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GENE TARGETING AND THE BIOLOGY OF LEARNING AND MEMORY

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ABSTRACT

The general goal of genetic studies of learning and memory is to develop and test theories that explain the animal's behavior in neuroanatomical, neurophysiological, cellular, and molecular terms. In this review we describe the role that gene targeting and other transgenic techniques have had in the study of mammalian learning and memory. We focus especially on the hippocampus, a brain structure that is thought to be central to the processing and temporary storage of complex information. We also discuss the main issues that confront this young field, as well as our vision for its future.

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INTRODUCTION

What Should Be Included in An Explanation of Learning and Memory?

Before we can argue that genetics will be central to developing explanations of learning and memory (L&M), it is useful to describe what such an explanation should include. Knowledge and insight into behavior are obviously essential in building and testing theories of L&M. Consequently, insights into animal behavior will continue to occupy a central place in this quest. Beyond behavioral analysis, studies of L&M must also include information about the neuroanatomy of the brain regions involved. Neuroanatomical results have often provided hints about the kinds of computations that a specific brain region could support. For example, the interconnective pattern of CA3 (4) suggests that it can be used as an association matrix. Such insights about the neuroanatomical structure of a brain region can also be extremely important in guiding physiological studies. The striking neuroanatomy of the cerebellum, for example, suggested models that led to the discovery of long-term depression (LTD) in the cerebellum (41).

In addition, studies of L&M will need to include information about the physiological properties of cellular networks in the brain. A number of techniques have been developed to study the output of these networks. A cluster of four very small electrodes can be used to record action potentials from single units (cells) during behavior. For example, single-unit recordings have shown that cells in the hippocampus of rodents tune their firing to certain places ("place" cells) in the animal's environment (62). Place cells have provided clear evidence that the hippocampus processes spatial information (61).

Information about molecular and cellular processes in the brain will complement and synergize with results from behavioral, neuroanatomical, and circuit studies. First, molecular techniques provide a way to manipulate the properties of complex circuits. The recent development of region-specific knockouts (KOs) in the brain (83), for example, allowed the study of CA1 place cells in mice lacking long-term potentiation (LTP) in the CA1 region of the hippocampus (57). The importance of N-Methyl-D-Aspartate Receptor (NMDAR)-dependent LTP for the generation of CA1 place cells remained untested until the development of the CA1-specific NMDAR KO mice (83). Thus, cellular mechanisms are not only an intricate part of theories of brain function (to understand L&M will require insight into molecuular and cellular mechanisms), they will also constrain and guide higher-order studies of brain function.

Formal models will also be an important part of studies of L&M (56). The complexity of the phenomena involved and their highly dynamic and nonlinear character will require formal representations of their properties (26). Most likely these models will not be replicas of functioning brains, but instead they will simply be useful abstractions of particular phenomena of interest. For example, in trying to understand how protein function regulates synaptic plasticity, it would be useful to model the complex biochemical interactions between the second-messenger pathways involved. Similarly, modeling has been crucial for our understanding of how the synaptic properties of the hippocampus could participate in the generation of place cells, and of how place cells may be used to generate a spatial map (56).

What will be the role of genetics? Genetic manipulations, such as gene targeting, allow studies that enable us not only to dissect complexity at several different levels, but also to integrate the resulting findings. The results obtained so far demonstrate that at each level, the mutations analyzed affect only a restricted subset of phenomena, leaving others relatively untouched. With this specificity, it is now possible to follow the effects of the mutation from level to level, all the way from molecular to behavioral analysis. Despite their complexity, this cross-level analysis is essential to developing and testing integrative explanations of L&M because it allows the simultaneous manipulation of phenomena at all the relevant levels of analysis. Although we may not understand the connections between each of these levels for each of the mutants analyzed, key patterns will emerge to provide biological explanations of L&M.

Memory Categories in Humans

Extensive behavioral and neuroanatomical studies have uncovered a number of different systems that process and store different types of information in the brain (79). For example, human cognitive studies have uncovered at least five different types of memory classified under two major categories: declarative and nondeclarative memory (91). Perhaps the most characteristic human form of memory is declarative or explicit memory, since it concerns memories that can be described to others, such as the recollection of facts and events. This form of memory depends on medial temporal lobe structures, such as the hippocampus (91).

