairs of neurons in similar spatial locations was decreased in the mutants (78), suggesting that NMDAR-dependent LTP in the CA1 region is required for the orchestration and coordination of place maps during learning. This was one of the first attempts to use circuit phenomena to bridge synaptic physiology with behavior. This illustrates an important feature of molecular and cellular cognition studies: the integration of findings at multiple levels of biological complexity, including biochemical, subcellular, cellular, network, and systems.

NR2A  CA1 cells from mice carrying a deletion of NR2A have smaller NMDA currents and, not surprisingly, show attenuated LTP (91). Stronger stimulation, however, can trigger LTP in the mutants that is comparable to WT mice, suggesting that this mutation did not disrupt the core mechanisms of LTP induction (63). Instead, it may have increased the threshold for LTP induction, a finding consistent with the idea that NR2A modulates NMDA currents during LTP induction. The increased threshold for LTP induction observed in these mutants may account for the higher thresholds for learning revealed in the NR2A mutant mice. In contextual fear conditioning, a hippocampal-dependent test, mice learn to fear a context (conditioned stimulus or CS) in which they receive a footshock (unconditioned stimulus
When placed back in the context in which they received the shock, mice show freezing, a cessation of all movement except for respiration. Freezing is a normal fear response used to evade natural predators since predators often use movement to detect their prey. While these NR2A\(^{-/-}\) mice were able to perform contextual fear conditioning normally, they were unable to process contextual information as readily as wild-type mice (63). The NR2A mutant mice can learn to associate the context with the shock, but they require longer exposures to it. Thus, just as more intensive LTP protocols revealed normal levels of potentiation, longer intervals to process the context allowed normal levels of learning in these mutants. These data suggest that the NR2A subunit’s ability to modulate NMDAR currents may be involved in the modulation of thresholds for LTP and learning.

Deletion of the NR2B subunit or its carboxy terminus results in neonatal lethality. Through intervention, the NR2B\(^{-/-}\) mice can be kept alive for a few days, at which time LTD was assessed in hippocampal slices. Although in wild-type mice LTD could be induced, it was absent from the NR2B\(^{-/-}\) mice (65). This subunit is normally expressed at high levels during hippocampal development, before the hippocampus is thought to be functional, and is down-regulated in the adult. Nevertheless, Tang et al. tested whether overexpression of this subunit, which should increase Ca\(^{++}\) flow through the NMDA receptor, affected plasticity and learning. Two-fold overexpression of the wild-type NR2B subunit with the \(\alpha\)CaMKII promoter, resulted in an increase in LTP and in enhanced performance on multiple learning tests. This is an important result in evaluating the role of LTP in learning because it demonstrates a positive correlation between these two phenomena. However, not all transgenetically modified animals with increased LTP result in enhanced learning due to deleterious effects of the mutation on other cellular processes.

A number of proteins are known to interact with NMDA receptor subunits and therefore are also likely to be involved in LTP, place cells, and memory formation. One such protein is PSD-95 (PostSynaptic Density 95), which binds NR2A and NR2B via PDZ domains. A dominant-negative mutation of PSD-95, which leaves the first two PDZ domains intact, results in enhanced paired pulse facilitation (PPF) and dramatically enhanced LTP. Inappropriate potentiation of synapses during learning may obscure the pertinent information, resulting in the apparent learning and memory deficits in these knockout mice (80).

Receptor tyrosine kinases (RTKs) are a large family of membrane-associated proteins that undergo autophosphorylation in response to activation by their respective ligands. Ephrin receptors comprise the largest RTK family. EphA receptors are membrane associated via GPI anchors, whereas EphB receptors have transmembrane domains; both contain PDZ protein interaction domains. Important during development as guidance cues, Ephs also function in the adult brain in learning and memory. EphA5 is expressed in the hippocampus, cortex, and olfactory bulb.
Sequestration of the EphA5 ligand, ephrin-A5, resulted in decreased LTP and impaired contextual fear conditioning (41, 43). Alternately, activation of this receptor enhanced paired pulse facilitation, LTP, and contextual fear conditioning (41, 43). The mechanism for these effects is unknown, but it may involve cytoskeletal regulation.

EphB2, another ephrin receptor, is also expressed in the hippocampus, amygdala, and neocortex. It physically interacts via its extracellular domain with the NR1, NR2A, and NR2B subunits of the NMDA receptor (27). In fact, activation of EphB2 results in clustering of NMDA receptors with other synaptic proteins, including αCaMKII (27). EphB2 activation enhances Ca\(^{++}\) influx through the NMDAR, and is dependent on the phosphorylation of specific tyrosines in NR2B (108). Furthermore, EphB2 activation results in phosphorylation of ERK and CREB, two other proteins involved in LTP and memory (see below). In two different transgenic lines, EphB2 deletion caused deficits in the late phases of LTP, LTD, and in depotentiation (47, 50). A corresponding deficit in the spatial version of the Morris water maze was also observed. Interestingly, these effects were not dependent on the kinase activity of EphB2 since mice carrying a kinase-deleted form of EphB performed similarly to wild-type mice (47). Instead, the lower levels of NR1 in the postsynaptic densities of EphB2\(^{-/-}\) mutants may account for their LTP and learning deficits (50).

Another RTK, TrkB, is activated by the growth factors NT-4/5 and BDNF (Brain-Derived Neurotrophic Factor). TrkB\(^{-/-}\) mice have increased apoptosis in the hippocampus and cortex. Regional postnatal deletion, using a floxed TrkB and Cre recombinase expressed from the αCaMKII promoter, resulted in no gross abnormalities, including normal levels of apoptosis. However, these mice displayed decreased LTP as well as impaired memory in a variety of hippocampal-dependent tasks (81).

TrkB requires processing by Presenilin 1 (PS1). This protease is involved in a multitude of functions, including intracellular trafficking, intracellular Ca\(^{++}\) handling, processing APP and Notch, and interaction with β-catenin in mediating apoptosis. In addition, PS1 mutations are commonly found in early onset familial Alzheimer’s disease. In the PS1\(^{+/−}\) heterozygous mouse, hippocampal LTP induction was normal but its maintenance was impaired (84). In two mouse lines with PS1 deleted specifically in regions of the forebrain, γ-secretase activity was impaired in the cortex and hippocampus, and no increase in apoptosis was observed. Deletion of PS1 in these two lines also did not appear to affect hippocampal LTP. However, one of the mouse lines exhibited mild spatial water maze deficits (123). The other mouse displayed enhanced contextual fear conditioning but only in circumstances in which neurogenesis was altered, suggesting a role for PS1 in coordinating these phenomena (37).

