Nonassociative Learning in Invertebrates

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The simplicity and tractability of the neural circuits mediating behaviors in invertebrates have facilitated the cellular/molecular dissection of neural mechanisms underlying learning. The review has a particular focus on the general principles that have emerged from analyses of an example of nonassociative learning, sensitization in the marine mollusk *Aplysia*. Learning and memory rely on multiple mechanisms of plasticity at multiple sites of the neuronal circuits, with the relative contribution to memory of the different sites varying as a function of the extent of training and time after training. The same intracellular signaling cascades that induce short-term modifications in synaptic transmission can also be used to induce long-term changes. Although short-term memory relies on covalent modifications of preexisting proteins, long-term memory also requires regulated gene transcription and translation. Maintenance of long-term cellular memory involves both intracellular and extracellular feedback loops, which sustain the regulation of gene expression and the modification of targeted molecules.

Learning can be divided into two general categories: associative and nonassociative. Associative learning includes classical conditioning and operant conditioning, which are discussed by Hawkins and Byrne (2015). Non-associative forms of learning include habituation and sensitization. Habituation, the simplest form of learning, is defined as the gradual waning of a behavioral response to a weak or moderate stimulus that is presented repeatedly. Following habituation, the response may be restored to its initial state either passively with time (i.e., spontaneous recovery), or with the presentation of a noxious stimulus. This latter phenomenon is called dishabituation, and its

presence distinguishes habituation from simple fatigue (Thompson and Spencer 1966). Sensitization is defined as the enhancement of a behavioral response by strong or repeated stimulation. In one form of sensitization (also referred to as pseudo-conditioning), the behavioral response to a nonhabituated stimulus is enhanced by presentation of a noxious stimulus to another site, similar to dishabituation. In another form of sensitization, a behavioral response is enhanced by the repeated presentation of a moderate to strong intensity stimulus to the same site. This is operationally the opposite phenomenon of habituation, and is referred to as site-specific sensitization.

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Invertebrates offer experimental advantages for analyzing the cellular and molecular mechanisms of learning. For example, many behaviors in invertebrates are mediated by relatively simple neural circuits, which can be analyzed with electrophysiological and optophysiological approaches. Once the circuit is specified, the neural locus for the particular example of learning can be found, and biophysical, biochemical, and molecular approaches can be used to identify mechanisms underlying the change. The relatively large size of some invertebrate neurons allows these analyses to take place at the level of individually identified neurons. In some cases, individual neurons can be surgically removed and assayed for changes in the levels of second messengers, protein phosphorylation, and RNA and protein synthesis. Moreover, peptides and nucleotides can be injected intracellularly or expressed in individual neurons via appropriate vectors.

This review will focus primarily on progress in understanding nonassociative learning in the marine mollusk *Aplysia*, but many other invertebrates have proven to be valuable model systems for the cellular and molecular analysis of learning and memory (for reviews, see Byrne 1987; Hawkins et al. 1987). Several of these model systems are described within. Each invertebrate model system has its own unique advantages. For example, *Aplysia* is excellent for applying cell biological approaches to the analysis of learning and memory mechanisms. Other invertebrate model systems, such as *Drosophila* and *Caenorhabditis elegans* offer tremendous advantages for obtaining insights into mechanisms of learning and memory through the application of molecular genetic approaches.

NEURAL AND MOLECULAR MECHANISMS OF NONASSOCIATIVE LEARNING IN *Aplysia*

The mechanisms of several simple forms of learning have been studied extensively in *Aplysia*, which has a number of advantages for a reductionist approach (for additional references, see Hawkins et al. 2006). The nervous system of *Aplysia* consists of ~10,000 neurons, many of which are uniquely identifiable across indi-

vidual animals. Studies of learning have focused primarily on defensive withdrawal reflexes, which have simple circuits consisting of only tens or perhaps hundreds of neurons. Many of those studies have examined the gill- and siphon-withdrawal reflex, in which a light touch to the siphon (an exhalant funnel for the gill) produces contraction of the gill and siphon, whereas others have examined the tail-withdrawal reflex or the tail-elicited siphon-withdrawal reflex. However, the results of all of these studies have generally been similar.

Despite their simplicity, the withdrawal reflexes undergo a variety of different forms of learning including habituation, dishabituation and sensitization. This review focuses on the mechanisms of sensitization. The memory for sensitization has multiple temporal domains that depend to a large extent on the training protocol. Typically, a single noxious stimulus, such as a shock produces short-term sensitization (STS) lasting minutes, whereas repeated shocks can produce long-term sensitization (LTS) lasting days. In addition, an intermediate-term stage has been identified that persists for hours. As we see in the literature, the different temporal phases of memory, the training contingencies that produce them, and even some of the underlying molecular mechanisms are conserved across species including humans.

