

Molecular Mechanisms of Memory Storage in *Aplysia*

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Abstract Cellular studies of implicit and explicit memory suggest that experience-dependent modulation of synaptic strength and structure is a fundamental mechanism by which these memories are encoded, processed, and stored within the brain. In this review, we focus on recent advances in our understanding of the molecular mechanisms that underlie short-term, intermediate-term, and long-term forms of implicit memory in the marine invertebrate *Aplysia californica*, and consider how the conservation of common elements in each form may contribute to the different temporal phases of memory storage.

Introduction

Modern studies in cognitive neuroscience have shown that memory is not a unitary process but consists of several forms that can be grouped into at least two general categories each with its own rules (Squire and Zola-Morgan, 1991; Polster *et al.*, 1991). Explicit, or declarative, memory is the conscious recall of knowledge about people, places, and things, and it is particularly well developed in the vertebrate brain. Implicit, or nondeclarative, memory is memory for motor skills and other tasks and is expressed through performance, without conscious recall of past experience; it

includes simple associative forms, such as classical conditioning, and nonassociative forms, such as sensitization and habituation. These two forms of memory have been localized to different neural systems within the brain (Milner, 1985). As first shown by the neuropsychological studies of the patient H.M., explicit memory is critically dependent on structures in the medial temporal lobe of the cerebral cortex, including the hippocampal formation. Implicit memory involves the cerebellum, the striatum, the amygdala, and in the simplest cases, the sensory and motor pathways recruited for particular perceptual or motor skills utilized during the learning process. As a result, implicit memory can also be studied in a variety of simple reflex systems, including those of higher invertebrates, whereas explicit forms can best (and perhaps only) be studied in mammals.

In the mammalian brain, the cellular and molecular changes that underlie both forms of memory are difficult to study because the effects are often modest and the contribution of individual synapses to the learning process is not yet well defined. To bridge this gap, the more tractable nervous systems of higher invertebrates have proven useful for the analysis of behavioral problems and have enhanced our knowledge about the synaptic loci and mechanisms that underlie various elementary forms of learning and memory (Carew and Sahley, 1986; Byrne, 1987; Hawkins *et al.*, 1993). One such model system is the gill- and siphon-withdrawal reflex of the marine invertebrate *Aplysia californica*. This reflex exhibits several forms of learning, including dishabituation, sensitization, and classical conditioning, that have many of the behavioral features of learning in mammals, suggesting that learning in *Aplysia* and mammals may share common mechanisms (Pinsker *et al.*, 1970; Carew *et al.*, 1971, 1981, 1983; Hawkins *et al.*, 1986, 1989, 1998; Colwill *et al.*, 1988a, b; Walters, 1989).

Recent studies of a variety of memory processes, ranging

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Abbreviations: AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AMPAR, AMPA receptor; C/EBP, CCAAT-box-enhanced binding protein; CamKII, calcium/calmodulin-dependent protein kinase type II; CPEB, cytoplasmic polyadenylation element binding protein; CRE, cAMP-responsive element; CREB, cAMP response element binding protein; CS, conditioned stimulus; EPSP, excitatory postsynaptic potential; LTF, long-term facilitation; LTP, long-term potentiation; MAPK, mitogen-activated protein kinase; NMDA, *N*-methyl-D-aspartate; PKA, protein kinase A; PKC, protein kinase C; PSP, postsynaptic potential; STF, short-term facilitation.

in complexity from simple forms of implicit memory in invertebrates to more complex forms of explicit memory in mammals, suggest that changes in the strength and structure of synaptic connections contribute critically to these diverse forms of memory storage (Kandel, 2001). For both implicit and explicit memory, two general types of storage mechanisms have been described: short-term memory lasting minutes and long-term memory lasting days, weeks, or longer. This temporal distinction in behavior is reflected in specific forms of synaptic plasticity that underlie each form of behavioral memory as well as in specific molecular requirements for each of these two forms of synaptic plasticity. The short-term forms involve the covalent modifications of pre-existing proteins by a variety of kinases and are expressed as alterations in the effectiveness of pre-existing connections. By contrast, in addition to PKA and MAPK, the long-term form also requires CREB-mediated gene expression and new mRNA and protein synthesis. Moreover, the long-term form often is associated with the growth of new synaptic connections. For both implicit and explicit memory storage, the synaptic growth is thought to represent the final and self-sustaining change that stabilizes the long-term process. In addition to short- and long-term memory, a family of intermediate processes that last one or more hours and often require translation but not transcription can be produced by various training protocols using repeated or prolonged stimulation.

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

Sensitization and Classical Conditioning of the Gill-Withdrawal Reflex in *Aplysia*: Two Elementary Forms of Implicit Memory Storage

The nervous system of *Aplysia* contains only about 20,000 large, identifiable nerve cells, clustered into 10 major ganglia. The ability to identify individual neurons and record their activity has made it possible to define the major components of the neuronal circuits of specific behaviors and to delineate the critical sites and underlying mechanisms used to store memory-related representations.

The molecular mechanisms contributing to implicit memory storage have been most extensively studied for the gill-and siphon-withdrawal reflex of *Aplysia* (Kandel, 2001). As is true for other types of defensive reflexes, the gill- and siphon-withdrawal reflex can be modified by several different forms of implicit learning. We begin by focusing on *sensitization*, an elementary form of nonassociative learning by which an animal learns about the properties of a single noxious stimulus and enables the formation of a learned fear

response. When a light touch is applied to the siphon of the snail, the snail responds by withdrawing its gill and siphon. This response is enhanced when the animal is given a noxious, sensitizing stimulus. As with other forms of defensive behaviors, the memory for sensitization of the withdrawal reflex is graded, and repeated tail shocks lead to a longer-lasting memory: A single tail shock produces short-term sensitization that lasts for minutes, whereas repeated tail shocks given at spaced intervals produce long-term sensitization that lasts for up to several weeks (Castellucci *et al.* 1986). The reflex also exhibits classical conditioning, an associative form of learning by which an animal learns about the predictive relationship between two stimuli. Enhancement of the withdrawal reflex is greater and longer lasting if the siphon is touched just before the noxious, sensitizing stimulus (paired training), compared to unpaired training or training with either stimulus alone (Carew *et al.*, 1981, 1983; Antonov *et al.*, 2001).

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

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

Short-term facilitation of synaptic connections involves enhanced transmitter release from the sensory neurons

Serotonin released *in vivo* during sensitization or applied directly to cultured neurons binds to cell surface receptors