In contrast, nondeclarative forms of memory—which include procedural memory, priming, classical conditioning, and nonassociative memory—do not involve awareness or conscious recall (91). For example, procedural memories involve skills and habits, such as learning to ride a bicycle, and they are thought to be dependent on the function of the basal ganglia (32).

Human and Animal Memory

The relation between human and animal learning is complex, but there are clear parallels between the two. For example, in humans the hippocampus has a critical and very specific role in declarative memory (78). Adult patients with lesions in this structure cannot recall facts and events that occurred months to years before the lesion (e.g. surgery). However, their remote declarative memories (for example, their childhood events) are usually unaffected by the lesions (78). These lesions have another devastating impact in patients: Without a functional hippocampus, patients are unable to create new declarative memories. Strikingly, there are close similarities between the effects of hippocampal lesions in humans and in other animals [e.g. primates and rodents (78)]. For example, in rodents hippocampal lesions block the formation of new contextual memories (43, 65). These lesions also affect the memory for recent events preceding the surgery more severely than remote ones (43, 85). Thus, although animals may lack explicit memories, their hippocampal systems support complex forms of memory that share the striking temporal gradient of declarative memory in humans (78).

Furthermore, the molecular and cellular mechanisms underlying different categories of memory may be similar, and even conserved across species. The disruption of the cAMP Responsive Element Binding protein (CREB) not only affects different forms of long-term memory in mice (spatial, contextual, cued, and socially transmitted), but it is also known to do so in several species (Aplysia, flies, rats, and perhaps humans) (81). These findings are consistent with the idea that fundamental biological mechanisms are conserved during evolution and implemented in a wide range of organisms and circumstances.

Why Study Memory with Mice?

Since there may be evolutionary conservation of L&M mechanisms, why not concentrate all efforts in studying very simple organisms? Undoubtedly, studies in these simple organisms, such as *Caenorhabditis elegans* (42) and *Drosophila* (84), will continue to yield interesting information on L&M. For example, gene discovery is more straightforward in flies than in mice, and there are advantages to studying behaviors modulated by an identifiable number of cells (*C. elegans* has a total of 302 neurons!). However, in addition to the similarities between molecular and cellular mechanisms across species, there will be differences that reflect the unique characteristics of the mammalian brain (e.g. neural circuit and organizational complexity). Furthermore, L&M is a multisystem problem with components that span several levels of complexity in the brain (systems, circuits, cells, and molecules). Information about the biology of each of these levels will be important for theories of learning and memory. This multisystem analysis is currently possible in mice but not in organisms like *C. elegans* and

Drosophila. Although simple organisms will continue to have an important role in the molecular and cellular studies of L&M, their usefulness as models for human cognition may be limited.

Probing Hippocampal Function

Neuroanatomical analysis has shown that the hippocampus receives inputs from all of the main neocortical and thalamic areas, as well as from other limbic structures such as the amygdala (3). This kind of connectivity is suggestive of a structure that integrates high-order information processed elsewhere. Single-unit studies suggest that the hippocampus functions as a comparing device that constantly assesses and contrasts incoming signals, working fundamentally as a novelty detector.

Important connections between hippocampal physiology and behavior were derived from studies of animals exposed to a variety of pharmacological compounds with known physiological effects. For example, NMDAR antagonists delivered to the hippocampus block LTP, and also impair some measures of hippocampal-dependent learning (21), reinforcing the suggestion that the Hebbian mechanisms of LTP might be required for learning. However, the involvement of this receptor in other electrophysiological mechanisms complicates the interpretation of these studies, and recent results show that under some conditions antagonists of this receptor do not seem to block hippocampal-dependent learning (6, 70).

Another important strategy to investigate neurophysiological models of behavior is to study directly the physiology of behaving animals (in vivo electrophysiology). For example, electrophysiological recordings of hippocampal neurons in rats during learning showed that they fire in ways known to be optimal for the induction of LTP in vitro (47, 63). This finding considerably strengthened the connection between LTP and learning. However, the physiological complexity of a working brain can obscure the relevance of any single physiological phenomenon. For example, short-term exploratory modulation or STEM, a neurophysiologic correlate of learning, also is partially affected by increases in brain temperature during active exploration (59). These concerns once again underscore the assertion that no single approach will suffice to unveil the exceedingly complex interactions between physiology and behavior.