SHORT- AND INTERMEDIATE-TERM SENSITIZATION

Mechanisms of Short-Term Sensitization

During STS, the withdrawal reflex elicited by a weak stimulus to one region of the animal's body is enhanced by a brief electric shock to another region (Carew et al. 1971; Walters et al. 1983b; Antonov et al. 1999; Philips et al. 2011). The neural circuits for many of the withdrawal reflexes consist in part of monosynaptic connections from sensory neurons (SN) to motor neurons (MN), as well as polysynaptic connections involving excitatory and inhibitory interneurons. It is possible to record the activity of these identified neurons and their synaptic connections during learning in semi-intact prepara-

tions, and thus to examine the contribution of plasticity at different sites in the circuit to behavioral learning. Such experiments have shown, for example, that homosynaptic depression and heterosynaptic facilitation at the SN–MN synapses contribute to habituation, dishabituation, and sensitization of the siphon-withdrawal reflex, and that plasticity at other sites also contributes (Antonov et al. 1999). During sensitization by tail shock, siphon stimulation produces increased siphon withdrawal and increased activity in siphon motor neurons as a result of, in part, two mechanisms (Fig. 1). First, the same peripheral stimulus evokes a greater number of action potentials in the presynaptic SNs (i.e., enhanced excitability). Second, each action potential fired by a SN produces a greater excitatory postsynaptic potential (EPSP) in the MN (i.e., short-term facilitation [STF]). The changes in excitability and synaptic potentials are induced in part by the neuromodulator serotonin (5-HT). Both of these changes are mimicked by application of 5-HT, and 5-HT is released from modulatory interneurons during training (Brunelli et al. 1976; Walters et al. 1983b; Glanzman et al. 1989; Mackey et al. 1989; Levenson et al. 1999; Marinesco and Carew 2002; Philips et al. 2011).

The mechanisms of STF at the SN–MN synapses have been examined more extensively in

neural analogs of learning in isolated ganglia or in cell culture, in which tail shock is replaced by either nerve shock or application of 5-HT. Early studies (reviewed in Byrne and Kandel 1996) found that STF produced by brief application of 5-HT to rested synapses (an analog of sensitization) involves cyclic adenosine-3-monophosphate (cAMP), protein kinase A (PKA), decreased K^+ current, and increases in spike width, Ca^{2+} influx, and transmitter release from the SNs (Fig. 2, short term [ST]). In contrast, STF at depressed synapses (an analog of dishabituation) involves protein kinase C (PKC), which acts by a spike broadening-independent mechanism, perhaps vesicle mobilization (Fig. 2, DIS). These results suggested that although dishabituation and sensitization both involve facilitation at the SN–MN synapses, they may involve fundamentally different mechanisms at the molecular level.

The Relationship between Short- and Long-Term Facilitation, and the Discovery of Intermediate-Term Facilitation

STF involves covalent modifications of proteins in existing synapses. In contrast, five tail shocks or five applications of 5-HT separated by 15 min produce long-term facilitation (LTF), which involves protein and RNA synthesis and

Figure 1. Heterosynaptic facilitation of the sensorimotor connection contributes to sensitization in *Aplysia*. (A) Sensitizing stimuli activate facilitatory interneurons (IN) that release modulatory transmitters, one of which is 5-HT. The modulator leads to an alteration of the properties of the sensory neuron (SN) and motor neuron (MN). (B) The enhanced synaptic input to the MN during sensitization results from enhanced sensory input, partly caused by two mechanisms. First, the same peripheral stimulus can evoke a greater number of action potentials in the presynaptic SN (i.e., enhanced excitability). Second, each action potential fired by an SN produces a stronger synaptic response in the MN (i.e., synaptic facilitation). A component of sensitization is also caused by the effects of 5-HT on the MN. (Based on data from Byrne and Kandel 1996.)

