Memory Suppressor Genes: Inhibitory Constraints on the Storage of Long-Term Memory

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Synaptic plasticity, the ability of neurons to alter the strength of their synaptic connections with activity and experience, is thought to play a critical role in memory storage. Molecular studies of gene expression during long-lasting synaptic plasticity related to memory storage initially focused on the identification of positive regulators. More recent work has revealed that the establishment of long-lasting synaptic plasticity and long-term memory also requires the removal of inhibitory constraints. By analogy to tumor suppressor genes, which restrain cell proliferation, we propose that these inhibitory constraints on memory storage, which restrain synapse growth, be termed memory suppressor genes.

Experience-dependent changes in the strength of neuronal connections are thought to play a critical role in memory storage and in the fine tuning of synaptic connections during the late stages of neural development. Recently, it has been possible to gain some molecular insights into the synaptic plasticity related to memory storage. These studies have revealed a surprising finding: Long-lasting forms of synaptic plasticity require not only the activation of positive regulatory mechanisms that favor memory storage but also the removal of inhibitory constraints that prevent memory storage. The importance of negative regulatory mechanisms for memory storage invites comparison once again between the study of memory and that of cellular differentiation, growth, and oncogenesis. On the basis of this comparison, we use the term "memory suppressor genes" to describe those genes whose products inhibit memory storage.

The initial molecular studies of development and tumor formation focused on positive regulators of growth: dominantly active oncogenes, such as *src*, whose protein products stimulate cell division (*1*). These oncogenes were originally identified as genes carried by retroviruses that cause cell transformation. The cellular progenitors of these retroviral oncogenes, the proto-oncogenes, were discovered to be parts of normal cellular signaling pathways required for growth and differentiation, and mutation or misexpression of proto-oncogenes contributes to a variety of human malignancies.

It was only after transforming, dominantly acting oncogenes had been well documented that biologists turned their attention to the molecular characterization of recessive mutations: tumor suppressor genes, whose products normally restrain growth. Henry Harris used somatic cell genetics to demonstrate that a malignant phenotype could be suppressed by fusion of a cancer cell with a normal cell and that reversion to malignancy was associated with chromosomal loss (*2*). The first genetic model describing a role for tumor suppressor genes in human disease came in 1971 with Alfred Knudson's remarkable study of retinoblastoma, a rare cancer with both sporadic and childhood forms (*3*). Knudson proposed a simple model for the two forms of this disease, according to which two distinct genetic alterations or mutational "hits" in the same target are required. The implication of this hypothesis was that malignancies occur as a consequence of loss or inactivation of both alleles (loss of heterozygosity) at a locus responsible for regulating normal growth and development. This idea suggested to Knudson that these genes normally suppress malignancies and, therefore, act as "anti-oncogenes" or tumor suppressor genes. Knudson's formulation laid the groundwork for the molecular characterization of tumor suppressor genes. We now know, for example, that more than half of all human tumors have mutations that inactivate the tumor suppressor gene *p53*, whose protein product is a nuclear phosphoprotein that, like wild-type retinoblastoma proteins, restrains growth (*1*).

The importance of negative regulatory mechanisms extends beyond growth and tumorogenesis to include many points in the normal development of the embryo. For example, Spemann's organizer induces neural tissue in *Xenopus* embryos, not by releasing positive inducers of neural cell fate, but rather by releasing inhibitory substances. These diffusible molecules, such as noggin and chordin, antagonize the action of a potent inducer of epidermal cell fate, bone morphogen protein 4 (*4*). Later in development the transcriptional regulation of neuron-specific genes, such as *SCG10* and *synapsin I*, appears to be mediated by a factor that binds to a *cis*-acting silencer element and represses transcription of these genes in nonneuronal cells (*5*). Finally, inhibitory mechanisms are crucial in axon outgrowth and guidance during the later development of neuronal connections (*6*).

Like the study of development and oncogenesis, the early molecular studies of long-term memory and synaptic plasticity also focused on positive regulators. Studies of long-term potentiation (LTP) in the mammalian hippocampus (*7*, *8*), long-term facilitation at the sensory-motor neuron synapse of the gill-withdrawal reflex in *Aplysia* (Fig. 1) (*9*, *10*), and odor avoidance conditioning in *Drosophila* (*11*, *12*) have revealed that the synaptic plasticity related to memory storage recruits a variety of protein kinase signaling cascades and positive regulators of transcription such as cyclic adenosine monophosphate (cAMP) response element–binding protein 1 (CREB1) and C/EBP. Activation of these positive regulators is important in the consolidation of short-term memory into long-term memory storage.