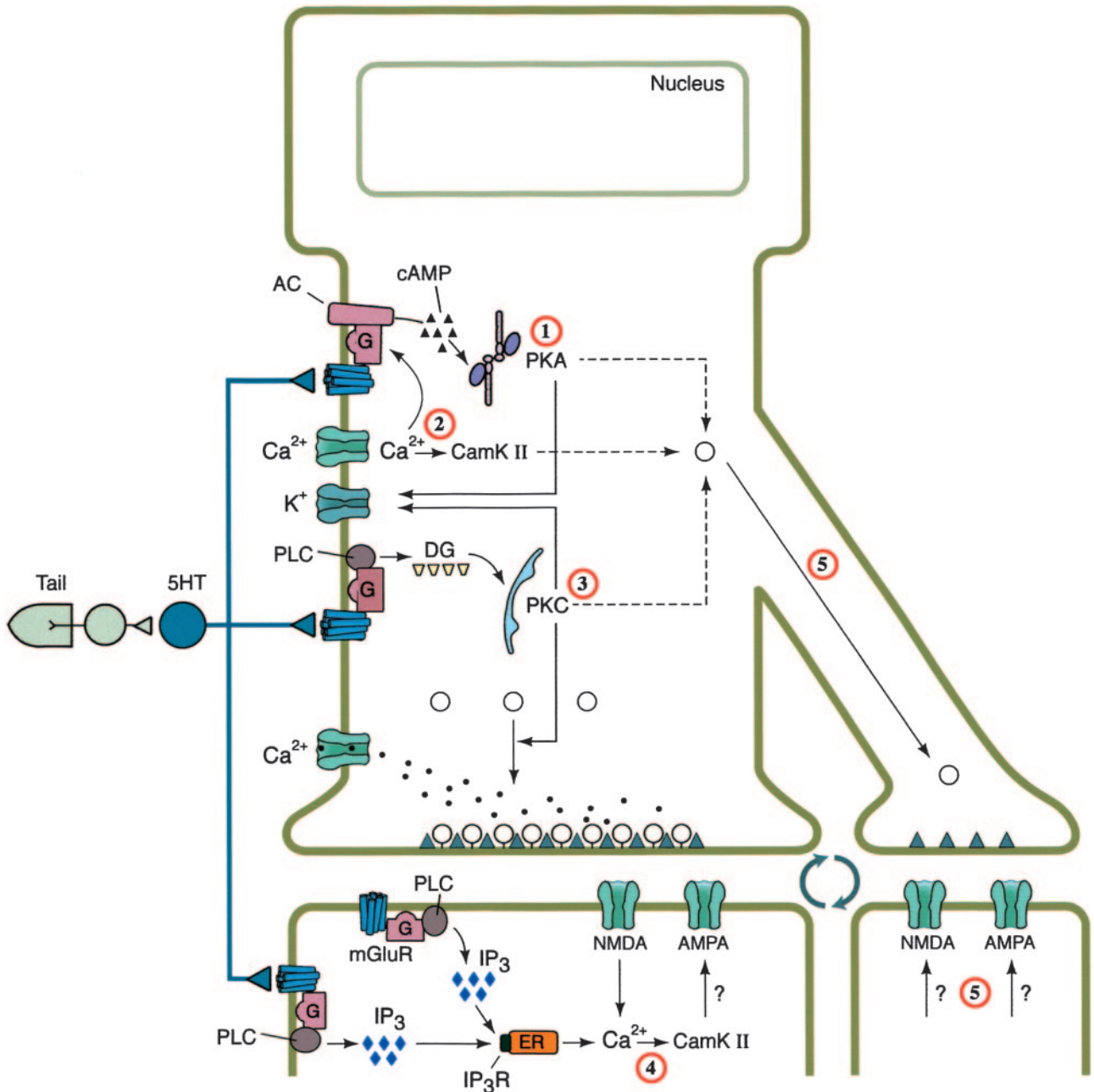


Figure 1. Mechanisms of short- and intermediate-term memory formation in *Aplysia*. Different forms of short- and intermediate-term synaptic plasticity contributing to learning in *Aplysia* involve different combinations of pre- and postsynaptic molecules including (1) presynaptic PKA, (2) presynaptic Ca²⁺ and CamKII, (3) presynaptic PKC, (4) postsynaptic Ca²⁺ and CamKII, and (5) recruitment of pre- and possibly postsynaptic molecules to new sites.

on the sensory neurons and promotes the production of the diffusible second messenger cAMP by activating the enzyme adenylyl cyclase. This increase in internal concentration of cAMP results in short-term behavioral sensitization lasting minutes and correlates with an increase in synaptic strength of the sensory-to-motor neuron connection referred to as short-term facilitation. This facilitation is partially due

to the enhanced release of the transmitter glutamate by the sensory neuron onto its follower cells and is accompanied by an increase in excitability of the sensory neuron attributable to the depression of specific sets of potassium channels (Klein *et al.*, 1982; Castellucci *et al.*, 1986; Dale *et al.*, 1988). In addition, the changes in cAMP and Ca²⁺ levels triggered by the activation of serotonin receptors and ionic

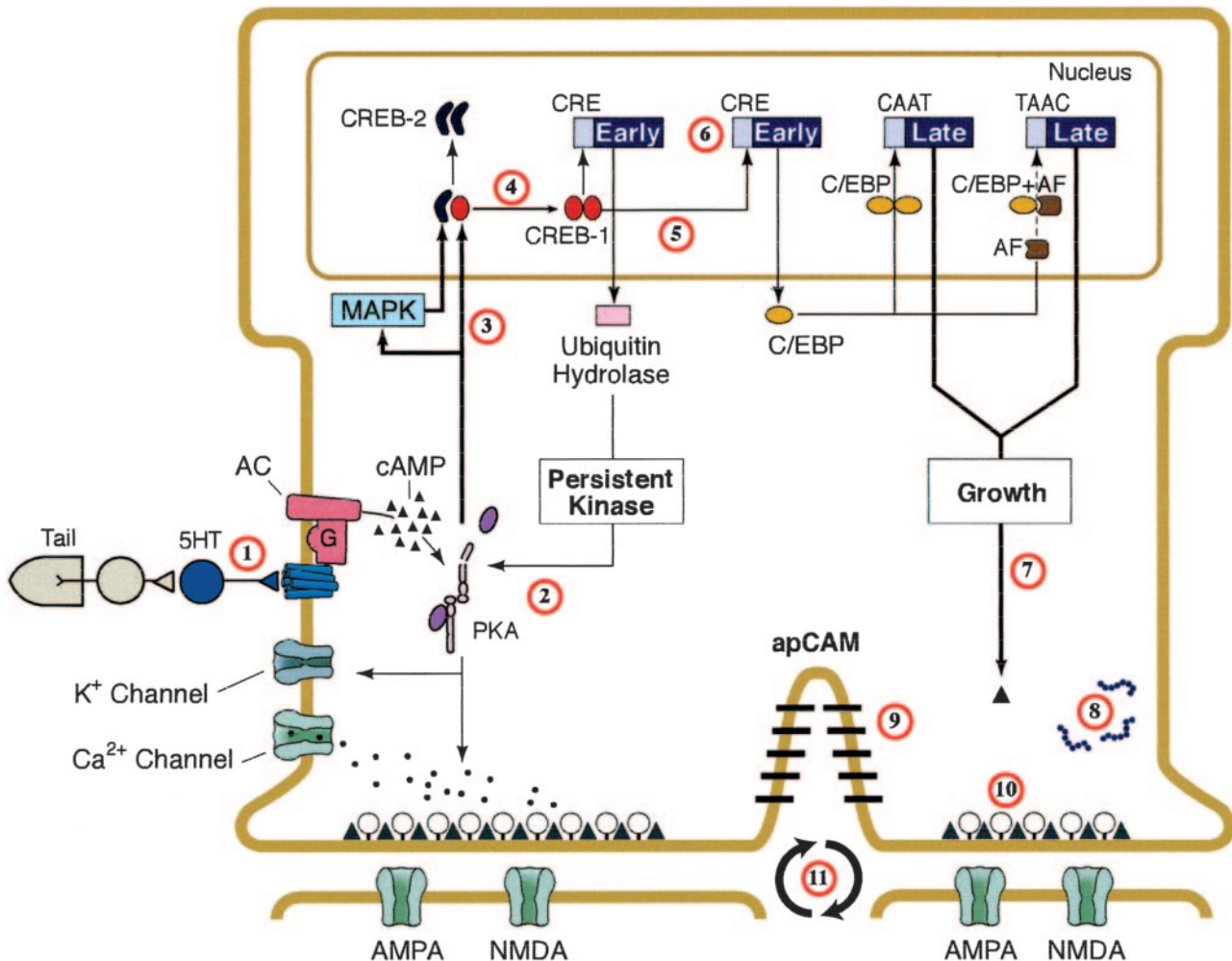


Figure 2. Mechanisms of long-term memory formation. Long-term synaptic plasticity contributing to learning involves a sequence of cellular and molecular mechanisms including (1) neurotransmitter release and short-term strengthening of synaptic connections, (2) equilibrium between kinase and phosphatase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

channels regulate different kinase and phosphatase activities that control the duration and strength of the changes in synaptic efficiency, as we will discuss later. These second messengers mediate the transient reinforcement of synaptic connections by covalent modifications of channel activation and the enhancement of neurotransmitter release at presynaptic terminals (Martin *et al.*, 2000; Kandel, 2001).