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Figure 2. Cellular and molecular mechanisms of facilitation at sensory-motor neuron synapses that contribute to short- and intermediate-term learning in *Aplysia*. Dishabituation (DIS) involves presynaptic protein kinase C (PKC). Short-term (ST) sensitization involves presynaptic protein kinase A (PKA) and calmodulin-dependent protein kinase (CaMKII). Intermediate-term (IT) sensitization involves presynaptic PKA and CaMKII or PKC, protein synthesis (prot syn), and spontaneous transmitter release. In addition, it involves postsynaptic mGluRs, CaMKII or PKC, protein synthesis, and membrane insertion of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-like receptors, as well as recruitment of pre- and postsynaptic proteins to new synaptic sites. In contrast, long-term (LT) sensitization involves gene regulation and growth of new synapses. AC, Adenyl cyclase; cAMP, cyclic adenosine-3-monophosphate; DG, diacylglycerol; NMDA, *N*-methyl-D-aspartate; PKM, protein kinase M; PLC, phospholipase C; RNA syn, RNA synthesis. (Based on data in Hawkins et al. 2013.)

the growth of new synapses, and is thus fundamentally different from STF (Fig. 2, LT). However, it was not clear whether the different stages of plasticity are independent and induced in parallel, or one induces the other in series. Initial studies of facilitation in isolated ganglia suggested that STF and LTF are induced in parallel (Emptage and Carew 1993; Mauelshagen et al. 1996), but similar experiments in culture have suggested that they can be induced, at least partly, in series (Ghirardi et al. 1995).

During the course of these parametric studies of STF and LTF, Ghirardi et al. (1995) also obtained evidence for a third stage that they called intermediate-term facilitation (ITF), which is typically induced by an intermediate level of 5-HT (four to five pulses of a low concentration), persists for hours, and involves PKA and protein synthesis but not RNA synthesis (Fig. 2, intermediate term [IT]). They also found that a higher concentration of 5-HT induces both ITF and LTF, and that the mechanisms of the facilitation depend not only on the time after 5-HT but also on the concentration of 5-HT. For example, facilitation 30 min after the 5-HT, which is in the intermediate-term time range, can depend on PKA only (with 1 or 10 nM 5-HT), PKA and protein synthesis (with 50 nM 5-HT), or PKA, protein synthesis, and RNA synthesis (with 100 nM or 10 μ M 5-HT). These results illustrate that ITF (like the other stages) is not a unitary entity but rather can involve a number of different mechanisms depending on the protocol and, therefore, suggest that it may be more meaningful to ask whether the mechanisms rather than stages are in parallel or series.

Mechanisms of Induction, Maintenance, and Expression of Intermediate-Term Facilitation

The experiments of Ghirardi et al. (1995) did not distinguish between mechanisms of induction, maintenance, or expression of the facilitation, nor did they examine whether those mechanisms are pre- or postsynaptic. Several groups have addressed those questions using different experimental protocols for ITE. Carew and colleagues found that induction of intermediate-

term sensitization (ITS) and ITF with a repeated pulses protocol (five spaced tail shocks or five pulses of 5-HT) requires MAP kinase and protein but not RNA synthesis, and maintenance involves persistent activation of PKA but not PKC (Sutton and Carew 2000; Sutton et al. 2001). In contrast, induction of ITS or ITF with a site-specific protocol (e.g., spike activity in a SN and simultaneous 5-HT) requires PKC and MAP kinase but not protein synthesis, and maintenance involves persistent activation of PKC rather than PKA (Sutton and Carew 2000; Sutton et al. 2004; Zhao et al. 2006; Shobe et al. 2009). Like mammalian PKC, PKC in *Aplysia* has three isoforms: conventional (Apl I), novel (Apl II), and atypical (Apl III), which can be cleaved by calpain to form a persistently active kinase, protein kinase M (PKM) (Kruger et al. 1991; Bougie et al. 2009), which is critical for the maintenance of ITF (Bougie et al. 2012). Thus, induction of ITF with either protocol involves multiple mechanisms including activation of MAP kinase, whereas maintenance involves persistent kinase activity, but of different kinases (PKA or PKM) depending on the induction protocol used.

Glanzman and colleagues found that ITF induced by a single 10-min application of 5-HT involves postsynaptic Ca^{2+} , protein synthesis, and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor insertion (Li et al. 2005; Villareal et al. 2007). To investigate those postsynaptic mechanisms independent of presynaptic mechanisms, they examined ITF of the response to focal application of glutamate (the glutamate excitatory potential or Glu-EP) in isolated MNs, and found that it also involves Ca^{2+} , protein synthesis, and AMPA receptor insertion (Chitwood et al. 2001; Villareal et al. 2007). Furthermore, induction of facilitation of the Glu-EP involves calpain-dependent proteolysis of PKC Apl III to form PKM, and maintenance involves persistent activation of PKM (Bougie et al. 2009; Villareal et al. 2009). Subsequent studies have shown that ITF of the SN–MN EPSP also involves postsynaptic PKC Apl III (Bougie et al. 2012).