The clearest evidence for repressive mechanisms that impede synaptic plasticity and memory storage has come from the molecular characterization of repressors of CREB in *Aplysia* and *Drosophila*. *Aplysia* sensory neurons constitutively express ApCREB2 (*13*), a leucine zipper transcription factor that is partially homologous to human CREB2 and murine ATF4. The observation that ApCREB2 can repress CREB1-mediated transcription suggested that the threshold for long-term facilitation could be regulated and that facilitation may require not only the activation of ApCREB1 but also the relief of ApCREB2-mediated repression (Fig. 1). If this is so, then relieving this repression would facilitate the activation process and lower the threshold for the long-term process. To test this idea, Bartsch *et al.* (*13*) injected antiserum to ApCREB2 (anti-ApCREB2) into sensory neurons 1 hour before exposure to single or multiple applications of serotonin. A single application of serotonin normally produces only short-term facilitation, but

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paired with injection of anti-ApCREB2, one application of serotonin produces a long-term facilitation that lasts 24 hours, which is accompanied by the growth of new synaptic connections.

The balance between CREB activator and repressor isoforms is also critically important in long-term behavioral memory, as first shown in *Drosophila*. Expression of an inhibitory form of CREB blocks long-term memory but does not alter short-term memory (*12*). Overexpression of an activator form of CREB increases the efficacy of massed training in long-term memory formation (*14*). Thus, in both *Aplysia* and *Drosophila*, blockading or overriding a memory suppressor gene has consequences that are analogous to interfering with the function of a tumor suppressor gene. It leads to a dramatic and, in the limit, abnormal exaggeration of normal cellular function.

In a larger sense, these studies reveal that memory formation is governed by both positive and negative regulators, as is the case with many biochemical processes. Beyond a certain optimum, however, altered expression of either the positive or the negative regulators of these pathways is deleterious. The importance of this optimum range is underscored by studies in *Drosophila* that have shown that the opposing biochemical effects of *dunce*, cAMP phosphodiesterase, and *rutabaga*, adenylyl cyclase, mutations on cAMP concentrations both produce deficits in synaptic plasticity and behavioral memory (*9*).

The cAMP-dependent protein kinase A (PKA) pathway, acting through CREB, appears to stimulate the growth of new synaptic connections in *Aplysia* (*15*). This formation of new synaptic connections between sensory and motor neurons of the gill-withdrawal reflex is seen during long-term memory after behavioral training in intact *Aplysia* as well as after repeated exposure to serotonin in dissociated *Aplysia* cell cultures (Fig. 1). After the injection of anti-ApCREB2, the long-term facilitation induced by one pulse of serotonin also leads to these same structural changes (*13*). Thus, ApCREB2 appears to act as a repressor of the morphological as well as the functional changes that accompany long-term facilitation.

An important clue to the molecular basis of these structural changes came from the identification of a second class of memory suppressor genes, the genes encoding proteins whose concentrations decrease after the exposure of *Aplysia* sensory neurons to serotonin. One group of down-regulated proteins, termed *Aplysia* cell adhesion molecules (apCAMs), is part of the immunoglobulin family of cell adhesion molecules, which includes mammalian neural cell adhesion molecule (NCAM) and *Drosophila* Fasciclin II (Fas II) (*16*). After repeated exposure to serotonin, the concentration of apCAM decreases as a result of the internalization of the transmembrane form of apCAM in the presynaptic sensory neuron (Fig. 1), a process that requires ongoing protein synthesis (*17*). Deletion of the entire cytoplasmic tail of the transmembrane isoform, removal of the PEST sequence, or mutations in the mitogen-activated protein kinase (MAPK) consensus sites block endocytosis (*18*). The selective down-regulation of the transmembrane isoform of apCAM decreases the interaction of sensory cell neurites with each other and leads to the formation of new synaptic connections (*19*).