Although behavioral studies of lesioned animals, imaging studies, and single-unit recordings have started to shed some light on the types of tasks that engage the hippocampus, little is known about the kind of information that reaches this structure, how this information is transformed there, and what exactly is the nature and destination of the output. These are immensely complex questions that current studies have not resolved. Before we can formulate reasonable hypotheses concerning the mechanisms of hippocampal-dependent learning, we

need more insight into how changes in the cellular and circuit mechanisms of this structure affect hippocampal-dependent L&M. For example, hippocampal neurons express a variety of different types of synaptic plasticity. Some enhance synaptic communication, others depress it; some do so for short periods of time (ms, s, min), others have a longer-lasting effect. Are all of these forms of plasticity required for learning and/or memory (see below)? Similarly, hippocampal neurons have an overwhelming array of K^+, Na^+, Ca^{2+}, etc, currents in their soma, dendrites, and axons. Is the modulation of these currents involved in hippocampal-dependent L&M?

Are There Learning or Memory Proteins?

Before reviewing the contributions of gene targeting to the study of L&M, we discuss first the crucial relation between single genes/proteins and behavior. Misunderstanding of this central issue leads to misleading preconceptions and to some avoidable confusion. Mutations of specific genes can have an enormous impact on behavior. For example, the loss of the α Ca^{2+} calmodulin kinase II (αCaMKII) leads to profound behavioral changes in mice, ranging from alterations in social responses to profound deficits in learning tasks (73, 18). However, the extensive evidence that the mutation of genes can cause specific behavioral disruptions cannot be used to conclude that single genes have well-defined behavioral functions.

Outside of the confines of biochemical reactions, and other cellular events, gene products may not have easily attributable functions. The major reason for this is that biological events are nonlinear and highly dynamic at all levels of organization. The well-known effect of genetic background on the phenotype of mutations is a powerful demonstration of the complexity of molecular interactions determining a phenotype. For example, even the simplest animal behavior involves multiple brain regions, within each region there may be several networks involving many cells, and the functional output of a single cell involves thousands of proteins working together. As outputs are integrated across many interacting systems (biochemical pathways, cells, circuits, brain regions, etc), the complexity of the emerging output can confound the initial contributions of single proteins.

But, if it is not possible to make direct connections between genes and behavior, then what is the point of using mutants in studies of L&M? First, mutations are a means to manipulate the molecular, electrophysiological, neuroanatomical, and behavioral characteristics of an animal. By contrasting mutants with their littermates, it is possible to unravel connections between the phenomena studied. Even in the relatively short time that mice mutants have been used in studies of L&M, interesting connections between molecular, cellular, and

behavioral function have already been uncovered. The following are a few representative examples of gene-targeting studies that provide exciting new insights into L&M.

GENE TARGETING AND THE STUDY OF LEARNING AND MEMORY

LTP: A Mechanism of Learning and Memory

Most of the genetic work on the basis of hippocampal-dependent L&M has focused on long-lasting changes in synaptic efficacy (i.e. LTP and long-term depression or LTD). These studies are based on the idea that information can be stored in neural circuits by adjusting the synaptic strengths or weights of neurons. This idea has been tested formally. Most of the computer modeling done on this subject is based on Hebbian mechanisms of L&M. With remarkable foresight, Donald Hebb predicted the existence of an L&M mechanism (36), Hebb's rule, with many of the properties found in LTP (10). In 1949, he proposed (36) that, "When an axon of cell A...excite(s) 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." Very few other ideas about mechanisms of L&M have captured the interest and imagination of neuroscientists to the extent that the Hebbian hypothesis and its derivatives have done.

Structures such as the hippocampus, with an acknowledged participation in L&M, express various forms of LTP and LTD, which have the very properties thought to be crucial for memory formation (associativity, specificity, reversibility, and stability) (10). Like memory, LTP is not a single phenomenon but a class of phenomena, with distinct sets of biochemical substrates and time courses. LTP has been most intensively studied in the CA1 region of hippocampal slices, because of the ease of recording in this region and because lesions of this structure in humans lead to memory impairments (72). Early-LTP (E-LTP) in CA1 lasts approximately one hour, and its induction involves the activation of NMDARs and a subsequent postsynaptic increase in Ca^{2+} (10, 38). This increase in Ca^{2+} activates a number of protein kinases and other enzymes (e.g. adenylate cyclase) thought to trigger the biochemical cascade of events that results in the long-lasting enhancement of synaptic transmission (10, 38).