Different in vitro training protocols elicit facilitation that may involve different mechanisms and sites

Previous studies have shown that facilitation of the sensory-to-motor neuron synaptic connection involves different

mechanisms depending on the duration of exposure to 5-HT and the state (rested or depressed) of the synapse (Byrne and Kandel, 1996). Facilitation at rested synapses by a relatively brief exposure to 5-HT involves activation of adenylyl cyclase and cAMP-dependent protein kinase A (PKA) in the sensory neuron, leading to reduced K^+ current, increased action potential duration, increased Ca^{2+} influx, and increased transmitter release, as described above. A longer exposure to 5-HT recruits activation of protein kinase C (PKC), which can also increase the duration of presynaptic action potentials. Facilitation at depressed synapses involves PKC as well, but in this case it acts through a

mechanism that is independent of spike broadening and is thought to involve vesicle mobilization. The spike-broadening-independent component of facilitation may also involve Ca^{2+} /camodulin-dependent protein kinase (CamKII) (Nakanishi *et al.*, 1997). In addition, longer exposure to 5-HT can induce intermediate-term facilitation, which requires protein synthesis and involves MAP kinase as well as PKA or (under some circumstances) PKC (Ghirardi *et al.*, 1995; Sutton and Carew, 2000; Sharma *et al.*, 2003b).

All of these mechanisms have been thought to be presynaptic. However, recent evidence suggests that, depending on the training protocol, intermediate-term facilitation by exposure to 5-HT lasting more than 5 min may also involve postsynaptic mechanisms including intracellular Ca^{2+} release from IP3-sensitive stores, activation of CamKII or PKC, and AMPA receptor insertion (Chitwood *et al.*, 2001; Roberts and Glanzman, 2003; Jin *et al.*, 2004, 2005; Li *et al.*, 2005). To investigate the precise roles of pre- and postsynaptic mechanisms with different protocols, Jin *et al.* (2004, 2005) have begun to examine facilitation induced by either a single brief 5-HT exposure (1 min, 50 μM) or a more prolonged 5-HT exposure (10 min, 20 μM) following a single pretest (rested) at sensory-motor neuron synapses in isolated cell culture. A 10-min exposure to 5-HT produced larger facilitation than a 1-min exposure, but facilitation with either protocol lasted more than 30 min compared to test alone controls. With the 1-min 5-HT protocol, bath application of an inhibitor of PKA (KT5720) or injection of a peptide inhibitor of PKA into the presynaptic sensory neuron reduced the facilitation. Similarly, bath application of an inhibitor of CamKII (KN93) or injection of a peptide inhibitor of CamKII (CamKII 281-301) into the sensory neuron also reduced the facilitation. By contrast, bath application of an inhibitor of PKC (Go6983) or injection of BAPTA into the postsynaptic motor neuron had no significant effects. None of the bath-applied inhibitors affected homosynaptic depression or the pretest EPSP amplitude. These results suggest that with a short application of 5-HT presynaptic PKA and CamKII play important roles, and that PKC and postsynaptic Ca^{2+} are not involved.

With the 10-min 5-HT protocol, bath application of an inhibitor of PKA (KT5720) or presynaptic injection of the PKA inhibitor did not have a significant effect, but bath application of inhibitors of either PKC (Go6983) or CamKII (KN93) reduced the facilitation. Presynaptic injection of a peptide inhibitor of PKC (PKC 19-31) also reduced the facilitation produced by 10-min exposure to 5-HT, but presynaptic injection of a peptide inhibitor of CamKII did not. By contrast, postsynaptic injection of PKC 19-31 did not reduce the facilitation by 10-min exposure to 5HT, but postsynaptic injection of either BAPTA or CamKII 281-301 did. These results suggest that with a longer 5-HT application, presynaptic PKC plays an important role, and postsynaptic Ca^{2+} and CamKII are also important. Thus, not only

the specific kinases involved but also their site of action may depend on the duration of 5-HT exposure. Furthermore, whereas facilitation with a short application of 5-HT is predominantly presynaptic, facilitation with a longer 5-HT exposure involves both presynaptic (PKC) and postsynaptic (Ca^{2+} and CamKII) mechanisms.

Behavioral dishabituation and sensitization may involve different molecular mechanisms of facilitation

A major question in psychology is how different forms of learning such as dishabituation, sensitization, and classical conditioning are related (Marcus *et al.*, 1988; Hawkins *et al.*, 2006). The sensory-motor neuron synapses exhibit cellular analogs of these forms of learning with temporal parameters similar to those of behavioral learning (Castellucci *et al.*, 1970; Carew *et al.*, 1971, 1984; Hawkins *et al.*, 1983; Walters and Byrne, 1983; Clark *et al.*, 1994; Murphy and Glanzman, 1996, 1997, 1999), making it possible to examine how the analogs are related at the molecular level. However, these studies of synaptic changes in the isolated nervous system have not been able to address the contribution of the different molecular mechanisms of plasticity to behavior.

For this reason, Antonov *et al.* (1999) developed a simplified preparation for studying the siphon-withdrawal reflex of *Aplysia*, with which it is relatively easy to record the activity of identified neurons and their synaptic connections simultaneously with behavioral learning. This preparation undergoes dishabituation, sensitization, and classical conditioning that are similar parametrically to learning in intact animals (Antonov *et al.*, 1999, 2001). The neural circuit mediating the withdrawal reflex in the simplified preparation has been well characterized. Monosynaptic connections from LE siphon sensory neurons (Byrne *et al.*, 1974) to LFS siphon motor neurons (Frost and Kandel, 1995) have been estimated to mediate about one-third of the reflex response, which corresponds to siphon flaring in the intact animal (Antonov *et al.*, 1999). The remainder of the response is mediated by peripheral motor neurons (Perlman, 1979), which also receive monosynaptic input from the LE neurons (Bailey *et al.*, 1979), other unidentified sensory neurons (Frost *et al.*, 1997), and polysynaptic inputs onto the LFS neurons from excitatory and inhibitory interneurons (Frost and Kandel, 1995).

To investigate cellular mechanisms contributing to dishabituation and sensitization of siphon withdrawal, Antonov *et al.* (1999) recorded evoked firing of LFS motor neurons, the siphon withdrawal produced by stimulation of an LFS neuron, the complex PSP in an LFS neuron, and the monosynaptic PSP from an LE sensory neuron to an LFS neuron during behavioral training. The various cellular measures, including monosynaptic PSPs from LE neurons that either fired during the siphon tap (on-field) or did not

(off-field), all changed approximately in parallel with changes in the behavior, supporting the idea that both dishabituation and sensitization involve heterosynaptic facilitation of sensory-motor neuron PSPs.

Antonov *et al.* (2005, 2006) have begun to investigate the relevance of the different molecular mechanisms of facilitation of sensory-motor neuron PSPs to behavioral dishabituation and sensitization. To minimize habituation by the test stimulation and obtain a better estimate of the time course of sensitization, they modified their previous behavioral protocol (Antonov *et al.*, 1999) by testing the reflex only once every 15 min, instead of every 5 min. A single shock to the tail produced sensitization that lasted about 20–30 min, and a train of four shocks produced sensitization that lasted about 1 h. The sensory-motor neuron PSP changed in parallel with the behavior during sensitization after either shock. Injecting BAPTA into the motor neuron reduced facilitation after the strong shock but not after the weak shock. Bathing the abdominal ganglion in an inhibitor of PKA, KT5720, blocked sensitization after the weak shock and the early part of sensitization after the stronger shock. Furthermore, injecting a peptide inhibitor of PKA into the sensory neuron blocked facilitation of the PSP during sensitization. Bathing the ganglion in an inhibitor of PKC, chelerythrine, did not have a significant effect, but bathing the ganglion in an inhibitor of CamKII, KN93, blocked both the early and late parts of sensitization with the stronger shock. These results suggest that both postsynaptic Ca^{2+} and CamKII and presynaptic PKA contribute to sensitization with the strong shock, whereas sensitization with the weak shock is entirely presynaptic. Ten closely spaced siphon stimuli produced habituation that lasted about 1 h, and strong tail shock produced dishabituation that was reduced by the PKC inhibitor chelerythrine but not by KT5720 or KN93. These results provide the first evidence that PKA and PKC contribute preferentially to behavioral sensitization and dishabituation, respectively, similar to the roles of PKA and PKC during facilitation at nondepressed and depressed synapses *in vitro* (Byrne and Kandel, 1996).