αCaMKII also interacts with NMDARs, and has a profound effect on LTP, place cells, and learning and memory. Furthermore, αCaMKII, the protein kinase A (PKA) pathway, and the Ras/MAPK pathway can all be activated by Ca\(^{++}\) influx through the NMDA channel (see Figures 2–5). These pathways culminate in new
Calcium plays a key role in synaptic plasticity and memory formation. Calcium influx via the NMDA receptor binds calmodulin, activating Ca\(^{2+}\)/calmodulin-dependent kinases. The role of the boxed proteins in LTP and in learning and memory is described.

gene transcription, frequently mediated by the CREB transcription factor. The role these pathways play in the processes underlying learning and memory is discussed below.

**αCaMKII: Triggering the Molecular Processes of LTP and Learning**

Once NMDA receptors are activated (e.g., during the induction of LTP and during learning), calcium enters dendritic spines and activates a number of kinases, including αCaMKII. αCaMKII is a Ca\(^{2+}\)/calmodulin-dependent kinase that is heavily expressed in postnatal forebrain structures such as the hippocampus and cortex. Autophosphorylation of αCaMKII at threonine-286 (T286) converts the enzyme into an active state that is Ca\(^{2+}\)/calmodulin independent, and stabilizes its association with proteins in the postsynaptic density (PSD), such as the NMDAR. Subsequent autophosphorylation at TT305/306 blocks activation by calcium/calmodulin and drives the kinase from the PSD. This kinase is thought to have a key role in synaptic transmission because its phosphorylation of AMPA receptors is thought to affect their conductance and/or stability [reviewed in (67)].
Evidence for an interaction between NMDARs and αCaMKII during LTP and learning was obtained using a novel method with considerable potential. A heterozygous αCaMKII null mutation was combined with a subthreshold dose of an NMDAR antagonist. The heterozygous αCaMKII null mouse does not exhibit deficits in acquisition of contextual fear conditioning. Similarly, subthreshold doses of CPP or MK801, two different NMDA receptor antagonists, do not affect contextual fear conditioning in wild-type mice. When the subthreshold doses of drugs are combined with the heterozygous mutation, however, a deficit in contextual fear conditioning is revealed. These data suggest that NMDAR-dependent activation of αCaMKII is required for learning and memory (87). A similar experiment also suggested that NMDAR-dependent activation of αCaMKII is required for LTP (87). These studies are applicable to other heterozygous recessive mutations, and provide a novel strategy to test gene function in a temporal and tissue-specific manner (regulated by where and when the drug is administered).

The NMDA receptor and αCaMKII were the first two molecules implicated in both LTP and spatial learning and memory. The first study using knockout mice to investigate the connection between LTP and learning examined the effect of deleting αCaMKII. In these mice, NMDA currents were unaffected, as would be predicted if the action of αCaMKII were downstream of calcium influx through this receptor. Even LTP induced by high-frequency stimulation (3 × 100 Hz) was severely decreased in these mice (103). The mutant mice also showed profound spatial learning deficits: they were unable to learn to find the hidden platform although they were able to master a version of the water maze (visible platform) that is insensitive to hippocampal lesions.

Similarly, deregulation of αCaMKII function with a transgenically expressed constitutively active mutant kinase also resulted in deficient LTP (6, 75). Under conditions where the controls showed LTP, these transgenics showed LTD, suggesting that this kinase modulates how stimulation frequency affects synaptic plasticity. Place cell studies revealed profoundly unstable place cells in these mutants, suggesting that this kinase is also required for place field stability. This study was one of the first to address the genetic basis of place fields. The effects on LTP and learning were not due to changes during development since this phenotype was reproduced with the same αCaMKII transgene under the control of the tTA system. With this inducible system, it was also possible to show that deregulating αCaMKII function affects not only learning, but also memory and/or recall (75).

In contrast to homozygotes, heterozygous αCaMKII−/+ mutants can acquire spatial and contextual information. Accordingly, they also show normal hippocampal LTP tested with a variety of protocols (39). LTP in various areas of the neocortex, however, was profoundly abnormal in these mutants. The lower levels of this kinase in the neocortex, compared to the hippocampus, may explain why the neocortex is more sensitive than the hippocampus to the loss of half of the levels of the kinase in the heterozygous mutant mice. Lesion studies have suggested that the hippocampus is initially involved in memory, but that the neocortex is the final site for memory storage. Since these mice had normal hippocampal but
abnormal neocortical LTP, it was predicted that memory would be stable as long as it was supported by hippocampal circuits, but that memory would weaken as it became more dependent on neocortical sites. Consistent with this idea, the heterozygous αCaMKII+/− mice showed normal spatial memory when tested 3 days after training, but revealed profound amnesia when tested at later time points (39). Imaging studies with 2-deoxyglucose had shown predominant hippocampal activation during spatial learning, but neocortical activation during spatial recall at approximately the same time point that the heterozygous αCaMKII+/− mice showed spatial memory deficits (17). These studies suggested that αCaMKII-dependent LTP is required for memory storage in the neocortex. Other studies had already involved this molecule in neocortical plasticity [reviewed in (67)].

Calcium influx leads to autophosphorylation of αCaMKII at T286, converting it to a Ca++/calmodulin-independent and active kinase. Induction of LTP results in the activation and concomitant autophosphorylation at this site (11). The importance of this site has been tested using knock-in mice with a T286A mutation that prevents phosphorylation. The T286A mice showed unaltered presynaptic plasticity, NMDA currents, GABA_A inhibition, and synaptic transmission. However, this mutation resulted in a clear disruption of CA1 LTP tested with a number of different induction protocols. This LTP deficit, which very likely affects other hippocampal and neocortical regions, also disrupted the stability of hippocampal place cells. The mutants showed place fields, but they tended to change their mapping locations every time the animal was removed from the experimental room (23). Not surprisingly, with unstable LTP and place maps, these animals revealed profound spatial learning deficits (44). These findings demonstrate the critical role that this kinase plays on LTP, place cell stability and learning.