Pharmacological blockers of LTP (e.g. NMDAR-blockers) were shown in many cases to also disrupt memory formation [see for example (21)]. However, seemingly contradictory results abound in this literature (16), which could easily be due to the incomplete specificity of the compounds used, and to differences

among the complicated behavioral procedures involved. Thus, in the early 1990s the time was ripe to apply the newly developed gene targeting to probe the connection between LTP and L&M.

A number of mutations affect both LTP and L&M. For example, genetic deletions of the α-Ca^{2+}-calmodulin kinase II (αCaMKII) (73), Fyn tyrosine kinase (31), type I adenylate cyclase (86), NMDA receptor $\varepsilon 1$ subunit (69), and metabotropic glutamate receptor 5 (mGluR5) (50) all affect E-LTP to some extent in the hippocampal CA1 region, and hippocampal-dependent L&M.

Many problems remain, and they fall into at least two categories. First, there is the possibility that LTP is not involved in learning or memory, but that it shares many biochemical components with the cellular mechanism(s) of L&M. To address this possibility, it is worthwhile reexamining the details of experiments associating LTP with learning, since one would expect to see some dissociation, even if subtle, between these two phenomena. Second, there is a fundamental theoretical problem with claiming that a single aspect of cellular physiology could be responsible for L&M in the hippocampus or in any other structure. Learning is a property of circuits and brains; thus it is extremely likely to involve a variety of mechanisms. The gene-targeting studies that follow address these issues.

αCaMKII and Plasticity

The first gene-targeting study of LTP and memory used mice with a null mutation for the αCaMKII (73, 75). The αCaMKII is mostly expressed postnatally in forebrain structures (15). Previous electrophysiological studies had shown that peptides that inhibit the activity of this kinase can block the induction of LTP in the CA1 region of the hippocampus (39, 51, 52). Consistent with these pharmacological findings, the genetic studies showed that the αCaMKII mutants had severe deficits in LTP and in LTD in hippocampus and neocortex (44, 75, 80).

Behavioral studies also uncovered clear deficits in these mutants (73). The mutant mice were severely impaired in the hippocampal-dependent version of the water maze. Importantly, they were able to learn the "visible-platform" version of this task, which shows that the αCaMKII mutants can learn that the platform is the only escape in the pool, and that they have the motivation to escape the water and the motor coordination and sensory perception required to efficiently swim to the escape platform. However, they seem to be unable to learn the spatial relationships required to guide them to the hidden platform. Further behavioral studies with other tests such as the "plus-maze" confirmed that this mutation did not affect all forms of learning (73).

The results described above suggest that the LTP/LTD deficit of αCaMKII mutants may be responsible for their learning abnormalities. Models of L&M

propose that learning triggers changes in neuronal connectivity such as LTP-driven structural plasticity. Even though these changes are difficult to observe directly, it is possible to measure their consequences by studying plasticity in neocortical sensory inputs. After closure of one eye for a few days, cells in the binocular area of the visual cortex appear to respond preferentially to the inputs from the nondeprived eye, as if deprivation triggered a shift of inputs from the deprived to the nondeprived eye. Although visual responses in the cortex developed normally in the αCaMKII-deficient mice (e.g. topography was normal), visual cortical plasticity was greatly diminished (29). Similar results were also obtained in the somatosensory cortex (28).

Just as the deletion of the αCaMKII gene in mice affects LTP and learning, adding a constitutively active αCaMKII transgene also disrupts hippocampal LTP and learning (5, 54, 55). Cloning of the promoter elements of this gene allowed the faithful expression of a mutant αCaMKII gene in transgenic mice. The substitution of a threonine for an aspartate (D) at position 286 of the protein constitutively activated the transgenic kinase (TαCaMKIID286). Electrophysiological analysis of the TαCaMKIID286 mice revealed that the range of tetanic frequencies that normally induce LTP or LTD had been changed in the mutants (55). Behavioral studies with the Barnes maze uncovered learning deficits in the αCaMKIID286 mice (5, 54). Altogether, the gene-targeting and the transgenic studies described above show that the αCaMKII plays a central role in mechanisms underlying cellular, circuit, and behavioral plasticity.