Activity-dependent plasticity in Aplysia may also involve both pre- and postsynaptic mechanisms

In addition to these neuromodulatory forms of plasticity, *Aplysia* sensory-motor neuron synapses also exhibit several activity-dependent forms of plasticity. Moreover, like the neuromodulatory forms, the activity-dependent forms can involve both pre- and postsynaptic mechanisms. Homosynaptic potentiation induced by moderate tetanic stimulation of the presynaptic neuron (20 Hz, 2 s) has similarities to both post-tetanic potentiation (PTP) and long-term potentiation (LTP) in other preparations. It depends on activation of metabotropic glutamate receptors, and is substantially reduced by injecting Ca^{2+} chelators (Bao *et al.*, 1997),

inhibitors of intracellular Ca^{2+} release, or inhibitors of CamKII (Jin and Hawkins, 2003) into either the sensory neuron or the motor neuron. These results suggest that the potentiation involves both pre- and postsynaptic mechanisms that are more than additive and therefore might interact in some way. The sensory-motor neuron synapses also exhibit two associative cellular mechanisms, NMDA-dependent LTP, which occurs when presynaptic spike activity is temporally paired with postsynaptic depolarization (Pre-post) (Lin and Glanzman, 1994a, b) and activity-dependent facilitation, which occurs when presynaptic spike activity is temporally paired with 5-HT (Pre-Mod) (Eliot *et al.*, 1994; Bao *et al.*, 1998; Schacher *et al.*, 1997). During activity-dependent facilitation, the spike activity is thought to cause an influx of Ca^{2+} that “primes” the adenylyl cyclase, leading to enhanced activation of the PKA pathway (Kandel *et al.*, 1983; Ocorr *et al.*, 1985; Abrams *et al.*, 1998). Consistent with that idea, activity-dependent facilitation can be blocked by injecting either EGTA or a peptide inhibitor of PKA into the sensory neuron (Bao *et al.*, 1998). However, it can also be blocked by injecting BAPTA into the motor neuron, suggesting that activity-dependent facilitation also involves both pre- and postsynaptic mechanisms that are more than additive.

Classical conditioning involves both pre- and postsynaptic mechanisms of plasticity

The siphon-withdrawal reflex in the simplified preparation also exhibits an associative form of learning, classical conditioning (Antonov *et al.*, 2001). Paired training in which the conditioned stimulus (CS; mechanical stimulation of the siphon) occurred just before the unconditioned stimulus (US; a shock to the tail) produced a greater increase in the withdrawal reflex than unpaired, CS alone, or US alone training. Compared with unpaired training, paired training also produced a greater increase in evoked firing of the LFS motor neuron and greater facilitation of the monosynaptic LE-LFS PSP. Moreover, the enhanced facilitation of monosynaptic PSPs was greater for LE neurons that fired during the siphon tap (on-field) and correlated significantly with the enhancement of siphon withdrawal and evoked firing of the LFS neurons. These results support the idea that associative, activity-dependent plasticity at sensory-motor neuron synapses contributes to behavioral conditioning.

In addition to changes in the PSP, there were also pairing-specific increases in both the evoked firing and input resistance of the LE sensory neurons during conditioning (Antonov *et al.*, 2001). Moreover, like facilitation of the monosynaptic PSPs, the increase in input resistance occurred only in the LE neurons that fired during the CS (on-field), and it correlated significantly with facilitation of the PSPs from those neurons. These results are all consistent with the idea that conditioning involves activity-dependent

presynaptic facilitation, but they are also not inconsistent with Hebbian LTP. Both mechanisms contribute to a cellular analog of conditioning (Hawkins *et al.*, 1983; Walters and Byrne, 1983; Clark *et al.*, 1994; Murphy and Glanzman, 1996, 1997, 1999). Both mechanisms might also contribute to behavioral conditioning because the CS causes firing of LE sensory neurons and the US causes firing of both facilitatory interneurons (Hawkins and Schacher, 1989; Mackey *et al.*, 1989) and the LFS motor neurons. To begin to distinguish between these possibilities, Antonov *et al.* (2003) bathed the abdominal ganglia in the PKA inhibitor KT5720 or the NMDA antagonist APV and found that either drug blocked behavioral conditioning. Similarly, either injecting three to six LE sensory neurons with a peptide inhibitor of PKA (PKAi) or injecting two to three LFS motor neurons with the Ca²⁺ chelator BAPTA significantly reduced behavioral conditioning. These results suggest that both activity-dependent presynaptic facilitation and Hebbian LTP are required for the conditioning. Because the sensory neurons fire a brief burst of only about three action potentials during the siphon tap CS, the LTP component might correspond to a mammalian form with minimal presynaptic activity such as theta-burst paired or spike-timing-dependent LTP.

To analyze the cellular mechanisms contributing to conditioning in more detail, Antonov *et al.* (2003) recorded evoked firing of an LFS motor neuron and an LE sensory neuron, the membrane resistance of each neuron, and the monosynaptic PSP between them after either injecting the LE neuron with PKAi or injecting the LFS neuron with BAPTA. Both procedures significantly reduced the pairing-specific facilitation of the PSP during conditioning, providing the strongest evidence to date that either activity-dependent presynaptic facilitation or Hebbian LTP contributes to synaptic plasticity underlying behavioral learning. Injecting an LE sensory neuron with PKAi also significantly reduced the effects of pairing on evoked firing and membrane resistance of the LE neuron, consistent with the idea that PKA mediates most of the changes in sensory neuron membrane properties during activity-dependent presynaptic facilitation (Eliot *et al.*, 1994; Bao *et al.*, 1998; Byrne and Kandel, 1996). Surprisingly, however, injecting an LFS motor neuron with BAPTA also significantly reduced the effects of pairing on evoked firing and membrane resistance of the LE sensory neuron. These results suggest that the pre- and postsynaptic mechanisms are not independent but rather interact through retrograde as well as orthograde signaling.

Collectively, the results of these experiments suggest that the synaptic plasticity during conditioning does not simply involve separate and independent pre- and postsynaptic mechanisms. Facilitation of the EPSP during conditioning can be almost completely blocked either by presynaptic injection of a peptide inhibitor of PKA or the nitric oxide scavenger myoglobin, or by postsynaptic injection of

BAPTA (Antonov *et al.*, 2003, 2004). Thus, if there were separate pre- and postsynaptic mechanisms, blocking either one must also block the other. Furthermore, postsynaptic injection of BAPTA also blocks the changes in presynaptic membrane properties (Antonov *et al.*, 2003), suggesting retrograde signaling. Thus, these results fit better with the idea that facilitation of the EPSP during conditioning involves both pre- and postsynaptic changes coordinated by trans-synaptic interactions at the sensory-to-motor neuron synapse.