GLUR1, AN αCaMKII SUBSTRATE A known substrate of αCaMKII is the GluR1 subunit of the AMPA receptor. Hippocampal pyramidal cells express mainly the GluR1 and GluR2 subunits. The GluR1 subunit is phosphorylated by αCaMKII and PKA after LTP induction (7, 11, 49), which is thought to result in a concomitant increase in AMPA receptor function. Deletion of the GluR1 subunit demonstrated that this subunit is required for LTP induced by high-frequency tetanization in the hippocampus (124). This deficit in LTP could be rescued by exogenous expression (<20% of endogenous GluR1) of GluR1 both during development and postnatally (69). Further studies of LTP in these mice revealed that a more physiologically relevant stimulus (theta burst stimulation) induced nearly normal LTP (52). This physiologically relevant LTP probably accounts for the apparent lack of spatial learning deficits in this mutant mouse (124). This study is a good example of the complexities of testing connections between physiology and behavior.

The PKA Pathway An influx of Ca++ through the NMDA receptor can also activate the Protein Kinase A (PKA) signaling pathway. When high cAMP levels are produced (e.g.,
Figure 3 The PKA pathway is required for LTP and memory. Ca^{++} influx via the NMDA receptor activates Ca^{++} sensitive adenyl cyclases 1 and 8, PKA and calcineurin (CN). The role of the boxed proteins in LTP and in learning and memory is described.

through Ca^{++} sensitive adenyl cyclases), cAMP binds to the regulatory subunit of PKA. This binding alters the conformation of the regulatory subunit, thereby releasing the catalytic subunit. PKA can then phosphorylate numerous substrates, including the NMDA receptor, Inhibitor-1 (I-1) and the cAMP Response Element Binding protein (CREB) (see Figure 3). Opposing the actions of PKA are the protein phosphatases PP1A and calcineurin (PP2B), since they dephosphorylate PKA substrates. Calcineurin (CN), a calcium-sensitive serine/threonine phosphatase, is highly expressed in the hippocampus and is enriched at synapses. The CN regulatory subunit (B) is required for the phosphatase activity of the catalytic subunit (Aα or Aβ) that contains an autoinhibitory domain. In brain, there is only one regulatory subunit, B1. Among its substrates are the NMDA receptor, I-1, and CREB, some of the same proteins that are targets of PKA phosphorylation.

Adenylyl Cyclases (AC) catalyze the production of cAMP, a classic second-messenger signaling molecule that activates PKA. Ten different ACs have been identified and most are expressed in all cell types, including nine different ones in the hippocampus. Of these nine, AC1 and AC8 are stimulated by Ca^{++}/CaM. Deletion of either AC1 or AC8 does not alter LTP; however, the double mutant, which retains essentially no Ca^{++}/CaM-stimulated activity, exhibits profound deficits in
the late phase of LTP (L-LTP) (122). Forskolin, a chemical activator of all ACs, rescues the L-LTP of these mutants, thereby suggesting that downstream components of L-LTP are intact in these mutants. In agreement with the hypothesis that L-LTP is required for long-term memory formation, the memory deficit of the ΔAC1/ΔAC8 double mutant mice is more profound when tested eight days after training. Finally, just as forskolin rescued the L-LTP deficits, it also reversed the memory impairments of these mice, demonstrating that both deficits were due to a decrease in cAMP production. The infusion of forskolin also increased expression of a CRE-lacZ reporter, showing that the increase in cAMP via ACs probably involves CREB activation (see below). These data show that Ca^{++} activation of adenylyl cyclases, and therefore cAMP signaling, is required for L-LTP and LTM formation, a finding first suggested in pioneering studies in flies and in the sea slug Aplysia (100).

DECREASING PHOSPHORYLATION OF PKA/CALCINEURIN SUBSTRATES The opposing effects of PKA and calcineurin on learning and memory have been studied in multiple transgenic mice using overexpression of a dominant negative mutant of the regulatory domain of PKA, R(AB), or overexpression of the calcineurin Aα subunit lacking its autoinhibitory domain, ΔCaM-AI. Both of these manipulations were expected to result in an overall decrease in the phosphorylation of PKA substrates.

Increased activity of calcineurin in the ΔCaM-AI mice affected LTP and memory. Pharmacological activation of PKA rescued these LTP deficits. This misregulation of phosphorylation also affected spatial memory tested in two separate hippocampal-dependent tasks. The mice displayed intact short-term memory but severely impaired long-term memory (24 h after training) (72).

Evidence for a role of PKA on LTP was also obtained from studies of mice over-expressing R(AB), an inhibitory form of the regulatory subunit of PKA. These transgenics show a decrease in both the basal and cAMP-stimulated activity of PKA with a concomitant decrease in L-LTP 1 to 3 h after high-frequency stimulation. Early stages of LTP, however, are not affected by this transgene. Similarly, although hippocampal-dependent memory was disrupted when tested 24 h after training, short-term memory was not (1). These results provide a compelling parallel between the time course of LTP and learning deficits.

Besides a requirement for PKA activity during training, pharmacological studies with Rp-cAMP, a PKA inhibitor, showed that PKA activity is also needed for memory 4 h after training. Blocking PKA activity at other times does not seem to have an effect on contextual memory. The requirement for PKA at 4 h was only observed in mice that received weak training. Strong training seemed to circumvent this requirement. Similar results were also obtained with protein synthesis inhibitors. Weak training revealed that protein synthesis is also required during training and 4 h later, but not at other times (18).

Electrophysiological studies showed that PKA is required for an intermediate phase of LTP that is insensitive to protein synthesis inhibitors. Mild synaptic
stimulation in wild-type mice results in an intermediate level of LTP that is insensitive to protein synthesis inhibitors, but sensitive to PKA inhibitors. In the \( \Delta \)CaM-AI mutant, this intermediate level of synaptic stimulation elicited decreased LTP compared to wild-type mice and was insensitive to PKA inhibitors (121). These data show a dissociation between the early phase of LTP (E-LTP), which is independent of protein synthesis and PKA/calcineurin regulation, intermediate LTP (I-LTP), which is dependent on PKA/calcineurin regulation, and L-LTP, which is dependent on both.

The I-LTP deficits in the R(AB) also resulted in unstable place fields (90), confirming the importance of stable synaptic changes for the stability of representations in hippocampal circuits. Note that like the LTP deficits in the \( \alpha \)CaMKII mutants, the LTP deficit in the R(AB) almost certainly affects brain regions other than CA1. Altogether, the experiments just reviewed defined an intermediate memory phase with behavioral and electrophysiological correlates.