These studies suggest that, although STF involves presynaptic mechanisms, ITF by 10-min

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5-HT involves postsynaptic mechanisms. However, in each case, only one side of the synapse was examined, and it was not known whether the same protocol might involve mechanisms on both sides. Hawkins and colleagues addressed that question, and found that facilitation during STS in the semi-intact siphon-withdrawal preparation involves PKA, calmodulin-dependent protein kinase (CaMK)II, and transient spike broadening in the SN, but it does not involve Ca^{2+} or CaMKII in the MN and, thus, appears to be entirely presynaptic. The facilitation during ITS also involves PKA, CaMKII, and transient spike broadening in the SN. However, it also involves Ca^{2+} and CaMKII in the MN and protein synthesis in both neurons, and is thus both pre- and postsynaptic (Antonov et al. 2010).

Similarly, facilitation by 1-min application of 5-HT in cell culture (an analog of STS) involves PKA and CaMKII in the SN, but it does not involve Ca^{2+} in the MN or PKC in either neuron (Jin et al. 2011). In contrast, facilitation by a 10-min application of 5-HT (an analog of intermediate-term sensitization) involves PKC (but not PKA or CaMKII) in the SN, in agreement with previous studies (Byrne and Kandel 1996). In addition, 10-min application of 5-HT involves Ca^{2+} and CaMKII in the MN and protein synthesis in both neurons and is, thus, both pre- and postsynaptic. Collectively, these results suggest that ITF is the first stage to involve both pre- and postsynaptic molecular mechanisms.

ITF also involves recruitment of synaptic proteins. ITF induced by multiple applications of 5-HT is accompanied by filling of empty presynaptic varicosities with the vesicle-associated protein synaptophysin within 3 h, but not by the formation of new varicosities (Kim et al. 2003). Like facilitation of the postsynaptic potential (PSP) with this protocol (Ghirardi et al. 1995), the increase in clusters of synaptophysin does not persist for 24 h, and does not require protein or RNA synthesis. In contrast, LTF is accompanied by both filling of varicosities and the formation of new varicosities within 12–18 h. Again, like facilitation of the PSP and the increase in varicosities (Bailey et al. 1992), the increase in clusters of synaptophysin during LTF

persists for 24 h and requires protein synthesis. ITF and LTF are also accompanied by increases in clusters of the postsynaptic proteins ApGluR1 and ApNR1 within 12 h, whereas STF is not (Li et al. 2009). These results suggest that the intermediate-term stage is also the first to involve recruitment of both pre- and postsynaptic proteins, which could be initial steps in the formation of new synapses during long-term facilitation.

Spontaneous Transmitter Release from the Presynaptic Neuron Recruits Postsynaptic Mechanisms of Intermediate- and Long-Term Facilitation

If STF is presynaptic but ITF and LTF involve both pre- and postsynaptic mechanisms, how are the postsynaptic mechanisms first recruited? There are at least two possibilities, which are not mutually exclusive: the pre- and postsynaptic mechanisms might be induced by activation of pre- and postsynaptic 5-HT receptors in parallel, or activation of presynaptic 5-HT receptors might increase spontaneous release of glutamate, which then activates postsynaptic glutamate receptors to induce the postsynaptic mechanisms in series (Fig. 2).

Partly because the postsynaptic mechanisms of ITF are similar to those induced by glutamate release during homosynaptic potentiation (Jin and Hawkins 2003), Jin et al. (2012a,b) investigated the possible role of spontaneous transmitter release from the presynaptic neuron as an anterograde signal for recruiting postsynaptic mechanisms of ITF and LTF. 5-HT produced a substantial increase in the frequency of spontaneous miniature excitatory postsynaptic currents (mEPSCs) and a more modest increase in their amplitude during, or shortly after, the induction of ITF or LTF in cell culture. Those increases correlated with subsequent facilitation of the evoked EPSP, consistent with the idea that spontaneous release contributes to the induction of the facilitation. In support of that idea, several manipulations that either reduced or enhanced spontaneous release (without affecting baseline evoked release) also reduced or enhanced ITF and LTF. These results

suggested that spontaneous release is necessary for the induction of ITF and LTF, and acts synergistically with additional mechanisms (activated, e.g., by presynaptic cAMP) to produce the facilitation.

Further experiments showed that spontaneous release from the presynaptic neuron activates metabotropic glutamate receptors, which stimulate IP₃ production and Ca²⁺ release in the postsynaptic neuron (Fig. 2). In addition, expression of the latter part of facilitation may involve up-regulation of AMPA-like receptors (see also Li et al. 2005). To examine that mechanism more directly, Jin et al. (2012b) expressed the *Aplysia* homolog of the AMPA receptor subunit GluR1 in the MN. The application of 5-HT for 10 min produced an increase in membrane insertion of ApGluR1 into existing puncta as well as increases in the number of puncta of ApGluR1 and overlap with puncta of the presynaptic protein synaptophysin. Furthermore, all of those increases depended on spontaneous release and/or mGluRs. The increase in ApGluR1 puncta during ITF preceded an increase in synaptophysin puncta (Kim et al. 2003), and, therefore, may be a first step in a sequence that can lead to new synapse assembly during LTF.