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

Gating signals at the synapse: a balance between the activities of protein kinase and phosphatase

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

In addition to protein kinases, synaptic protein phosphatases also play a key role in regulating the initiation of long-term synaptic changes. Various protein phosphatases, such as PP1 and calcineurin, counteract the local activity of PKA, acting as inhibitory constraints of memory formation. For example, recent experiments in cultured *Aplysia* neurons indicate that calcineurin may act as a memory suppressor for sensitization in this organism (Sharma *et al.*, 2003a). Thus, an equilibrium between both kinase and phosphatase activities at a given synapse gates the synaptic signals that reach the nucleus and thus, can regulate both memory storage and retrieval (Abel *et al.*, 1998).

Retrograde signaling from the synapse to the nucleus

One of the features that fundamentally distinguishes the storage of long-term memory from short-term cellular changes is the requirement for the activation of gene ex-

pression. Given this requirement at the nucleus, one might expect that LTF would have to be cell-wide. However, experiments using local applications of 5-HT in a culture preparation of a single bifurcated *Aplysia* sensory neuron to two motor neurons (Martin *et al.*, 1997b; Casadio *et al.*, 1999), as well as parallel experiments by Frey and Morris in the hippocampus (1997), demonstrated that each synapse could be modified independently in a protein-synthesis-dependent manner. Thus, LTF and the associated presynaptic changes are synapse-specific. This implies that there must be not only anterograde signaling from the nucleus to the synapse, but also retrograde signaling from the synapse back to the nucleus. Recently, Thompson *et al.* (2004) have found that 5-HT stimulation that produces LTF triggers the nuclear translocation of importins, proteins involved in carrying cargos through nuclear pore complexes, in *Aplysia* sensory-motor neuron co-cultures. Similarly, in hippocampal neurons, NMDA activation or LTP induction, but not depolarization, leads to translocation of importin (Thompson *et al.*, 2004). Although details underlying the translocation of these retrograde signals remain unknown, the effector molecules identified thus far appear to be conserved in both invertebrates and vertebrates. The future identification of the molecular cargoes of importin and its signaling role in the nucleus are likely to increase our understanding of how transcription-dependent memory is regulated.

Activation of nuclear transcription factors

Long-term memory is represented at the cellular level by activity-dependent modulation of both the function and the structure of specific synaptic connections that, in turn, depends on the activation of specific patterns of gene expression (Kandel, 2001). As mentioned above, the inhibition of transcription or translation blocks the formation of long-term memory in a variety of model systems, but does not affect short-term memory.

Studies in *Aplysia* first revealed the participation of the cAMP/PKA-signaling pathway in LTF and sensitization (Brunelli *et al.*, 1976). In mammalian cells, PKA activates gene expression by the phosphorylation of transcription factors that bind to the cAMP-responsive element (CRE). The CRE is one of the DNA response elements contained within the control region of a gene. The binding of different transcription factors to these response elements regulates the activity of RNA polymerase, thereby determining when and to what level a gene is expressed. One of the major transcription factors that recognizes the CRE is a protein called CRE-binding protein (CREB1), which functions as a transcriptional activator only after it is phosphorylated by either PKA, MAPK, or CamK. However, evidence for a direct role of CRE-driven transcription, downstream of the cAMP pathway, in memory-related synaptic plasticity was provided more than a decade later. During LTF in *Aplysia*

neurons, PKA activates gene expression *via* an *Aplysia* CREB (Dash *et al.*, 1990). If CREB1 is essential for LTF, then blocking the binding of CREB1 to its DNA response element should selectively eliminate the long-term process. Dash *et al.* (1990) first tested this idea by microinjecting CRE oligonucleotides into sensory neurons co-cultured with motor neurons. This oligonucleotide inhibits the function of CREB1 by binding to the CREB1 protein within the cell, thereby preventing it from binding to CRE sites in the regulatory regions of cAMP-responsive genes and activating gene expression. While injection of the CRE oligonucleotide had no effect on STF, it selectively blocked LTF. Several studies by a number of laboratories have now revealed that different members of the CREB family of transcription factors participate in the molecular switch that regulates LTF formation (Lonze and Ginty, 2002; Barco *et al.*, 2003). Both the CREB activator ApCREB1 and the repressor ApCREB2 contribute to this process. The formation of LTF requires the activation of ApCREB1 by PKA and the concomitant down-regulation of ApCREB2 by MAPK (Guan *et al.*, 2003). Injection of anti-ApCREB2 antibodies into *Aplysia* sensory neurons causes a single pulse of 5-HT, which normally induces STF lasting minutes, to evoke LTF that lasts several days (Bartsch *et al.*, 1995). Conversely, the injection of pApCREB1 can by itself trigger facilitation lasting 24 h, and this can be stabilized by a single pulse of 5-HT (Bartsch *et al.*, 1998; Casadio *et al.*, 1999).

These studies reveal that long-term synaptic changes are governed by both positive and negative regulators, and that the transition from STF to LTF requires the simultaneous removal of transcriptional repressors and activation of transcriptional activators. These transcriptional repressors and activators can interact with each other both physically and functionally. It is likely that the transition is a complex process involving temporally distinct phases of gene activation, repression, and regulation of signal transduction. The CREB-mediated response to extracellular stimuli can be modulated by a number of kinases (PKA, CamKII, CamKIV, RSK2 MAPK, and PKC) and phosphatases (PP1 and calcineurin). The CREB regulatory unit may therefore serve to integrate signals from various signal transduction pathways. This ability to integrate signaling, as well as to mediate activation or repression, may explain why CREB is so central to memory storage.

Recent studies by Guan *et al.* (2002) have directly examined the role of CREB-mediated responses in long-term synaptic integration by studying the long-term interactions of two opposing modulatory transmitters important for behavioral sensitization in *Aplysia*. Using chromatin immunoprecipitation techniques to investigate how opposing inputs are integrated in the nucleus of sensory neurons, they showed that both the facilitatory and inhibitory modulatory transmitters activate signal transduction pathways that alter

promoter occupancy by activator or repressor CREB isoforms and subsequently affect nucleosome structure bidirectionally through acetylation and deacetylation of histone residues in chromatin.

The first genetic screenings designed to identify learning mutants in *Drosophila* rendered two interesting mutants, *dunce* and *rutabaga*, with specific defects in memory formation (Dudai *et al.*, 1976; Duerr and Quinn, 1982) that were subsequently identified to be affected in genes mapped to the cAMP signaling pathway (Byers *et al.*, 1981; Waddell and Quinn, 2001). Experiments in transgenic flies have confirmed that the balance between CREB activator and repressor isoforms is critically important for long-term behavioral memory. Overexpression of an inhibitory form of CREB (dCREB-2b) blocked long-term olfactory memory but did not alter short-term memory, whereas the overexpression of an activator form of CREB (dCREB-2a) had the opposite effect and increased the efficacy of training in long-term memory formation (Yin *et al.*, 1994, 1995). It should be noted, however, that a recent study in flies by Perazzona and colleagues could not replicate the enhancing effect of the CREB activator in learning (Perazzona *et al.*, 2004).

Most of the upstream signaling cascade leading to CREB activation appears to be conserved through evolution, and many aspects of the role of CREB in synaptic plasticity described in invertebrates have also been observed in the mammalian brain, although the role of CREB in explicit forms of memory appears to be more complicated than in implicit forms of memory in invertebrates (see reviews by Lonze and Ginty, 2002, and Barco *et al.*, 2003)).

Finally, although we have focused on CREB-dependent gene expression because of its conserved role in memory formation through evolution, other transcription factors, such as SRF, *c-fos*, *EGR-1*, or *NF- κ B* (Tischmeyer and Grimm, 1999; Albenis and Mattson, 2000; Izquierdo and Cammarota, 2004; Ramanan *et al.*, 2005) are also likely to contribute to the transcriptional regulation that accompanies long-lasting forms of synaptic plasticity.