CREB and Memory

The mechanisms mentioned above are likely to be involved in early stages of memory formation (i.e. during learning). However, little is known about the molecular and cellular processes required to support long-term memory (LTM). Previous pharmacological studies showed that the synthesis of new proteins is a pivotal requirement of long-term memory (20). Elegant studies with Aplysia cultured neurons suggested that CREB is one of the transcription factors activated during the induction of LTM (19). It is noteworthy that CaMKII may be one of the kinases in the signaling pathway that eventually activates CREB (9).

Guided by the results in Aplysia, studies in mice (12) and Drosophila (88) also uncovered evidence for the involvement of CREB in LTM. For example, work in Drosophila showed that a dominant-negative mutant form of CREB can block LTM, without having a measurable effect in other memory stages (88). Strikingly, induction of a transgenic CREB activator under a heat shock promoter triggered LTM with a single trial, whereas in control flies the induction of LTM required multiple spaced trials (87). Similarly, in Aplysia, removing CREB repression with an antibody against a natural CREB blocker also avoids the requirement for multiple spaced serotonin applications in the induction of

long-term synaptic facilitation (8). These results argue for a role of CREB in the induction of LTM, and suggest that the requirement for spaced training during the induction of LTM in Aplysia and in flies is related to the levels of CREB activator (88).

Studies in mice also demonstrated the requirement for CREB in several different forms of memory (12, 45). The CREB mutation affects several different forms of memory. Recent studies with anti-sense oligonucleotides against the CREB gene have confirmed the involvement of this transcription factor in memory formation (35), suggesting that the effects observed in the mutant mice are not due to developmental abnormalities caused by the CREB mutation.

In parallel with the Drosophila and Aplysia results, spaced training in mice overcomes the memory impairments detected in contextual conditioning, spatial memory (water maze) and in the social transmission of food preferences tasks (45). For example, after a single trial normal mice can form a long-lasting contextual memory of the cage in which they were shocked. Despite normal sensory perception, the same single trial can only trigger a transient ($<$60 min) memory in CREB mutant mice (12). Not even training that produces maximal long-term memory in WT mice (5 trials with 1 min intervals; massed training), can compensate for the profound contextual amnesia of these mutants. In contrast, two spaced trials with a 1-h interval (spaced training), which in control mice do not trigger higher levels of conditioning than a single trial, can nevertheless induce robust 24-h memory in CREB mutants (45).

All together these results indicate that CREB-mediated transcription has an impact on the number of trials and intertrial interval required for memory formation in mice. Flies and mice with presumably more active CREB require less training than flies and mice with less active CREB. Therefore, in both mice (45) and flies (87), manipulations of CREB function affect directly the amount of training required for the induction of LTM. Related electrophysiological results from Aplysia (8) also suggest that these effects apply to nonassociative forms of memory such as habituation and sensitization.

Protein synthesis is not only essential for long-term memory (LTM), but it is required also for late LTP (L-LTP) (27, 53). LTP induced by a train of 100 pulses (250 μsec pulse width) at 100 Hz was stable for at least 2 h in controls. In contrast, LTP returned to baseline levels within 1.5 hours in CREB mutant mice (12). Moreover, CRE-mediated transcription is induced after a tetanus-evoking LTP (22, 40). Furthermore, transgenic mice expressing a mutant form of a regulatory subunit of PKA, a kinase that activates CREB, also show L-LTP deficits (1). Consistent with the idea that LTP underlies memory, LTM is specifically impaired in these transgenic mice (1).

Undoubtedly, CREB is not the only transcription factor modulating the induction of protein synthesis required for LTM (2). For example, CREB functions

in association with other factors, and there are other transcription factors whose activity is influenced by cellular changes in cAMP and Ca^{2+}, the two upstream signals that activate CREB (48).

The Connection between LTP and Learning

The results presented above suggest that CREB-dependent transcription is not only required for memory, it is also required for long-term synaptic changes. This finding is consistent with others suggesting that LTP has a role in L&M. However, the connection between LTP and memory is not a simple one. First, it is unlikely that LTP is the only cellular mechanism required for L&M. Second, LTP is a complex phenomenon present in many brain regions, and it is possible that some forms of LTP are not essential for L&M, or that LTP in certain regions is not required for certain L&M tasks. Finally, LTP may be involved in behavioral responses other than memory. Not surprisingly, evidence shows that some forms of LTP may be eliminated without completely disrupting L&M, and that manipulations intended to disrupt LTP and learning seem to affect other behaviors such as stress, attention, and motor coordination. Additionally, the agents used to disrupt LTP also affect other systems that may themselves disrupt learning, seriously confounding many of the experiments testing the connection between LTP and learning.