LONG-TERM SENSITIZATION

Neuronal Correlates of Long-Term Sensitization

In addition to STS and ITS, withdrawal reflexes can also display LTS, lasting from several hours to weeks (Pinsker et al. 1973). Although STS (or its in vitro analogue STF) can be induced by brief treatments (lasting a few seconds to minutes), LTS- and 5-HT-induced LTF require more extensive training involving multiple trials spaced over hours or days.

One approach to investigating mechanisms of LTS is to examine its neural correlates—that is, to train an animal, remove the nervous system some time later, and test for differences in cellular properties compared with controls. Such studies have shown that mechanisms supporting LTS generally resemble the mechanisms sup-

porting STS including changes in SN excitability (long-term enhanced excitability [LTEE]) and facilitation of sensorimotor synapses (i.e., LTF) (Frost et al. 1985; Scholz and Byrne 1987; Cleary et al. 1998). However, although STS is correlated with SN spike broadening, LTS is correlated with SN spike narrowing (Antzoulatos and Byrne 2007). The functional implications of spike narrowing are not obvious, but may involve an increase in the fidelity of the neuronal response to peripheral stimuli by decreasing the probability of spike failures. Another key difference between cellular correlates of STS and LTS is that LTS is associated with structural modifications of SNs, namely, outgrowth of neurites and remodeling of active zones, whereas STS is not. These data suggest that enhanced transmission is mediated by an increase in transmitter release and in number of synapses (Bailey and Chen 1983; Wainwright et al. 2002). LTS and LTF are also correlated with enhanced uptake of glutamate (Levenson et al. 2000), the endogenous transmitter of SNs (Dale and Kandel 1993; Antzoulatos and Byrne 2004). In addition, LTS is correlated with changes in the biophysical properties of MNs (Cleary et al. 1998), and an increased synthesis of postsynaptic receptors (Trudeau and Castellucci 1995; Zhu et al. 1997; Cai et al. 2008).

Molecular Mechanisms Contributing to the Induction of LTF—cAMP-Response Element-Binding (CREB) and Gene Regulation

The molecular mechanisms of LTF have been examined more extensively in neural analogs in isolated ganglia or cell culture (Fig. 3). Such studies have shown that STF and LTF share some common cellular pathways during their induction. For example, both forms activate the cAMP/PKA cascade. However, although STF involves PKA-dependent covalent modifications of proteins involved in increasing spike width, excitability, and transmitter release, LTF involves the PKA-dependent regulation of gene transcription and new protein synthesis. Multiple training trials or repeated applications of 5-HT lead to a translocation of PKA to the nucleus, where it phosphorylates the transcriptional ac-

Figure 3. Simplified scheme of the mechanisms that contribute to long-term sensitization in *Aplysia*. Sensitization training leads to cyclic adenosine 3-monophosphate (cAMP)-dependent regulation of cAMP-response element binding (CREB)1. Serotonin also leads to activation of extracellular signal-regulated kinase (ERK), which regulates CREB2. Although CREB1 acts as an initiator of gene transcription, CREB2 acts as a repressor of gene transcription. The combined effects of activation of CREB1 and suppression of CREB2 lead to regulation of the synthesis of at least 10 proteins, only some of which are shown. *Aplysia* tolloid/BMP-like protein (ApTBL) is believed to activate latent forms of transforming growth factor (TGF)- β , which can then bind to receptors on the sensory neuron (SN). TGF- β activates ERK, which may act by initiating a second round of gene regulation by affecting CREB2-dependent pathways. Serotonin can also increase the local synthesis of the *Aplysia* homolog of cytoplasmic polyadenylation element-binding protein (ApCPEB) and the peptide sensorin through phosphoinositide-3-kinase (PI3K). ApCPEB can exist in two conformations, one of which dominates and allows ApCPEB to self-perpetuate. Sensorin release is dependent on type II protein kinase A (PKA). Sensorin binds to autoreceptors leading to further activation of ERK. Because increased synthesis of sensorin requires elevation of postsynaptic calcium, a retrograde signal is also postulated. In addition to the retrograde signal, 5-HT-induced postsynaptic signaling also leads to an increased number of glutamate receptors. AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ApCAM, *Aplysia* cell adhesion molecule; ApTrk, *Aplysia* tyrosine kinase autoreceptor; ApUch, *Aplysia* ubiquitin hydrolase; MEK, MAPK/ERK kinase; PKM, protein kinase M. (Based on data from Liu et al. 1997.)

dition is defined as the time window during which RNA and protein synthesis are required for converting short- to long-term memory. For example, treatment of ganglia with five pulses of 5-HT over a 1.5-h period to mimic sensitization training leads to the binding of CREB1 to the promoter of its own gene and induces CREB1 synthesis, giving rise to a CREB1-positive feedback loop that supports memory consolidation (Liu et al. 2011).