Activity-dependent induction of cAMP-responsive genes

The identification of the consensus palindromic sequence recognized with high affinity by CREB (namely, TGACGTC) occurred nearly 20 years ago. However, the presence of this sequence does not guarantee CREB regulation. For example, different cell types express different sets of target genes in response to CREB activation, suggesting that the availability of the promoter for CREB binding is likely different in different neurons. Moreover, the promoters of many cAMP-responsive genes known to be regulated by CREB often contain only a half-site TGACG sequence. For all these reasons, the complete set of

genes regulated by this transcription factor in a specific cell type is still unknown.

In *Aplysia* sensory neurons, the activity of ApCREB1 leads to the expression of several immediate-response genes, such as ubiquitin hydrolase, that stabilize STF (Hegde *et al.*, 1997), and the transcription factor CCAAT-box-enhanced binding protein (C/EBP), whose induction has been shown to be critical for LTF (Alberini *et al.*, 1994). This induced transcription factor (in concert with other constitutively expressed molecules such as ApAF; Bartsch *et al.*, 2000) activate a second wave of downstream genes that lead to the growth of new synaptic connections. These genes represent only two of a family of physiologically relevant examples of gene products generated by CREB activity.

Chromatin alteration and epigenetic changes in gene expression with memory storage

Although epigenetic mechanisms were widely known to be involved in the formation and long-term storage of cellular information in response to transient environmental signals, the discovery of their putative relevance in adult brain function is relatively recent (Guan *et al.*, 2002; Levenson and Sweatt, 2005). The epigenetic marking of chromatin, such as histone modification, chromatin remodeling, and the activity of retrotransposons, may have long-term consequences in the transcriptional regulation of specific *loci* involved for long-term synaptic changes (Hsieh and Gage 2005).

The contribution of histone tail acetylation, a modification that favors transcription and is associated with active *loci*, to LTF formation was first revealed by the study by Guan *et al.* (2002) in *Aplysia* neurons. This study found that both facilitatory and inhibitory stimuli bidirectionally alter the acetylation stage and structure of promoters driven by the expression of genes involved in the maintenance of LTF, such as C/EBP. It also demonstrated that enhancing histone acetylation with deacetylase (HDAC) inhibitors facilitates the induction of LTF (Guan *et al.*, 2002). These results indicate that critical chromatin changes occur during the formation of long-term memory and that these changes are required for the stable maintenance of these memories.

Synaptic capture of learning-induced gene products

Following transcriptional activation, newly synthesized gene products, both mRNAs and proteins, have to be delivered specifically to the synapses whose activation originally triggered the wave of gene expression. To explain how this specificity can be achieved in a biologically economical way in spite of the massive number of synapses in a single neuron, Martin *et al.* (1997b) and Frey and Morris (1997) proposed the synaptic capture hypothesis. This hypothesis, also sometimes referred to as synaptic tagging, proposes

that the products of gene expression are delivered throughout the cell, but are only functionally incorporated in those synapses that have been tagged by previous synaptic activity. This synaptic tag model has been supported by a number of studies both in the rodent hippocampus (Frey and Morris, 1997, 1998; Barco *et al.*, 2002; Dudek and Fields, 2002) and *Aplysia* (Martin *et al.*, 1997b; Casadio *et al.*, 1999).

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

Local protein synthesis at active synapses

A number of distinct mRNAs have now been localized in the axons of *Aplysia* and the dendrites of mouse hippocampal neurons (for review, see Steward and Schuman, 2001, 2003). The molecular mechanisms that carry these mRNAs to the synapse are largely unknown, but they are thought to involve the recognition of *cis*-acting elements in their 3' untranslated region by specific RNA-binding proteins that interact with the cytoskeleton. Once translocated to synaptic compartments, these mRNAs are efficiently translated only after docking at active synaptic sites, a process frequently referred to as synaptic, or local, protein synthesis. Recent reports indicate that regulation of local protein synthesis may play a major role in the control of synaptic strength.

Martin *et al.* (1997b) investigated the role of local protein synthesis in the maintenance of synapse-specific LTF in *Aplysia* using a culture system in which a single bifurcated sensory neuron of the gill-withdrawal reflex was plated in contact with two spatially separated gill motor neurons. In this culture system, repeated application of 5-HT to one synapse produces a CREB-mediated, synapse-specific LTF that can be blocked by the local application of inhibitors of translation, suggesting that local protein synthesis at the synapse is required for the initiation of synapse-specific LTF.

The control of translation at the synapse is likely to be complex and involve several different mechanisms, including mRNA transport and docking, cytoplasmic polyadenylation, and the phosphorylation of different translation factors (see recent review by Sutton and Schuman, 2005). Many of the molecules contributing to the regulation of this

process have been found to be required for both LTF in *Aplysia* and late-phase LTP in the hippocampus. This is the case for kinase mTOR, which is the target of the selective protein synthesis inhibitor rapamycin (Cammalleri *et al.*, 2003; Purcell *et al.*, 2003); the brain derived neurotrophic factor (BDNF), which promotes local protein synthesis (Aakalu *et al.*, 2001; Purcell *et al.*, 2003); and the cytoplasmic polyadenylation element binding protein (CPEB), which activates dormant mRNAs (Huang *et al.*, 2002; Si *et al.*, 2003b).

Synaptic growth and the formation of new synapses

Synaptic remodeling and the growth of new synaptic connections have been found to accompany various forms of long-term memory. This is particularly well documented at the connections of identified neurons in *Aplysia* (Bailey and Kandel, 1993). Long-term sensitization has been extensively studied in this respect and is associated with the growth of new synaptic connections between the sensory neurons and their postsynaptic cells (Bailey and Chen, 1983, 1988a, b, 1989). The storage of long-term memory for sensitization (lasting several weeks) is accompanied by the learning-induced remodeling of presynaptic sensory neuron varicosities at two levels of synaptic organization: (a) alterations in focal regions of membrane specialization of the synapses that mediate transmitter release—the number, size, and vesicle complement of sensory neuron active zones are larger in sensitized animals than in controls (Bailey and Chen, 1983); and (b) a parallel but more pronounced and widespread effect involving modulation of the total number of presynaptic varicosities per sensory neuron (Bailey and Chen 1988a). Sensory neurons from long-term sensitized animals exhibit a twofold increase in the total number of synaptic varicosities, as well as an enlargement in the size of each neuron's axonal arbor.

By comparing the time course for each morphological change with the behavioral duration of the memory, Bailey and Chen (1989) were able to determine which class of structural changes at sensory neuron synapses might contribute to the retention of long-term sensitization. They found that the increase in the size and synaptic vesicle complement of sensory neuron active zones present 24 h after the completion of behavioral training was back to control levels when tested 1 week later; in contrast, the changes in varicosity and active zone number persisted for at least 1 week and were only partially reversed at the end of the 3-week experiment.

These findings indicated that clear structural changes accompany long-term behavioral modifications in *Aplysia* and demonstrated, for the first time, that these changes could be detected at the level of identified synaptic connections known to be critically involved in the behavior. These initial studies also provided evidence for an intriguing no-

tion: that active zones are plastic rather than immutable components of the synapse and that even elementary forms of learning can alter the organization and number of presynaptic transmitter release sites to modulate the functional expression of synaptic connections. In addition, results from these studies indicated that the growth of new sensory-neuron synapses is likely to represent the final and perhaps most stable phase of long-term memory storage in *Aplysia* and suggested that the stability of the long-term process may be achieved, at least in part, because of the relative stability of synaptic structure.