In addition to their role during long-term facilitation in *Aplysia*, cell adhesion molecules act as negative constraints during development and synaptic plasticity in a variety of other species (*20*). At the *Drosophila* neuromuscular junction, the presynaptic down-regulation of Fas II, a cell adhesion molecule related to NCAM and apCAM, is both necessary and sufficient for activity-dependent synaptic sprouting (*21*). Flies with reduced amounts of Fas II have an increased number of synaptic terminals, and the increased sprouting that normally occurs in the *Drosophila* mutants *ether-a-go-go*/*Shaker* or *dunce* is suppressed by transgenes that maintain Fas II concentrations. Although Fas II down-regulation results in synaptic sprouting, CREB-mediated transcription is required for changes in the functional strengths of these connections.

In forms of learning and memory in other animals, such as passive avoidance learning, in which chicks learn to suppress pecking behavior toward a bead that is coated with a bitter-tasting liquid, the synthesis of new cell adhesion molecules appears to be critical. Relatedly, spatial learning and LTP are impaired in NCAM knockout mice (*20*). Furthermore, increases in the expression of polysialylated NCAM and in the extracellular concentration of NCAM have been observed after hippocampal LTP, and LTP is inhibited by the application of NCAM antibodies, by NCAM-blocking peptides, or by the removal of polysialic

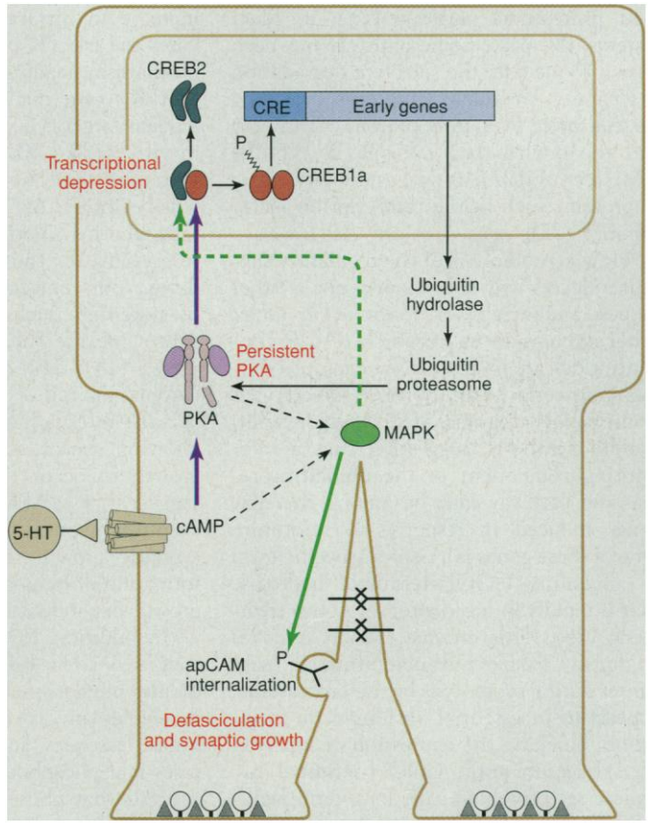

Fig. 1. Three inhibitory constraints on memory formation. During sensitization of the gill-withdrawal reflex of *Aplysia*, facilitatory interneurons release serotonin (5-HT) onto the presynaptic terminals of sensory neurons. Binding of serotonin to a serotonin receptor stimulates adenylyl cyclase, leading to an increase in intracellular cAMP concentrations. This increase in turn activates PKA, and the PKA catalytic subunit then translocates to the nucleus, where it may phosphorylate CREB1. In addition to the activation of CREB-mediated transcription, turning on the long-term process requires the removal of three inhibitory constraints. First, MAPK, which is also activated by serotonin, translocates to the nucleus, where it may phosphorylate the transcriptional repressor CREB2, thereby allowing CREB1-mediated transcription to proceed. Second, COOH-terminal ubiquitin hydrolase, an immediate early gene product, increases ubiquitin-mediated proteolysis, leading to the degradation of the regulatory subunit of PKA and producing persistent PKA activity. Finally, the phosphorylation of apCAM by MAPK triggers the internalization of apCAM, thereby allowing sensory cell defasciculation and synaptic growth to occur.

acid by neuraminidase (*22*). Addition of polysialic acid to NCAM may be functionally equivalent to the internalization of apCAM that occurs in *Aplysia* neurons in that both processes promote defasciculation, thereby allowing for the growth of new synaptic connections.