Although most gene-targeting experiments have provided data supporting the hypothesis that LTP is involved in L&M, results in several experiments seem to question this hypothesis. For example, mutants that lack either the β1 isoform of the catalytic subunit (C β1-) of PKA (66) or the β isoform of its inhibitory subunit (RIβ) (13) lack CA3 LTP, but have normal learning (37). Also, both mutants show a loss of LTD and LTP depotentiation in the CA1 region (13, 66). Studies with RIβ-mice found that LTD is also blocked in the perforant path–dentate gyrus synapse (13). Similarly, mGluR2-deficient mice are impaired in mossy fiber LTD, and also have no learning impairments (89). These results were unexpected since LTD-like phenomena are thought to have a key impact on the storage capacity of neuronal networks, and on fine tuning synaptic weights during learning (26). Without the ability to reverse LTP, all synapses in an active network should become quickly potentiated and, therefore, unable to store additional information.

These studies underscore the complexity of the association between long-term synaptic changes (LTP/LTD) and L&M. Note that the measurements of LTP/LTD described above were done in hippocampal slices, and that the mutations did not completely block LTP. Thus, the effects of the mutations in vivo may be much milder than the effects measured in vitro. This is a serious concern that may be circumvented by electrophysiological studies in vivo. For example, mice mutant for the neuronal glycoprotein Thy-1 show abnormal dentate gyrus

LTP (both in vivo and in slices) but normal learning in the water maze (60). The LTP deficit seems to be due to abnormally high inhibition mediated by $GABA_A$ receptors. When this inhibition is removed by adding bicuculline, an antagonist of $GABA_A$ receptors, LTP can be induced normally in these mutants (60). Additionally, recent studies with Thy-1 mutants showed that LTP measured in vivo in freely moving mice is reduced, but not absent (23).

The problems with the interpretation of these experiments illustrate the need to understand the events that surround "natural" LTP induction. Without insight into these events it will be very hard to interpret data concerning the relation between LTP lesions and learning. Nevertheless, LTP is probably not sufficient for memory formation. Understanding how other electrophysiological mechanisms modulate L&M will have an important impact on the connection between LTP and memory.

A Role for Short-Lived Plasticity in Learning

In addition to long-term plasticity, synapses express other computationally interesting properties. For example, synapses adjust their properties according to their recent (ms–min) history of activation. A single action potential may or may not induce release of neurotransmitter from a CA3 axon terminal (11). However, in the next 400 ms this single action potential dramatically increases the probability that neurotransmitter will be released after stimulation by another action potential (11). If a CA3 axon terminal is activated by a train of action potentials, however, the probability that a single action potential will induce neurotransmitter release will be increased for seconds or even minutes following the tetanic stimulation (even in the absence of LTP induction). These properties seem ideal to implement complex functional algorithms, and it is possible that the brain uses them for processing information (25). For example, within milliseconds to seconds of exposure to a complex new object, an animal may mount a behavioral response to it. The speed and complexity of these cognitive events implies the existence of mechanisms of information processing capable of working in the ms–s time domain. Thus, it is very possible that short-lived mechanisms of synaptic plasticity (SLP) have a key role in these events (25).

Studies with a variety of organisms suggest that SLP may allow simple circuits to adapt quickly to changing environments. For example, short-term changes in synaptic efficacy seem to underlie activity-dependent regulation of the siphon-withdrawal reflex in Aplysia (25). In addition, some Drosophila learning mutants show abnormal short-term plasticity in the neuromuscular junction, suggesting that similar CNS deficits may underlie their learning impairments (90).

Computational studies have also suggested that SLP may have a role in L&M (14, 49). With elements of SLP, a continuous time neuronal network model was

able to discriminate different temporal patterns, suggesting that time-dependent synaptic properties may enable networks to transform temporal information into a spatial code (14), perhaps a critical element in many forms of learning.