As illustrated by the CREB1 feedback loop, the phosphorylation of CREB1 by PKA and CREB2 by ERK is not a simple serial cascade starting with binding of 5-HT to membrane receptors.

Indeed, the transduction process involves multiple feedback pathways. For example, levels of *Aplysia* ubiquitin hydrolase (ApUch) in SNs are increased, possibly via a CREB-based increase in ApUch transcription. The increased levels of ApUch increase the rate of degradation of proteins, via the ubiquitin–proteasome pathway, including the regulatory subunit of PKA (Chain et al. 1999). The catalytic subunit of PKA, when freed from the regulatory subunit, is highly active. Thus, increased ApUch triggered by the initial treatment of SNs with 5-HT will lead to an increase in PKA activity and a more protracted phosphorylation of CREB1 (Fig. 3). This phosphorylated CREB1 may act to further prolong ApUch expression, thus closing a positive feedback loop. Protein degradation, in general, and the role of ubiquitination in particular, is an emerging theme in recent studies on the neural basis of long-term memory (Fioravante and Byrne 2011).

Several extracellular feedback cascades also appear to operate to activate ERK and, thereby, regulate CREB2 (Martin et al. 1997; Guan et al. 2002). 5-HT stimulates secretion of a recently identified endogenous *Aplysia* neurotrophin (ApNT) from the SN (Kassabov et al. 2013), leading to the activation of an *Aplysia* tyrosine kinase autoreceptor (ApTrk) and subsequent activation of ERK in the SN (Fig. 3) (Purcell et al. 2003; Ormond et al. 2004; Sharma et al. 2006). A similar feedback loop occurs through the release of an *Aplysia* cysteine-rich neurotrophic factor (ApCRNF) (Pu et al. 2014). 5-HT also acts through feedback pathways involving the peptides sensorin and transforming growth factor (TGF)- β . A role for TGF- β was originally hypothesized based on the finding that LTF is associated with an increased expression of *Aplysia* tolloid/BMP-like (ApTBL)-1 protein. Tolloid and the related molecule BMP-1 appear to function as secreted Zn^{2+} proteases, which activate members of the TGF- β family in some systems. In SNs, application of TGF- β mimics the effects of 5-HT in that it produces LTF (Fig. 3) (Zhang et al. 1997). Interestingly, TGF- β activates ERK in the SNs and induces its translocation to the nucleus. Thus, TGF- β could be part of an extracellular positive feedback loop,

possibly leading to another round of protein synthesis to further consolidate the memory (Zhang et al. 1997).

Another extracellular positive feedback loop that affects ERK involves the 5-HT-induced regulation of the release of the SN-specific neuropeptide sensorin (Fig. 3). Synthesis of sensorin is stimulated by 5-HT in a PI3 (phosphatidylinositol-3)-kinase–dependent manner, and sensorin binding to presynaptic autoreceptors activates ERK. Sensorin synthesis also requires elevation of postsynaptic calcium (Cai et al. 2008). The mechanism through which postsynaptic calcium regulates the local protein synthesis of sensorin in the presynaptic terminal is not fully understood, but presumably involves the release of a retrograde signal (Cai et al. 2008). Although the presence of a postsynaptic neuron seems to be required for long-term facilitation, it is not required for another correlate of long-term sensitization, increased SN excitability (Cleary et al. 1998; Liu et al. 2011). Thus, accumulating evidence suggests that expression of long-term memory in this simple system does not rely on a unitary mechanism, but on multiple mechanisms at multiple sites.