Although a number of molecular components that underlie the functional changes (*i.e.*, changes in synaptic strength) associated with the storage of long-term memory have been characterized, little is known about how these are regulated by and coupled to the signaling pathways that give rise to the synaptic structural changes (Bailey *et al.*, 2004). Recent *in vitro* studies of the sensory-to-motor neuron synapse have begun to delineate the molecular mechanisms that underlie these learning-related structural changes and the functional contribution of these changes to the different temporal phases of memory storage. For example, the long-lasting growth of new synaptic connections between sensory neurons and their follower cells (both interneurons and motor neurons) during long-term sensitization can be reconstituted in dissociated sensory-motor neuron co-cultures by repeated applications of 5-HT (Glanzman *et al.*, 1990). In culture, the structural change can be correlated with the long-term enhancement in synaptic effectiveness and, like the synapse formation that occurs during development, depends upon the presence of an appropriate target cell. The 5-HT-induced synaptic growth in culture requires new macromolecular synthesis (Bailey *et al.*, 1992b) and is associated with a cellular program of gene expression (Alberini *et al.*, 1994; Bartsch *et al.*, 1995), the modulation of NCAM-related cell adhesion molecules (Bailey *et al.*, 1992a, 1997; Mayford *et al.*, 1992; Han *et al.*, 2004), and reorganization of the presynaptic actin network (Hatada *et al.*, 2000; Udo *et al.*, 2005).

Activation of silent synapses

These earlier studies did not examine the functional contribution of presynaptic structural changes to the different time-dependent phases of LTP; thus, they could not determine whether the increase in synaptic strength resulted from the conversion of pre-existing but nonfunctional (silent) synapses to active synapses or from the addition of newly formed functional synapses or both.

Kim *et al.* (2003) have addressed these issues by monitoring functional and presynaptic structural changes continuously during LTP using time-lapse confocal imaging of individual presynaptic varicosities of sensory neurons labeled with three fluorescent markers: the whole cell marker

Alexa-594 and two presynaptic marker proteins—*synaptophysin-eGFP*, which monitors changes in the distribution of synaptic vesicles within individual varicosities, and *synapto-Phluorin*, a monitor of active transmitter release sites (Miesenbock *et al.*, 1998). They found that repeated pulses of 5-HT induce two temporally, morphologically, and molecularly distinct classes of presynaptic changes: (1) a rapid activation of silent presynaptic terminals through the filling of pre-existing empty varicosities with synaptic vesicles, which requires empty translation but not transcription, and (2) a generation of new synaptic varicosities, which occurs more slowly and requires both transcription and translation. The enrichment of pre-existing but empty varicosities with synaptophysin is completed within 3 to 6 h, parallels intermediate-term facilitation, and accounts for about 32% of the newly activated synapses evident at 24 h. By contrast, the new sensory-neuron varicosities, which account for 68% of the newly activated synapses at 24 h, do not form until 12–18 h after exposure to 5 pulses of 5-HT. The rapid activation of silent presynaptic terminals suggests that, in addition to its role in long-term facilitation, this modification of pre-existing synapses may also contribute to the intermediate phase of synaptic plasticity and memory storage (Ghirardi *et al.*, 1995; Mauelshagen *et al.*, 1996; Sutton *et al.*, 2001)

These findings, the first to be made on individually identified presynaptic varicosities, suggest that the duration of the changes in synaptic effectiveness that accompany memory storage may be reflected by the differential regulation of two fundamentally disparate forms of presynaptic compartment: (1) nascent (empty) silent varicosities that can be rapidly and reversibly remodeled into active transmitter release sites and (2) mature, more stable and functionally competent varicosities that after long-term training may undergo fission to form new stable synaptic contacts.

The activation of silent synapses also seems to play a major role in LTP in the hippocampus. In mammals, the term *silent synapse* refers to a very specific molecular configuration found in synapses in different regions of the central nervous system of vertebrates (Malinow *et al.*, 2000; Malinow and Malenka, 2002). It is used to define those excitatory glutamatergic synapses whose postsynaptic membrane contains NMDARs but no AMPARs. These synapses are so named because the lack of AMPAR-mediated signaling renders the synapse inactive, or silent, under normal conditions. As in the case of presynaptically silent synapses in *Aplysia*, synaptic stimulation activates these postsynaptically silent synapses, but by a different molecular mechanism, the insertion of AMPARs into the postsynaptic membrane, a phenomenon sometimes referred to as *AMPA-fication*. In the silent synapse model for LTP, CamKII is activated by high-frequency stimulation and phosphorylates AMPARs, triggering their insertion into the postsynaptic membrane. Once AMPARs are inserted, the

synapse is no longer silent and the postsynaptic responses are consequently enhanced. Conversely, synapses can be made to be silent (for example, after LTD induction) by removing AMPARs (Malinow and Malenka, 2002). This important LTP mechanism seems to be exclusive of glutamatergic synapses. A similar regulation involving the insertion and clustering of postsynaptic neurotransmitter receptors following long-term training at the sensory-motor neuron synapse in *Aplysia* has recently been described (Li *et al.*, 2004).

Self-perpetuating mechanisms and the molecular basis of memory persistence

As outlined above, the stability of LTF seems to result from the activation of a nuclear program and the persistence of structural changes at sensory-neuron synapses, the decay of which parallels the decay of the behavioral memory. This raises two fundamental questions in the cell biology of memory storage. First, the activation of a nuclear program suggests that long-term memory could potentially be cell-wide. On the other hand, there might be a cellular mechanism to utilize a cell-wide process in a synapse-specific way. Second, if a change in synaptic strength and structure is indeed the underlying mechanism of long-term memory storage, then the experience-dependent molecular changes at the synapse must also be maintained for the duration of the memory. Since biological molecules have a relatively short half-life (hours to days) compared to the duration of memory (days, weeks, even years), how is the altered molecular composition of a synapse maintained for such a long time? What is the nature of the enduring molecular changes that underlie long-term memory storage?

One possible answer to these elusive questions is that some type of self-sustained alteration at the synaptic level is required to confer stability to memory storage. Several molecular mechanisms have been proposed as candidates for this process. We will discuss here one recent model to account for the stable maintenance and persistence of LTF in *Aplysia*.

Prion-like proteins represent auto-replicative structures that may serve as a persistent form of information (Tompa and Friedrich, 1998). Kandel and Si have recently proposed a model based on the prion-like properties of *Aplysia* neuronal cytoplasmic polyadenylation element binding protein (CPEB) (Si *et al.*, 2003a). Neuronal CPEB can activate the translation of dormant mRNAs through the elongation of their poly-A tail. *Aplysia* CPEB has two conformational states: one is inactive or acts as a repressor; the other is active. In a naive synapse, the basal level of CPEB expression is low, and its state is inactive or repressive. According to the model of Si *et al.*, serotonin induces an increase in the amount of neuronal CPEB and, if a given threshold is reached, causes the conversion to the prion-like state, which

is more active and lacks the inhibitory function of the basal state (Si *et al.*, 2003b). Once the prion state is established at an activated synapse, dormant mRNAs, made in the cell body and distributed cell-wide, would be translated only at the activated synapses. Because the activated CPEB can be self-perpetuating, it could contribute to a self-sustaining, synapse-specific long-term molecular change and provide a mechanism for the stabilization of learning-related synaptic growth and the persistence of memory storage.

A structurally similar neuronal isoform of CPEB, CPEB-3, has been found in mouse hippocampal neurons, and is induced by the neurotransmitter dopamine. This raises the possibility that dopamine-dependent regulation of mouse CPEB-3, similar to serotonin-mediated regulation of CPEB activity in *Aplysia*, acts as a synaptic mark that triggers the molecular switch for memory stabilization at mammalian synapses (Theis *et al.*, 2003).

An Overall View

Two general types of storage mechanisms have been described for both implicit and explicit processes. Short-term memory, lasting minutes to hours, involves an alteration in the effectiveness of pre-existing synaptic connections as a result of the covalent modification of pre-existing proteins. By contrast, long-term memory, lasting days, weeks or years, is associated with the growth of new synaptic connections initiated by a program of cAMP-inducible gene expression and the synthesis of new proteins.