A third category of memory suppressor genes is revealed by the dynamics of PKA activity in *Aplysia*. Behavioral training in *Aplysia*, or exposure of sensory neurons to repeated pulses of serotonin, leads to long-term facilitation lasting 24 hours or more, but the increases in cAMP concentrations last for only about 2 hours. These cAMP increases, however, lead to a persistent increase in the activity of PKA, which continues for up to 24 hours even in the absence of cAMP or serotonin (*23*). This persistent activation of PKA bridges the transition from short- to long-term facilitation and is the result of decreased protein concentrations of the regulatory subunit of PKA after training (*23*) or treatment with serotonin (*24*). This degradation of the regulatory subunit requires adenosine triphosphate and ubiquitin and is not blocked by inhibitors of serine proteases, suggesting that it is mediated by the ubiquitin-dependent proteasome pathway (*25*). In other systems, the proteasome pathway has been shown to mediate the selective degradation of a variety of regulatory proteins, including the cell cycle regulatory protein cyclin (*26*) and the nuclear factor kappa B (NF-κB) inhibitor IκB (*27*), as well as the processing of proteins such as the transcription factor NF-κB1 (*27*).

How is proteolysis of the regulatory subunit induced, why does it not occur in other tissues, and why does it not occur under other circumstances in which cAMP concentrations are increased (for example, during short-term facilitation)? A clue comes from the fact that degradation requires new protein synthesis, suggesting that a rate-limiting component of the ubiquitin-proteasome pathway may be among the new genes induced in response to serotonin. One of these genes is a neuron-specific form of ubiquitin COOH-terminal hydrolase that is rapidly induced after serotonin treatment (*28*). This enzyme, which removes ubiquitin from multiubiquitinated substrates during proteolysis by the proteasome, appears to be essential for long-term facilitation: blocking the expression or function of *Aplysia* ubiquitin COOH-terminal hydrolase selectively impairs long-term facilitation induced by repeated serotonin treatments. Thus, as a result of the increased expression of the ubiquitin COOH-terminal hydrolase and the subsequent activation of the ubiquitin pathway, learning induces proteolysis, thereby removing a third inhibitory constraint on memory storage: the regulatory subunit of PKA (Fig. 1).

The genetic switch from short- to long-term plasticity can be divided into three components: (i) initiation (the removal of CREB2 and activation of CREB1), (2) consolidation (the induction of immediate response genes, including ubiquitin COOH-terminal hydrolase and the transcription activator ApC/EBP), and (3) stabilization (the growth of new connections). Each of the three inhibitory constraints that we have considered here acts in one of these three phases.

Is there a common pathway for the removal of these inhibitory constraints? Probably not, but one signaling system for removing inhibitory constraints that has been identified is the MAPK pathway. During training for sensitization in *Aplysia*, serotonin is released onto the presynaptic neuron by a facilitatory interneuron. Binding of serotonin to the sensory cell serotonin receptor stimulates adenylate cyclase, leading to an increase in intracellular cAMP concentration (Fig. 1). This increase in turn activates PKA, and the catalytic subunit of PKA then translocates to the nucleus, where it may phosphorylate CREB1. The increase in intracellular cAMP also activates the MAPK pathway by an unknown mechanism, leading to the translocation of MAPK to the nucleus (*29*). One potential nuclear target is CREB2, a MAPK substrate in vitro (*13*). CREB2 is phosphorylated in vivo after serotonin treatment (*13*), and it will be interesting to examine experimentally whether phosphorylation by MAPK is responsible for the derepression of CREB2 during long-term facilitation. In addition to its potential nuclear targets, MAPK substrates include the cytoplasmic tail of apCAM (*18*). The phosphorylation of the cytoplasmic tail of apCAM by MAPK triggers the internalization of apCAM, thereby allowing sensory cell defasciculation and growth to occur (Fig. 1). These findings suggest that MAPK may relieve inhibitory constraints on both gene expression and synaptic growth, thereby facilitating the formation of long-lasting transcription- and growth-dependent synaptic plasticity.

In addition to the regulatory proteins that have so far been described, other candidate memory suppressor genes are the molecules involved in regulating protein kinase cascades, such as protein phosphatases and phosphodiesterases. Recent work suggests that phosphatases provide one inhibitory constraint in the hippocampus modulating the transition from short- to long-term memory storage, acting as a gate regulating the activity of a variety of kinases (*30–32*). Transgenic mice overexpressing a truncated, active form of calcineurin exhibit specific deficits in hippocampus-based long-term memory (*31*) and in a novel intermediate phase of LTP (*32*). These findings underscore the importance of the balance between kinases and phosphatases in regulating memory storage and suggest that calcineurin may function as a memory suppressor gene in mammals.