Molecules Involved in the Maintenance and Expression of LTF

The combined effects of activation of CREB1 and removal of the repression of transcription by CREB2 lead to changes in the synthesis of specific proteins that allow for the maintenance and expression of LTF. The down-regulation of a homolog of a neuronal cell adhesion molecule (NCAM) shows that ApCAM plays a key role in the expression of long-term facilitation. This down-regulation has two components. First, the synthesis of ApCAM is reduced (Fig. 3). Second, preexisting ApCAM is internalized via increased endocytosis (not shown). The internalization and degradation of ApCAM allow for the restructuring of the axon arbor (Bailey and Kandel 2008). This restructuring allows the SN to form additional connections with the MN or with other cells. Structural changes associated with LTF also involve the presynaptic cell-adhesion protein neuexin, along with its postsynap-

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Figure 4. Cascade model of mechanisms contributing to the different stages of synaptic plasticity in *Aplysia*. In cascade models (Fusi et al. 2005), synapses have two levels of strength (weak and strong) and several increasingly long-lasting states. In *Aplysia*, relatively weak stimulation produces short-term facilitation (STF) that lasts minutes, stronger stimulation produces intermediate-term facilitation (ITF) that lasts minutes to hours, and even stronger stimulation produces long-term facilitation (LTF) that lasts days. The different stages of facilitation may involve a series or cascade of pre- and postsynaptic mechanisms that is initiated by spontaneous transmitter release during STF, progresses through two stages of ITF, and can culminate in synaptic growth during LTF. The mechanisms in this growth cascade are a subset of all mechanisms involved in facilitation, and some other mechanisms (not shown) may act in parallel and contribute only to specific stages. Thus, the idea of a cascade applies to the mechanisms and not the stages per se. In addition to this linear cascade, facilitation also involves feedforward and feedback loops. Dashed lines, transitions that are initiated by different durations or patterns of MN; solid lines, spontaneous transitions; red, extracellular signaling molecules; blue, structural modifications. MN, motor neuron; SN, sensory neuron.

tic counterpart neuroligin and their transsynaptic interaction (Choi et al. 2011).

In addition, the persistence of LTF depends on two molecules that can maintain a change in functional state for long periods of time. First, the stabilization of new structures depends on the *Aplysia* homolog of cytoplasmic polyadenylation element-binding protein (ApCPEB) (Miniaci et al. 2008), which regulates local translation in the SNs (Fig. 3). ApCPEB has prion-like properties, meaning that it can exist

in two conformations, one of which dominates and allows ApCPEB to self-perpetuate. Conversion of ApCPEB to the self-perpetuating state is enhanced by 5-HT and is required for the persistence of LTF (Si et al. 2010). Second, as discussed earlier for ITF, the persistence of LTF also depends on the persistent activation of PKC by cleavage to the PKM form (PKM Apl III) (Cai et al. 2011), which is thought to regulate the membrane insertion of AMPA receptors in the MN (Fig. 3).

Possible Relationships between Mechanisms Contributing to STF, ITF, and LTF

Collectively, the results on STF, ITF, and LTF in *Aplysia* suggest that, like synapse formation (McAllister 2007), the different stages of synaptic plasticity may involve different pre- and postsynaptic mechanisms coordinated by back-and-forth signaling in a chain or cascade that can culminate in growth (Fig. 4). Consistent with that idea, spontaneous transmitter release from the presynaptic neuron during STF recruits postsynaptic molecular mechanisms of ITF including IP₃, Ca²⁺, and formation of clusters of AMPA-like glutamate receptors. Postsynaptic Ca²⁺ is, in turn, necessary for LTF, perhaps through retrograde signaling to presynaptic ApCAM, neurexin, or Trk receptors (Purcell et al. 2003; Ormond et al. 2004; Sharma et al. 2006; Cai et al. 2008; Hu et al. 2010; Choi et al. 2011; Kassabov et al. 2013). The new postsynaptic clusters of AMPA-like receptors may also participate in retrograde signaling, and recruit presynaptic clusters of synaptophysin during a later stage of ITF and growth of presynaptic varicosities during LTF (Kim et al. 2003; Ripley et al. 2011; Lee et al. 2012). These ideas are similar to theoretical “cascade” models of memory storage that can show plasticity as well as long-term stability (Fusi et al. 2005), which would seem to be mutually exclusive but are both essential features of memory. In addition to this linear cascade, the mechanisms of plasticity also form feedforward loops involved in synaptic “tagging,” which allows transcription-dependent LTF to be synapse specific (Casadio et al. 1999), as well as feedback loops involved in the long-term persistence of the functional and structural changes (Fig. 3).