Increasing phosphorylation of PKA/calcineurin substrates The data discussed above support the idea that calcineurin and PKA regulate key substrates for LTP and learning and memory, using manipulations that decrease overall phosphorylation. Alternately, phosphorylation can generally be increased by inhibition of calcineurin via overexpression of the inhibitory domain of A\( \alpha \), deletion of A\( \alpha \) or deletion of the brain-specific regulatory domain, B1.

The A\( \alpha \) subunit is predominantly expressed in the hippocampus, cerebral cortex, cerebellum, and striatum. The rtTA system was used to overexpress the carboxy terminus of the A\( \alpha \) subunit containing the autoinhibitory domain (AI). Biochemical studies showed that this resulted in decreased phosphatase activity in the hippocampus and cortex. These mutants displayed an increase in E-LTP that was
blocked by PKA inhibition (71). Thus, overexpression of the catalytic domain in ΔCaM-AI mice (decreasing overall phosphorylation) impaired LTP, whereas overexpression of the inhibitory domain in AI mice (increasing overall phosphorylation) enhanced LTP. In the AI mice, enhanced LTP was also observed in vivo in anaesthetized and awake mice.

Consistent with enhanced LTP, the AI mutant mice also showed enhanced memory in hippocampal-dependent tasks. For example, in the Morris water maze, the mutant mice searched selectively for the hidden platform after only five days of training, whereas wild-type mice required ten days of training to attain the same level of performance (71). These data suggest that calcineurin normally acts as a negative regulator of LTP and memory. This may serve as a filter so that only strong and important memories are stored. There are several psychological studies that suggest that the inability to filter information is as debilitating as the inability to store it.

The overexpression of the auto-inhibitory domain of Aα reduced phosphatase activity, but did not eliminate it. To completely eliminate calcineurin activity, Zeng et al. deleted the B1 subunit (CNB1−/− mice), the only regulatory subunit in the brain. In the CNB1−/− mice, LTD was diminished, whereas LTP induced by one train of 100 Hz was intact. Depotentiation was also normal in the mutant mice. This is in contrast to the CNAα−/− mice that had normal LTD and LTP but a diminished ability to depotentiate LTP (125). Since phosphatases and kinases affect the thresholds of LTP and LTD induction, Zeng et al. investigated these thresholds in the CNB1−/− mice. Consistent with the idea that increased kinase activity favors LTP, while increased phosphatase activity favors LTD, the CNB1−/− mutation lowered the threshold for LTP while increasing the threshold for LTD. These mice showed normal performance in cued and contextual conditioning as well as in spatial learning tested in the Morris water maze. However, CNB1−/− showed severe deficits in both a working memory version of the water maze and the radial arm maze, suggesting that CNB1 is required for working memory in which fast, one-trial learning is required. These one-trial-learning tasks require that the animal disregard previous trials and focus its attention on information relevant to the current trial. The inability to reverse or erase information pertaining to prior trials could conceivably interfere with performance in future trials. Thus, the deficits in LTD in the CNB1−/− mice may account for their disrupted performance in the working memory tasks used (125). This is a good illustration of the complex relation between LTP/LTD and learning and memory. To successfully interpret studies testing the connection between LTP and learning, it is critical to consider the details of the experiments performed, including the possible presence of other electrophysiological changes and the specific requirements of the tasks used. Note also that changes in synaptic function may represent only one class of physiological changes involved in learning and memory, and that interaction with other relevant mechanisms may further complicate the interpretation of experiments testing the role of LTP in learning.
Figure 4  The Ras/MAPK pathway plays a role in LTP and memory. Ca^{++} influx via the NMDA receptor activates the Ras pathway. The receptor tyrosine kinase TrkB and the Ephrin receptor also activate the Ras pathway. The role of the boxed proteins in LTP and in learning and memory is described.

The Ras/Raf/MAPK Pathway is Involved in Learning and Memory

The Ras/Raf/MAPK signaling pathway is another pathway that figures prominently in LTP and learning and memory. Ras is the head of the superfamily of small GTPases that bind GTP. Ras signaling is stimulated by tyrosine kinase receptors, G-protein–coupled receptors, NMDA receptors, and some voltage-dependent Ca^{++} channels. The activity of Ras is further regulated by GTPase Activating Proteins (GAP) and Guanine Exchange Factors (GEF or GRF) (see Figure 4). Although Ras is primarily thought of as an oncogene with a key role in cellular proliferation, it also plays a role in key biological processes in post-mitotic cells, such learning and memory. Pioneering pharmacological work from Sweatt and colleagues implicated MEK/MAPK signaling in LTP and learning [reviewed in (106)].
Nf1, a RasGAP, is predominantly expressed in adult neurons. Mutations in this gene lead to neurofibromatosis I, the commonest single-gene genetic disorder resulting in learning disabilities (see below). A homozygous deletion of this gene is lethal whereas heterozygotes are viable. These Nf1⁺/− mice display learning deficits in the water maze; they perform poorly but with additional training are able to reach the same performance levels as their littermate controls (102). The Nf1 gene has multiple splice forms and this diversity has been explored in learning and memory studies. For example, an isoform containing exon23a (Nf1 type II) has increased affinity for Ras, but decreased GAP activity, thereby competing with the type I isoform and thus decreasing the conversion of Ras into its GDP-bound state. Analysis of the Nf1 23a⁺/− mice showed normal life expectancy, normal levels of Nf1 type I, seemingly normal development and no hints of increased tumor predisposition similar to the Nf1⁺/− heterozygotes. They do display, however, clear hippocampal-dependent learning deficits. These mice showed impairments in both the water maze and contextual discrimination, which can be partially rescued with extended training (24). These findings show that the learning deficits associated with Nf1 mutations are not due to tumors or to deficits in development.