Consequently, synaptic and computational studies suggest that SLP may have a significant role in information processing. Recently, this hypothesis has also been tested with mutants that have normal long-term potentiation (LTP), but abnormal SLP. Mice heterozygous for a null mutation of αCaMKII (17, 76), synapsin II mutant homozygotes (SyII$^{-/-}$) (67), and mice homozygous for both synapsin mutations (SyI/II$^{-/-}$) (67, 77) have deficits in SLP, although studies of LTP revealed no abnormalities in these mutants. However, these mutants show marked impairments in hippocampal-dependent learning, as measured by contextual conditioning and the water maze (74). These data strongly suggest that hippocampal SLP is involved in learning.

SLP is not a single discrete phenomenon, but reflects a number of distinct mechanisms with distinct time frames and biochemical substrates (25). For example, paired-pulse facilitation (PPF) refers to the facilitation observed in the second of a pair of stimuli separated by 30–400 ms. Augmentation refers to the presynaptic increase in release observed seconds after a period of high-frequency stimulation (e.g. 100 Hz, 1 s). Studies in the CA1 region demonstrate that the SyII$^{-/-}$ mice show a decrease in augmentation, but normal PPF (67), whereas the αCaMKII$^{+/-}$ mutants reveal a decrease in PPF and an increase in augmentation (17). Additionally, mice lacking synapsin I show an increase in PPF, no change in augmentation, and no learning deficits (74). These genetic studies constitute the first concrete evidence that PPF and augmentation are not regulated by similar presynaptic molecular mechanisms, as previously thought. Furthermore, these studies open up the possibility that SLPs are involved in mammalian learning (74).

Limitations of Current Approaches

Despite their power and usefulness, there are clear limitations in gene-targeting studies of L&M. Many genes are expressed to some extent during development, and therefore mutations in these genes could have developmental effects that could confound studies of L&M. Additionally, it is difficult to restrict the mutations to desired regions or cell populations in the brain. As a consequence, it may be problematic to attribute behavioral deficits to functional disruptions in a specific brain region. Experimental solutions to these problems are discussed in the following section.

THE PRESENT FUTURE

In addition to adding, replacing, or removing either genes, domains of genes, or even single amino acids of interest, it is now feasible to restrict mutations

to certain tissues (83) and, possibly, to specific times. Recent developments have also expanded the tools and techniques that can be used in the analysis of mutant mice. For example, single-unit recording techniques have been applied to the study of mouse mutants (see below). All together, these new approaches address earlier concerns with gene targeting (e.g. developmental effects), and they also promise to open exciting new doors into the analysis of brain systems underlying behavior.

Tissue-Specific KOs

A strategy recently developed for the restricted inactivation of genes in the mouse is based on the bacteriophage P1-derived Cre/loxP recombination system (33, 34). The Cre recombinase recognizes loxP sites (34-bp sequences), and specifically excises loxP-flanked gene segments from the genome (71). For example, Cre expression regulated by the αCaMKII promoter was used to delete the NMDAR-gene from the CA1 region of the hippocampus (83). The CA1-specific NMDAR mutants specifically lack NMDAR-dependent LTP in this region. In addition, they also have pronounced spatial learning deficits in the Morris water maze (83). These results indicate that NMDAR-dependent LTP in the CA3/CA1 synapse is required for spatial L&M.

It may be possible to control the expression or activity of Cre-recombinase in the brain with other systems. For example, fusing Cre with a mutant ligand-binding domain of the estrogen receptor (LBD) should place Cre-activity under the regulation of ligands of the mutant receptor [e.g. tamoxifen (24, 58)]. These ligands should turn Cre on and in their absence Cre should be off. It may also be possible to regulate expression of Cre in mice with the tetracycline-regulated (tet) system (30). In this case, the expression of Cre is regulated by tetracycline-sensitive promoters. There are two versions of this system: In one version, tetracycline-derivatives activate the expression of Cre, whereas in the other they repress it. The tet system was first shown to regulate the activity of a luciferase reporter gene in transgenic mice (30), but has recently been shown to regulate the expression of a mutant form of αCaMKII (54). Conditional, cell type–specific inactivation of an endogenous gene has only been accomplished in nonneuronal tissues (46).

Inducing the Expression of Transgenes in the Brain

Inducible gene targeting can irreversibly delete a gene of interest. After the deletion of the gene, the protein remaining has to be naturally degraded before the mutant can be studied. Depending on the stability of the protein, this may take a few days or a few weeks. This slow time course may be problematic for many L&M experiments where faster time resolutions may be needed to separate the effects of the mutation on acquisition versus retrieval of information.