NEURAL AND MOLECULAR MECHANISMS OF NONASSOCIATIVE LEARNING IN OTHER INVERTEBRATE MODEL SYSTEMS

Analyses of nonassociative learning in other invertebrates have confirmed and extended the work on *Aplysia*. Thus, as in *Aplysia* (Castellucci et al. 1970; Castellucci and Kandel 1974), habituation in crayfish (Zucker 1972) and several oth-

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er species (see below) appears to be caused by a progressive decrease in the amount of transmitter released by primary sensory neurons. In addition, there is apparent conservation of molecular mechanisms for sensitization, which often involve the engagement of 5-HT and the cAMP cascade. For example, the opisthobranch *Tritonia diomedea* initiates stereotypical rhythmic swimming to escape a noxious stimulus. The behavior shows both habituation and sensitization (Frost et al. 1996). Habituation appears to involve plasticity at multiple loci, including decrement at the first afferent synapse. Sensitization appears to involve enhanced excitability and synaptic strength in one of the central pattern-generating (CPG) interneurons. Modulation of interneurons can be mediated by 5-HT, which has diverse effects on multiple loci of the circuit (Sakurai et al. 2007). Sensitization of withdrawal reflexes of the land snail *Helix* also appears to be mediated by serotonergic modulatory cells whose spiking frequency increases following noxious stimulation (Balaban 2002). These serotonergic cells are electrically coupled so that they are recruited and fire synchronously in response to strong excitatory input.

The shortening reflex of the leech *Hirudo medicinalis* shows habituation and sensitization, and the neuronal changes underlying both occur in the pathway from mechanosensory neurons to the S cells (Sahley et al. 1994). Habituation correlates with decreased S-cell excitability (Burrell et al. 2001) and the reflex can be restored (dishabituation) following application of a single noxious stimulus (Boulis and Sahley 1988). The potentiation of the shortening reflex observed during sensitization is mediated by 5-HT through an increase of the levels of cAMP (Belardetti et al. 1982; Burrell and Sahley 2005), which also increases the excitability of S cells and spike after hyperpolarization (AHP) (Burrell et al. 2001; Burrell and Crisp 2008).

Habituation of the shortening reflex also involves depression of the synapses of touch (T) sensory neurons onto their follower target neurons. This synaptic depression is associated with an increase in the amplitude of the T-cell AHP that follows their discharge (Brunelli et al. 1997;

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Scuri et al. 2002). The persistent increase in AHP amplitude, following low-frequency stimulation of T cells, has been attributed to increased activity of the electrogenic Na^+ pump, and requires activation of phospholipase A2 (Scuri et al. 2005; Zaccardi et al. 2012).

Nonassociative learning has been studied extensively in *C. elegans* by Catharine Rankin and colleagues. *C. elegans* is a valuable model system for cellular and molecular studies of learning because it has an extremely simple nervous system that consists of a total of 302 neurons, the anatomical connectivity of which has been described at the electron microscopy level. *C. elegans* responds to a vibratory stimulus applied to the medium in which they locomote by swimming backward. This reaction, known as the tap withdrawal reflex, shows habituation, dishabituation, sensitization, and long-term (24 h) retention of habituation training. Laser ablation studies have been used to elucidate the neural circuitry supporting the tap withdrawal reflex and to identify likely sites of plasticity within the network. Plastic changes during habituation appear to occur at the chemical synapses between presynaptic sensory neurons and postsynaptic command interneurons. Analysis of several *C. elegans* mutants has revealed that synapses at the locus of plasticity in the network may be glutamatergic (Ardiel and Rankin 2010; Bozorghmehr et al. 2013). CREB is required for long-term but not short-term habituation (Timbers and Rankin 2011).

SUMMARY AND CONCLUSIONS

The simplicity and tractability of the neural circuits mediating behaviors in invertebrates have facilitated the cellular/molecular dissection of the underlying neural mechanisms of nonassociative learning and has illuminated several basic principles:

- Learning and memory rely on multiple mechanisms of plasticity at multiple sites of the neuronal circuits.
- The relative contribution to memory of the different sites varies as a function of the extent of training and time after training.

- Although the target proteins are different, the same intracellular signaling cascades that induce short-term modifications in synaptic transmission can also be used to induce long-term changes.
- Short-term memory relies on covalent modifications of preexisting proteins, but long-term memory also requires regulated gene transcription and translation.
- The induction of long-term memory requires both the activation of inducing signals, and the inhibition of inhibitory constraints imposed by other molecular pathways.
- Maintenance of long-term cellular memory involves both intracellular and extracellular feedback loops, which sustain the regulation of gene expression and the modification of targeted molecules.
- As described by Hawkins and Byrne (2015), associative forms of learning and memory can arise from the neural mechanisms that are used for nonassociative learning.

Since the 1960s, research on nonassociative learning in invertebrates has provided a wealth of information on the mechanisms of simple forms of learning. Although the learning studied is of the simplest type, the mechanisms have proven to be extremely rich and complex. The studies have provided important general principles that have proven applicable to all animals. Despite the great progress, the knowledge of memory mechanisms is still in its infancy. A more mature understanding will come from continued analyses of these model systems.

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