Neurofibromin, the protein encoded by the Nf1 gene, is a large protein with multiple biochemical functions, including GAP activity, adenylyl cyclase modulation, and microtubule binding. However, rescue experiments based on manipulations that decrease Ras function showed that the learning deficits of the Nf1⁺/− mice (and by implication NF1 patients) are due to increased Ras signaling. Deleting N-ras, for example, rescued the spatial learning deficits of the Nf1⁺/− mutant mice. A K-ras heterozygous null deletion, which on its own causes spatial learning deficits, was also able to rescue the learning deficits of the Nf1⁺/− mutant mice (25). Furthermore, a farnesyl transferase inhibitor, which affects the farnesylation and hence the activity of Ras, was also able to rescue the learning deficits of the Nf1⁺/− mutant mice, suggesting that these deficits are not due to irreversible developmental problems. Importantly, the K-ras⁺/− mutation, which rescued the spatial learning impairments of the Nf1⁺/− mutants, also rescued their LTP impairments, a result that suggests a causal connection between the LTP deficits of the Nf1⁺/− mutants and their learning impairments. Further electrophysiological studies indicated that the LTP deficits of the Nf1⁺/− mutants are caused by increased GABA-mediated inhibition, and that the K-Ras⁺/− mutation could also rescue these deficits. These data suggest that Ras/Nf1 signaling modulates inhibition and that inhibition may have a role in regulating LTP during learning.

While RasGAPs inactivate Ras-GTP by catalyzing the hydrolysis of GTP to GDP, GRFs (Guanine nucleotide Releasing Factors) stimulate the release of GDP thereby promoting the active, GTP-bound state of Ras. Ras GRF1 and GRF2 both couple Ras activation to Ca²⁺/CaM, G protein, cAMP, and receptor tyrosine kinase signaling. Ras-GRF1 is expressed almost exclusively in brain (97). It is found primarily in the PSD, suggesting a role in plasticity and learning. Two separate deletions of this gene were generated, one disrupting the nucleotide exchange
activity domain (ex) and the other, a more N-terminal insertion of neo, disrupting the Dbll domain (dbl) that activates Ras (20, 45). Both, however, show a complete loss of GRF1 protein in the mutant mice. Although both manipulations affected learning and memory, they differ in important ways. The GRF1 ex−/− mutant strain had normal hippocampal, but impaired amygdala-dependent memory (20, 45), whereas the dbl-mutant showed normal amygdala, but impaired hippocampal-dependent learning (20, 45). It is difficult to reconcile these data because the mice were studied in seemingly identical genetic backgrounds and with very similar procedures. However, the GRF1 locus is imprinted and differential disruption of imprinting by these two different mutations could account for the disparities in their behavioral phenotypes. Nevertheless, these discrepancies could be due to a number of other factors, such as the differential disruption of nearby genes by the neomycin selectable marker inserted into this locus during gene targeting. These studies underscore the importance of studying multiple alleles of the same mutation. Allelic series of point mutations, complete deletions, and overexpression of wild-type or mutant alleles will give a richer understanding of the role of GRF1 in learning and memory. Although the history of biology has clearly demonstrated the enormous power of genetics, the most compelling evidence usually comes from convergent data obtained with different approaches (e.g., KO, transgenics, viral vectors, pharmacology). Taken together, these studies show that regulation of Ras activity through perturbation of either its GAP or GRF affect learning and memory. Why one GRF allele affects learning and the other memory remains unclear, but further genetic and genomic analysis should shed light on this interesting difference.

Members of the MAPK pathway, ERK1 and ERK2, are highly expressed in postmitotic neurons. Their activation via phosphorylation is required for theta-frequency induced LTP and for hippocampal-dependent learning. For example, phosphorylated ERK is seen after electrical corticostriatal stimulation, high-frequency stimulation in living rats, after amygdala-dependent CTA training and after hippocampal-dependent contextual fear conditioning and water maze training. Furthermore, these effects are blocked by ERK kinase (MEK) inhibitors (5, 13, 31, 36, 95). Increased ERK1/2 phosphorylation and nuclear translocation were observed after learning in the water maze, but not after a single training trial that results in no obvious learning (16). This phosphorylation was observed only in the CA1 and CA3 regions of the dorsal hippocampus. As inhibition of ERK phosphorylation after training blocked recall, it was suggested that MAPK activation in the CA1/3 regions is required for memory consolidation.

The role of ERK1 in learning and memory was further analyzed in two different ERK1−/− mice (77, 94). Behavioral analyses in these mice gave somewhat contradictory results. One of the ERK1−/− mouse strains showed no alterations in ERK2 protein levels or phosphorylation, LTP, general activity, motor skills, or learning and memory as assessed by fear conditioning and passive avoidance tasks (94). These results were later challenged in a second ERK1−/− mouse line in which alterations in the phosphorylation levels of ERK2 were enhanced, and LTP in the nucleus accumbens, but not the hippocampus or amygdala, was enhanced (77).
Similarly, these mice showed enhanced learning or memory observed in active and passive avoidance tests.

Dopaminergic stimulation resulted in enhanced ERK2 phosphorylation in these ERK1\(^{-/-}\) mutant mice (77). Consistent with the idea that this mutation led to a potentiation of the reward system, the mice displayed enhanced place preference for a context associated with a drug stimulus. These results support the hypothesis that MAPK activity, and specifically ERK1, is required for synaptic plasticity and learning and memory, as well as in drug reward behavior. Unfortunately, ERK2 is an essential gene (2) so further studies of its role in LTP and learning and memory have to wait for the generation of conditional alleles.

These studies have shown that Ras and its modulators, the GAP (Nf1) and the GEFs (GRF1 and GRF2), as well as downstream signaling molecules (ERK1 and potentially ERK2) have functional roles in both synaptic plasticity and learning and memory. How this signaling pathway interacts with either NMDAR/CaM Kinase or PKA/calcineurin signaling may lead to a better understanding of the molecular events underlying memory formation. Another important question concerns the behavioral relevance of the transcriptional events known to be downstream to these signaling pathways.