Tumor suppressor genes often function as checkpoints, allowing a variety of signals to be integrated into a single cellular response—cell proliferation. Similarly, cascades of gene activation are switched on during memory consolidation, and it is important that this switch be tightly controlled. In this way, memory suppressor genes may provide a checkpoint for memory storage to ensure that only salient features are learned. This checkpoint is especially important because one of the hallmarks of human cognition is the ability to remember a signal from a noisy background by attending to and remembering only the most critical details. It is an evolutionary advantage for individuals to learn only facts that are important for survival, rather than storing in long-term memory everything that is encountered. Thus, as was shown by Kamin and Rescorla and Wagner, temporal contiguity is not the only important variable in associative learning; the functional importance and salience of the stimulus as well as the relevance of the association are also crucial (*33*). Memory suppressor gene products may be a central part of the integrative mechanisms that allow for the formation of these associations.

Although our discussion has focused on synaptic plasticity and learning, memory—the storing of new information—is likely to reflect some sort of a balance between learning and forgetting. If information is continually stored in existing synapses, retraining may be difficult. In this context, memory suppressor genes may decrease synaptic strength in much the same way that tumor suppressor genes stop or limit growth. These limitations imposed by memory suppressor genes may provide an important condition for subsequent learning.

One of the hallmarks of memory storage is that spaced training produces stronger, longer lasting memory than massed training (*12–14*, *34*, *35*). As shown in the work on *Drosophila* and mice, the modulation of memory storage by CREB is particularly sensitive to the spacing and repetition of training trials (*12*, *35*), and altering the balance between activating and repressing isoforms of CREB can cause single events to be stored for longer periods (*13*, *14*). These observations suggest that memory suppressor genes may be particularly important in spaced training paradigms. Positive and negative regulatory mechanisms may be ac-

tivated with different kinetics, and these distinct time courses may help to orchestrate the balance between these pathways during spaced training. In addition, the products of memory suppressor genes may play a role in the modulation of memory storage by emotional stimuli (*36*), as occurs in "flashbulb memory" and in memories that are emotionally charged.

Does the finding that both positive and negative regulatory mechanisms play a role in memory storage—as they do in cell division, differentiation, and development—provide other insight into future studies of the molecular basis of synaptic plasticity and memory storage? In particular, what are its implications for our search for potential memory suppressor genes in mammals?

The key lesson may be that we need to search in different ways. The central observation that long-term memory storage required protein and RNA synthesis focused attention on the importance of gene-inductive events (*37*). As a result, many screens were carried out to identify genes whose expression was increased by neuronal activity. The importance of negative regulatory mechanisms in memory storage in invertebrates suggests that functional screens that focus on changes in phenotype may be critical in the future for elucidating the molecular basis of long-term memory in mammals. Such screens might directly use classical mutagenesis to identify important genes or might take advantage of the wide variety of available mouse strains to identify quantitative trait loci or modifier loci. The fact that p53 was identified as a host protein that interacted with the tumor antigens of several DNA tumor viruses suggests that these functional screens should also use the variety of molecular techniques available to identify proteins that interact with molecules, such as PKA and CREB, which play a central role in long-term memory storage. ApCREB2 was identified with such a screen. The identification of differences in gene expression after learning needs to be expanded to focus on genes that are downregulated during memory storage by the use of large-scale approaches such as differential display, DNA chip, and serial analysis of gene expression technology.

In a broader sense, it is becoming increasingly clear that the molecular components involved in long-term memory storage, including PKA and CREB, are important for other long-term adaptive changes in the brain, such as those associated with addiction to alcohol, cocaine, and other drugs of abuse (*38*). If memory suppressor genes act at key control points in these processes, then they might serve as the targets of novel pharmaceuticals useful in the treatment of drug abuse as well as memory disorders. Because these suppressor genes modulate or gate other signaling pathways rather than directly activating them, drugs targeted at memory suppressor gene products may prove to be more therapeutically precise than those pharmaceuticals targeted at positive regulators of memory storage.

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