These findings raise two questions central to an understanding of the relationship between the mechanisms that underlie the storage of short- and long-term memory: (1) How are the covalent modifications of pre-existing proteins and the alterations of the strength of pre-existing connections during short-term memory transformed into the growth of new functional synaptic connections and memory persistence during the long-term form? (2) What are the relative contributions of the presynaptic and postsynaptic neuron in this conversion?

As we have discussed above, long-lasting forms of learning-related synaptic plasticity are often associated with changes in both the number and structure of synapses (Bailey and Kandel, 1993; Lamprecht and LeDoux, 2004). Some of the best evidence for changes in synapse number has come from studies of long-term (days to weeks) plasticity in *Aplysia*, where long-term sensitization *in vivo* and LTF *in vitro* are both accompanied by increases in the number of presynaptic sensory-neuron varicosities (Bailey and Chen, 1988b; Glanzman *et al.*, 1990). The persistence of the learning-induced growth of new synaptic connections in *Aplysia* parallels both the behavioral duration of the memory *in vivo* and the enhancement of synaptic strength *in vitro*. In sensory-motor neuron co-cultures, the synaptic growth requires new macromolecular synthesis and is asso-

ciated with a cellular program of gene expression, the modulation of NCAM-related cell adhesion molecules, and the reorganization of the presynaptic actin network. The late (hours) protein-synthesis-dependent phase of hippocampal LTP is also accompanied by increases in both postsynaptic (Toni *et al.*, 1999) and presynaptic (Bozdagi *et al.*, 2000) structures, consistent with an increase in synapse number.

By contrast, short-term (up to about 1 h) plasticity and the early phases of long-term plasticity in *Aplysia* and hippocampus have been thought to involve a completely different type of mechanism—covalent modification of existing proteins in either presynaptic (*Aplysia*) or postsynaptic (hippocampus) structures (Kandel, 2001; Malinow and Malenka, 2002). However, the discovery of intermediate (2–3 h) forms of plasticity that involve elements of the short-term and long-term mechanisms in both *Aplysia* (Ghirardi *et al.*, 1995) and hippocampus (Winder *et al.*, 1998) called this dichotomy into question, and in recent years it has been challenged by two additional types of evidence. First, at least under some conditions, pre- as well as postsynaptic mechanisms have been found to contribute to early-phase LTP in hippocampus (Arancio *et al.*, 1995, 1996, 2001; Ninan and Arancio, 2004; Malgaroli *et al.*, 1995; Ryan *et al.*, 1996; Zakharenko *et al.*, 2003; Lu and Hawkins, 2006) and to a variety of types of short-term plasticity in *Aplysia* including homosynaptic potentiation (Bao *et al.*, 1997; Jin and Hawkins, 2003), heterosynaptic facilitation by 5-HT (Jin *et al.*, 2004; Li *et al.*, 2005), activity-dependent facilitation (Bao *et al.*, 1998), and the synaptic plasticity during behavioral conditioning (Antonov *et al.*, 2003, 2004). In many cases, inhibitors injected into the pre- and postsynaptic neurons have more than additive effects (Bao *et al.*, 1997, 1998; Jin and Hawkins, 2003; Antonov *et al.*, 2003; Lu and Hawkins, 2006), suggesting that the pre- and postsynaptic mechanisms are not independent but rather act synergistically. Furthermore, in some cases, postsynaptic inhibitors have been shown to affect presynaptic properties, suggesting transynaptic signaling (Antonov *et al.*, 2003).

Second, imaging studies have shown that even the earliest phases of hippocampal LTP are accompanied by pre- and postsynaptic structural alterations. Tens of minutes after the induction of LTP, there is an outgrowth of new pre- and postsynaptic processes (Maletic-Savatic *et al.*, 1999; Engert and Bonhoeffer, 1999; Nikonenko *et al.*, 2003); even earlier (minutes), there are increases in spine size (Matsuzaki *et al.*, 2004), clusters of postsynaptic glutamate receptors (Malinow and Malenka, 2002), and clusters of presynaptic vesicle-associated proteins and sites where the pre- and postsynaptic clusters colocalize (Antonova *et al.*, 2001). Moreover, the presynaptic alterations appear to depend on retrograde signaling from the postsynaptic cells (Nikonenko *et al.*, 2003; Wang *et al.*, 2005).

Similar to clustering of synaptic proteins during LTP,

intermediate-term facilitation in *Aplysia* is accompanied by filling of pre-existing empty presynaptic varicosities with synaptic vesicles (Kim *et al.*, 2003). Kim and colleagues employed a reduced 5-HT protocol that selectively induced facilitation in the intermediate-term time domain without inducing long-term facilitation (Ghirardi *et al.*, 1995). They found that isolated intermediate-term facilitation was accompanied by the redistribution and clustering of synaptic vesicle proteins into empty sensory-neuron varicosities at 0.5 h and 3 h; this effect was similar to what occurred when intermediate- and long-term facilitation were recruited together by the long-term protocol. However, the presynaptic structural changes induced by the reduced 5-HT protocol differed from those induced by long-term training in at least two ways. First, there was no growth of new sensory neuron varicosities in the isolated intermediate phase. Second, unlike the filling of pre-existing empty varicosities during the intermediate-term phase induced by the long-term protocol, the newly filled varicosities did not persist for 24 h and were unaffected by inhibitors of protein synthesis, suggesting that the structural remodeling induced by the reduced 5-HT protocol involved only a simple rearrangement of pre-existing synaptic components. This may reflect a fundamental difference in the molecular mechanisms recruited by the two 5-HT protocols. Although both protocols induce intermediate-term facilitation, the long-term protocol may activate additional molecular events (including the machinery for translational activation) required to set up the long-term phase, perhaps by stabilizing the intermediate phase. In preliminary studies, Li *et al.* (2004) found that the long-term protocol also induced clustering of postsynaptic proteins, including the *Aplysia* homologs of NMDA and AMPA receptors, within 12 hours. It is not yet known whether intermediate-term facilitation is accompanied by similar postsynaptic changes.

Jin *et al.* (2003) have begun to explore whether there are microstructural changes during even earlier phases of learning-related synaptic plasticity in *Aplysia*, using imaging of GFP fusion proteins in living neurons. They found that homosynaptic potentiation induced by moderate tetanic stimulation of the presynaptic neuron in isolated cell culture is accompanied by rapid (less than 10 min) aggregation of the vesicle-associated protein synaptophysin into new clusters, or puncta, as occurs during the early phase of LTP in hippocampal neurons and in 5-HT-induced intermediate-term facilitation in *Aplysia*.

Combined, these results suggest that even the early phases of learning-related synaptic plasticity can involve rapid and coordinated pre- and postsynaptic microstructural alterations that may lead to the (eventual) formation of new functional synapses, as occurs during synaptic development (Cohen-Cory, 2002). This hypothesis is consistent with the current view of intercellular communication that incorporates the biology of nerve cells and, specifically, signaling in

the nervous system, into the broader field of cell biology. Whereas, until about two decades ago, chemical synapses were thought to convey information in only one direction—from the presynaptic to the postsynaptic neuron—it is now clear that synaptic transmission is a bidirectional and self-modifiable form of cell-cell communication (Jessell and Kandel, 1993). The bidirectional nature of signaling across synapses has been demonstrated by biophysical studies of synaptic transmission and by the assembly of synapses both during development and, as we have outlined here, during learning-related plasticity of synapses in the mature brain. This emerging view of the relative contributions of the pre- and postsynaptic neuron and their reciprocal signaling interactions during learning-related synaptic plasticity is likely to supplement (rather than replace) the more established hypothesis that early-phase plasticity involves either pre- or postsynaptic covalent modifications. In some ways, this new appreciation represents a paradigm shift in our thinking about the cellular and molecular bases of learning and memory because it suggests that aspects of the pre- and postsynaptic mechanisms that underlie both early- and late-phase plasticity may not be fundamentally different and independent. Rather, these mechanisms appear to have certain features in common that may contribute to the transition between short-term and long-term memory storage.

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