### Transcriptional Regulation and Memory: CREB and Long-Term Memory

The signaling pathways required for learning are thought to ultimately drive the transcription of genes required for memory consolidation, a process that filters and stabilizes information in the brain. Early work using pharmacological inhibitors showed the requirement for protein synthesis in long-term memory. In contrast, protein synthesis inhibitors did not affect short-term memory (30). One of the transcription factors activated during learning and required for memory consolidation is the transcription factor cAMP Response Element Binding protein (CREB). In response to elevated levels of cAMP and Ca\(^{++}\), this transcription factor is phosphorylated at S133 (as well as S142 and S143), and then directs transcription of genes containing cAMP Response Elements (CRE) in their promoters. CREB is phosphorylated by multiple kinases including PKA, RSK-2, CaMKII, and CaMKIV (see Figure 5). Note that CREB has roles in multiple cellular processes from metabolism to apoptosis. Thus, CRE sites are found in the promoters of many genes (76), not just learning and memory genes. The CREB gene encodes several isoforms, derived from alternate splicing, that act as either activators or inhibitors. The CREB family of transcription factors forms functional heterodimers. Previous studies in numerous species and brain systems have demonstrated that CREB has a clear role in the formation of long-term memory (LTM): behavioral and electrophysiological protocols that induce LTP and LTM also activate CREB, lesions of CREB function block long-term plasticity and long-term memory, and overexpression of CREB can facilitate plasticity and learning [reviewed in (99)]. Thus, CREB plays a pivotal role in the transition between short- and long-term memory.
Figure 5  The CREB transcription factor is required for L-LTP and long-term memory. The Ca^{++}/calmodulin-dependent kinases, the PKA pathway and the Ras pathway in part regulate the phosphorylation state of CREB. Phosphorylated CREB is required for long-term memory formation.

Early studies showed that injection of CRE-containing oligomers in cultured Aplysia neurons specifically blocked long-term facilitation, a cellular model of synaptic changes thought to underlie behavioral sensitization (28). Later studies in Drosophila and mice suggested that CREB was universally required for memory. The deletion of the CREB α and δ isoforms (CREB^{α,δ−}) resulted in deficits in the stability of hippocampal LTP, place cells, and place memory (18, 23). Follow-up studies showed that CREB affected long-term memory for a large number of other tasks, irrespective of the brain system involved. In contrast, short-term memory was unaffected in these mice. The loss of the CREB α and δ isoforms led to up-regulation of the β isoform, as well as to changes in the expression of specific CREM isoforms, which may explain why the severity of the phenotype of the CREB^{α,δ−} mutants appears to be so sensitive to genetic background (42, 46). Variations in the compensatory expression of other CREB-family genes may account for the variable penetrance of the CREB^{α,δ−} phenotype in different genetic backgrounds. Importantly, transgenic expression of a dominant-negative form of CREB that heterodimerizes with CREB, CREM, and ATF1 but inhibits DNA binding, also impaired hippocampal-dependent long-term memory. This transgenic manipulation also attenuated forms of LTP that were thought to be sensitive to CREB manipulations, such as forskolin-induced and dopamine-regulated LTP (89).
The analysis of the CREB\(^{\alpha1-}\) mice also revealed a role for this transcription factor in regulating the training required for LTM formation. Pioneering studies by Ebbinghaus in the nineteenth century established that the schedule of training affects long-term memory formation: Longer intervals between training sessions (spaced training) are more effective than shorter intervals (massed training) (33). Strikingly, the long-term memory deficits of the CREB\(^{\alpha1-}\) mice (i.e., fear conditioning and spatial learning) could be rescued by spaced training (58). Accordingly, increases in CREB levels with viral delivery systems eased training requirements for the formation of long-term memory (58). With increased CREB levels, animals were able to show LTM formation under training conditions (massed training) that are ineffective in controls, suggesting that CREB regulates how training schedules affect memory formation. Similarly, overexpressing CREB in mice enhances LTP, suggesting that the level of this transcription factor correlates with the ease of induction of synaptic changes underlying memory formation (10). Similar results were also obtained with Drosophila and Aplysia [reviewed in (99)]. These results suggest that complex behavioral phenomena (i.e., the effect of training schedules on memory) can be directly modulated by single molecular mechanisms (i.e., CREB activity), a finding that supports the idea that integration of some aspects of behavioral experience can occur at the molecular level.

The studies with the CREB\(^{\alpha1-}\) mice indicated a role for CREB in LTM. However, it was difficult to pinpoint exactly when CREB function was required for memory formation. To circumvent this limitation, a new CREB mutant was derived with the inducible LBD system (88). A mutant ligand-binding domain of the estrogen receptor (LBD), which binds tamoxifen instead of estrogen (68), was fused to a dominant-negative CREB gene (S133A substitution). This fusion gene (LBD-CREB) was placed under the regulation of the \(\alpha\)CaMKII promoter, which restricted its expression to postnatal excitatory neurons. With this system, the fusion protein is inactive until tamoxifen binds the receptor. Injections of tamoxifen activated the dominant negative CREB-fusion protein and repressed the transcription of 14-3-3\(\eta\), a CREB-dependent gene whose transcription is activated during learning (62). With this inducible system, it was possible to show that CREB is crucial for the consolidation of long-term memory, but not for the storage or retrieval of memory.

The studies with the LBD-CREB mice also showed that CREB is required for the stability of memory after retrieval (62). Retrieved memories can be fine-tuned and changed, and there is extensive evidence that some of the same mechanisms involved in memory formation may also be required for the stabilization of retrieved memories, a process referred to as reconsolidation (92). For example, blocking protein synthesis during re-exposure to the conditioning context can attenuate fear conditioning (85). These studies showed that memory not only depends upon CREB function during training, but also requires it during retrieval. Interestingly, C/EBP\(\beta\), another transcription factor required for memory consolidation is not required for reconsolidation after retrieval (110).
In addition to experiments demonstrating a requirement for CREB function in memory, there is also evidence indicating that CREB-dependent transcription is activated during learning. For example, experiments with antibodies specific to the phosphorylated version of CREB showed that behavioral training as well as memory retrieval can induce the phosphorylation (activation) of CREB (48). Elegant transgenic experiments with a transgenic $\beta$-galactosidase gene under the regulation of a CREB-dependent promoter showed that both LTP and learning can activate CREB-dependent transcription (55).

**CREB AND CaMKIV**

CaMKIV counts among its substrates many transcription factors including CREB and its cofactor, CBP, as well as proteins that interact with actin and microtubules. This kinase is found in adult cortical, cerebellar, hippocampus and amygdala neurons and is both cytoplasmic and nuclear. Because of its nuclear localization, this kinase is hypothesized to phosphorylate and activate CREB. Furthermore, CREB-dependent transcription requires phosphorylation of CBP at S301 by CaMKIV and this phosphorylation is dependent on NMDA activation (54).