Thus, in addition to inducible gene targeting, it will be useful to derive mice with inducible transgenes (e.g. tet system) for the study of brain function.

The advantages of using inducible transgenic approaches is that the effects of the mutant protein can be reversed by simply turning off the transgene (inducible KOs cannot be reversed). Furthermore, the time required to turn on the expression or activity of the mutant protein can be relatively fast. With inducible KOs, the protein remaining after the genetic deletion may take weeks to degrade and disappear. Inducible transgenics only involve a single type of mutant mice, whereas the inducible KOs require the derivation of at least two different types of mice (the "floxed" line and the inducible Cre lines).

The disadvantage of the inducible transgenics is that the normal protein is present and may confound the effects of the induced mutant protein. Additionally, transgenic constructs almost never faithfully reproduce the expression of the endogenous proteins. As a result, the mutant proteins are expressed in inappropriate places, thus creating the potential for artificial and misleading results. In addition, it is often difficult to express the right amount of the mutant protein. Too little may not have a measurable effect, and too much may result in nonspecific effects (e.g. an overexpressed kinase with a wide substrate range may phosphorylate inappropriate substrates when overexpressed). Nevertheless, inducible transgenics are a powerful tool that will have many valuable applications. Although many technical elements are already available, this approach has not yet been applied to the study of brain function.

In addition to the inducible expression of transgenes, it is also possible to trigger the suppression of the transgene. So far the tet system has been used to inducibly suppress the expression of a transgene encoding a constitutively active form of CaMKII (54). Before suppression of the transgene, the mutants were impaired in CA1 LTP, in spatial learning and in fear conditioning (54). Remarkably, the inducible suppression of the transgene reversed the phenotype (54). This surprising result showed that the expression of the mutant CaMKII affected LTP and L&M, but it did not cause irreversible developmental damage (54), as may have been expected.

Observing Function in the Living Brain

Recent developments in imaging techniques (PET, MRI) have revolutionized human cognitive studies (64), since they have allowed the identification of the brain regions engaged during specific behavioral tasks. Although similar studies with smaller animals currently lag far behind, the required technology is advancing rapidly. Thus, it may be possible in the near future to use MRI to image the brain of mutants during behavioral tasks that do not require head movements (e.g. trace-eye blink conditioning). Other techniques can also be used to image function in neuronal networks, such as electroencephalogram

(EEG) recordings (7), in vivo Ca^{2+} imaging (82), and deoxyglucose activity studies (76).

An important tool used to observe neuronal activity in behaving mice is single unit recordings (57, 68). With this tool, neuronal firing can be recorded during behavior. There are even techniques that allow simultaneous recording from many neurons (57). Single-unit recordings have been used to study place cell activity in mice (57, 68). Place cells are hippocampal neurons (e.g. CA1 or CA3 pyramidal cells) whose firing is tuned to specific places in the animal's environment (61). A CA1-specific deletion of the NMDAR1, which results in deficits in LTP and in spatial learning (83), alters the properties of place cells in this region: The place fields of these mutants are enlarged, and they have decreased spatial selectivity (57).

Transgenic mice expressing a constitutively active form of CaMKII under the control of the αCaMKII promoter also have abnormal CA1 LTP and impaired spatial learning (5, 55). Interestingly, place cells are less abundant in these mutants, and their firing is less specific (68). Unlike wild-type place fields, the place fields measured in mutant hippocampal neurons are unstable and shift to new areas in subsequent recording sessions (68). This indicates that LTP is required for the stability of place fields. The mutants found to have abnormal place cells are also impaired in spatial learning, confirming that CA1/CA3 place cells are critical for hippocampal-dependent spatial learning. These studies mark the beginning of what will undoubtedly be a very active area of research.

CONCLUSION

The use of gene targeting has already contributed valuable insights into the mechanisms underlying L&M. A key feature of these studies is the integration of results from several levels of biological analysis (e.g. molecular, cellular, neural circuit, and behavioral), which is essential for developing explanations of L&M. Here, we have discussed several studies demonstrating the experimental fecundity of this approach. Recent developments both in transgenic technology and in the tools used for the analysis of the mutants demonstrate the exciting future that awaits this young field.

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