Sustained low-frequency stimulation of hippocampal neurons results in increased expression of immediate early genes (IEGs) and a long-lasting increase in phosphorylated CREB. Phosphorylation of CREB in this system is specifically blocked by decreasing $\alpha/\beta$ CaMKIV levels (14). In addition to its role in CREB phosphorylation, CaMKIV is required for hippocampal, amygdala, and cortical L-LTP (51, 120). Deletion of CaMKIV $\alpha$ and $\beta$ isoforms, as well as overexpression of a dominant negative form of CaMKIV, both result in decreased L-LTP (51, 59). The CaMKIV$^{-/-}$ mouse displays deficits in both context and cued fear conditioning, whereas mice overexpressing the dominant negative protein show deficits in both water maze and contextual fear conditioning (59, 120). These studies suggest that CaMKIV is required for L-LTP and for long-term spatial memory formation.

**DOWNSTREAM OF CREB**

Although CREB clearly plays a role in memory consolidation, the downstream effectors that mediate this role are less well understood. C/EBP and Zif268 are two transcription factors with a role in learning and memory and whose transcription is regulated by CREB.

The C/EBP bZIP transcription factor family consists of 5 isoforms that bind to CCAAT promoter elements. C/EBPs can heterodimerize with C/ATF (CREB family member) and bind specific CRE sites, suggesting that it could competitively inhibit CREB function. Furthermore, C/EBP itself is regulated at least in part by CRE sites in its promoter. C/EBPs in Aplysia are regulated by cAMP and are required for long-term facilitation (3). Consistent with the idea that C/EBP isoforms play a role in memory consolidation, $\beta$ and $\delta$ mRNA levels are upregulated after inhibitory avoidance training in rats (111). Deletion of the $\alpha$ isoform results in perinatal lethality whereas the health of mice with a deletion of the $\beta$ isoform declines in the weeks following birth. Deletion of the $\delta$ isoform, however,
produces viable, healthy animals with no gross histological neuronal abnormalities (105). Consistent with a possible CREB inhibitory role, C/EBP$\delta^{−/−}$ mice display a specific enhancement in context conditioning 24 h, but not 30 min after training. Similarly, the mutant mice seemed to acquire spatial information faster than controls. Thus, these data suggest that C/EBP$\delta$ has an inhibitory role in LTM formation. Perhaps, this molecule is part of a feedback mechanism that downregulates CREB-mediated transcription.

While CREB is required for memory consolidation and reconsolidation, the role of C/EBPs in memory consolidation does not parallel that of CREB. Disruption of C/EBP$\beta$ via injection of antisense oligomers into the hippocampus blocks memory consolidation. However, the same injection into the hippocampus does not affect memory reconsolidation (110). These data suggest that reconsolidation is independent of hippocampal expression of C/EBP$\beta$. They also suggested that the role of CREB in reconsolidation is not mediated by C/EBP$\beta$.

A second protein up-regulated after LTP is Zif268, a zinc finger transcription factor. In monkeys, Zif268 is up-regulated after associative learning. In rats, novel stimuli activate the transcription of this gene in the hippocampus. Mice with a deletion of Zif268 show no obvious histological differences, but exhibit deficits in L-LTP and hippocampal-dependent memory; short-term memory is normal in these mice. In awake, freely moving mice, stimulation of the perforant path induced normal LTP in the dentate gyrus for at least one hour post-stimulation. However, LTP in these mutants was dramatically reduced one day and three days later (56). Performance in a number of behavioral tasks confirmed that these mice exhibited normal short-term memory but decreased long-term memory, consistent with the idea that Zif268 is acting downstream of CREB during memory formation. As with the CREB$\alpha^+\Delta^−$ mice, spaced training was able to rescue the memory deficits of the Zif268$^{−/−}$ mice. Taken together, these data support the hypothesis that Zif268 acts as part of a cascade of transcription factors required for protein-synthesis-dependent L-LTP and long-term memory formation.

Both C/EBP$\delta$ and Zif268 are transcription factors that in turn regulate expression of other genes. The identity of these genes that relate to LTP and learning and memory remains unknown. Careful phenotypic characterization combined with expression profiling and promoter element analyses may yield the identity of these genes, and thus further our understanding of the molecular mechanisms underlying long-term memory.

Restructuring Synapses

So far, we have only discussed intracellular mechanisms of learning and memory. The synaptic cleft, however, is also a target for modification during short-term and long-term memory formation. Two mediators of extracellular remodeling are urokinase-plasminogen activator (uPA) and tissue-type-plasminogen activator (tPA). These serine proteases convert zymogens into their active, proteolytic state (e.g., conversion of plasminogen into plasmin, which in turn activates...
metalloproteases). These proteases are thought to be involved in structural alterations triggered by synaptic plasticity. Both uPA and tPA are expressed in the CNS although tPA is the more abundant of the two.

These proteases also play a role in synaptic plasticity and learning and memory. Mice overexpressing tPA show enhanced PPF as well as enhanced early and late LTP. These mice show a corresponding enhancement in spatial memory tested with the Morris water maze (70). If enhanced function of tPA results in enhanced synaptic plasticity and memory, presumably increasing the malleability of the synaptic milieu, then disruption of tPA would be expected to conversely affect these two processes. Abrogation of tPA activity via genetic or pharmacological intervention blocks L-LTP (9, 40, 53). Accordingly, tPA null mice show memory deficits. However, they show a complex behavioral phenotype that includes a deficit in active avoidance but normal performance in the water maze as well as a female-specific enhancement in contextual fear conditioning (53). Barring sex differences, regulation of uPA may provide some functional redundancy in the tPA−/− mice, thereby confounding interpretation of the direct role of tPA in learning and memory. Mice over-expressing uPA, however, show impairments in spatial learning in the water maze (79). Thus, these studies indicate that while uPA and tPA may share some degree of functional redundancy, they do not have identical roles in learning and memory. Other extracellular proteases, such as metalloprotease, are also induced upon neuronal activation, suggesting that multiple proteases remodel synaptic connections and thus affect synaptic plasticity and learning (107).

MOUSE MODELS OF COGNITIVE DISORDERS

An important facet of the new field of molecular and cellular cognition is transgenic mouse models of cognitive disorders. The study of these models will be key in understanding molecular and cellular mechanisms of human cognition, since they will enable us to apply ideas and findings derived from animal studies to human cognitive disorders. These studies may also bring us closer to designing rational therapies for these problems. For example, some mouse models of hemostatic diseases have effectively recapitulated hemophilic diseases and have been used to design effective clinical therapies [reviewed in (65a)].

However, a model is a simplified representation amenable to in-depth studies. Therefore, to be useful, mouse models do not have to recapitulate every feature of the human disease. Actually, simpler models often point to natural subdivisions of a more complex cluster of phenotypes associated with the human disorder. For example, mouse models of Alzheimer’s disease do not show the neurodegeneration characteristic of the human disorder. Nevertheless, they show accelerated age-related cognitive decline, a finding that supports the idea that cognitive decline cannot be solely attributed to neurodegeneration [reviewed in (4)]. Some mouse models of NF1 do not have any tumors, but show clear learning deficits, confirming that the learning disabilities of the patients are probably unrelated to their tumors (24).
There are also cases where mouse models do not exhibit any signs of the disorder, suggesting that functional redundancy or alternate pathways exist. For example, mice deficient for HPRT, the gene mutated in Lesch-Nyhan syndrome, have no overt phenotype. Since purine metabolism differs in mice and men, other purine monophosphate synthesizing enzymes may play a more important role in disease formation. The HPRT-APRT double mutant, however, showed no self-injurious behavior (35). Regardless of the results, modeling disorders in mice always leads to new insights into the biology associated with the gene or pathways studied. It is possible that careful study of the HPRT mouse, for example, will lead to strategies to circumvent the problems associated with the human condition.

Despite these caveats, mouse models for human learning and memory disorders have been very successful. Insights from studies of animals models of Neurofibromatosis I, the most common hereditary form of learning disabilities, Fragile X mental retardation, the most common hereditary form of mental retardation, and Alzheimer’s disease, the most common cause of age-related dementia, have already furthered our understanding of these disorders and figure prominently in our efforts to develop cures. An in-depth treatment of this topic is outside of the scope of this review.

CONCLUSIONS

Memory is a multilevel problem that spans the whole organizational complexity of biology from molecular mechanisms to the properties of interactions in complex societies. So far, the new field of molecular and cellular cognition has focused its studies on the interface between molecular, cellular, circuit, and behavioral processes. Although the majority of the studies reviewed here involved knockouts and transgenic mice, a growing number of other gene-based approaches also show considerable promise, including antisense and viral approaches. Their potential clinical value makes these approaches quite appealing. Additionally, emerging genomic approaches, such as expression studies with microarrays, promise to complement genetic studies, since it is problematic to imply function solely from studies of dysfunction (i.e., genetics experiments).

Molecular and cellular cognitive studies came of age with the advent of gene knock-out technology. This technology allowed molecular biologists, neurophysiologists, and psychologists to work side by side with the same animals. This led to an unprecedented explosion of multidisciplinary studies that tested the role of key physiological mechanisms in cognitive phenomena, such as learning and memory. Although the current review focused on hippocampal-dependent learning and memory, studies in this emerging field have encompassed many other memory systems (cerebellum, amygdala, striatum, cortex) and other cognitive functions (fear, attention). Interestingly, as transgenic analysis of mechanisms of learning and memory developed, the field tapped into classic, tried and true genetic methods such as determining epistatic interactions. The field is also beginning the
process of archiving all genes involved in learning and memory. This process is in its infancy and will involve the collection and characterization of all learning and memory genes. This will entail the complementary and systematic characterization of transgenic mice, as well as forward genetic searches for novel allelic variants derived from mutagenic screens. We estimate that more than 5000 genes have already been knocked out, representing a vastly underused resource. Cataloging the learning and memory phenotypes of these mice will undoubtedly identify novel signaling pathways and cellular processes affecting learning and memory. Mutagenic screens, on the other hand, will be used to scan for mutations with robust effects on learning and memory. The challenge will not be in identifying these genes, as this will assuredly happen. Rather, the difficulty will be to determine when, where and how these novel genes affect memory formation. This is a challenge that will require the full range of expertise of this emerging field, since it will involve extensive genetic, molecular, biochemical, electrophysiological, and behavioral studies. It is rewarding to see a growing number of young scientists trained in molecular and cellular cognition labs who have developed considerable expertise in all of these areas. These scientists and their students may be able to synthesize the complex body of molecular, cellular, circuit, and psychological data resulting from these studies.

Besides educating a new generation of interdisciplinary neuroscientists, what has been the major contribution of the first 10 years of this young field? The single most important contribution of this young field to neuroscience is the demonstration that changes in synaptic function are critical to learning and memory. This is not to say that the field accomplished this alone. Instead, the overwhelming amount of data provided in favor of this hypothesis by this young field synergized with and complemented existing electrophysiological and behavioral studies carried out in a number of other species, ranging from the sea slug Aplysia to monkeys and people. In the past ten years, the sheer volume and surprising internal consistency of the transgenetic experiments testing the idea that synaptic changes are required for learning and memory, established the validity of this hypothesis beyond a reasonable doubt. This is not to say that there are no results that question this hypothesis. The complexity of both the biological phenomena and the experimental approaches involved are such that it is surprising that there are not more apparent contradictions of this simple hypothesis. Recent studies have already identified other mechanisms that work in parallel and synergize with synaptic plasticity in memory formation (for example, see (25).

Although, molecular and cellular cognition studies have provided compelling evidence that synaptic plasticity is required for learning, it is still unclear how this happens, what other cellular mechanisms are involved, and where and how they act. The field is full of major questions that are even difficult to frame, such as the nature of the molecular and cellular mechanisms that encode, retrieve, edit, orchestrate, and use stored information. However, the last ten years have prepared us for the exciting journey ahead. We know that mechanisms of plasticity are central to cognitive function and thus, we have a starting point. We have an experimental
and theoretical framework to guide and instruct our searches, and perhaps most important, we are armed with the invincible optimism of a young field.

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LITERATURE CITED

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in forebrain-specific presenilin-1 knock-out mice is associated with reduced clearance of hippocampal memory traces. 


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Figure 1  Behavioral tasks used to test learning and memory. (A) The hidden version of the Morris water maze is used to test hippocampal-dependent spatial learning. Picture courtesy of Ype Elgersma. (B) Contextual fear conditioning is used to test associative learning in which a footshock is associated with the context in which the footshock occurred. Close-up picture courtesy of Paul Frankland, train and test images reproduced with permission from Anagnostaras et al. 2000 Learning and Memory vol 7:58.
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