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New Tricks for an Old Slug: The Critical Role of Postsynaptic Mechanisms in Learning and Memory in *Aplysia*

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Abstract

The marine snail *Aplysia* has served for more than four decades as an important model system for neurobiological analyses of learning and memory. Until recently, it has been believed that learning and memory in *Aplysia* were due predominately, if not exclusively, to presynaptic mechanisms. For example, two nonassociative forms of learning exhibited by *Aplysia*, sensitization and dishabituation of its defensive withdrawal reflex, have been previously ascribed to presynaptic facilitation of the connections between sensory and motor neurons that mediate the reflex. Recent evidence, however, indicates that postsynaptic mechanisms play a far more important role in learning and memory in *Aplysia* than formerly appreciated. In particular, dishabituation and sensitization depend on a rise in intracellular Ca^{2+} in the postsynaptic motor neuron, postsynaptic exocytosis, and modulation of the functional expression of postsynaptic AMPA-type glutamate receptors. In addition, the expression of the persistent presynaptic changes that occur during intermediate- and long-term dishabituation and sensitization appears to require retrograde signals that are triggered by elevated postsynaptic Ca^{2+} . The model for learning-related synaptic plasticity proposed here for *Aplysia* is similar to current mammalian models. This similarity suggests that the cellular mechanisms of learning and memory have been highly conserved during evolution.

1. Introduction

When Eric R. Kandel was awarded the Nobel Prize in Physiology or Medicine in 2000, the field of *Aplysia* learning and memory appeared to have reached its apogee. In his Nobel Prize lecture, published as an essay in *Science*, Kandel (2001) masterfully summarized the general insights gained from over forty years of work by his laboratory and others on the cellular mechanisms of memory in the marine snail, *Aplysia*. As illustrated in Figure 1, taken from the *Science* essay, Kandel's scheme for learning-related synaptic changes in *Aplysia* focused almost entirely on presynaptic mechanisms of plasticity. Specifically, Kandel proposed that during behavioral sensitization, the form of learning that has been the most studied in *Aplysia*, a monoaminergic transmitter, serotonin (5-HT), is released within the central nervous system (CNS) of *Aplysia*; 5-HT then binds to receptors on the sensory neurons that innervate the siphon and gill of *Aplysia*; and, as a result, a cascade of intracellular changes are initiated in the sensory neurons. These changes involve activation of protein kinases, and, in some cases,

activation of genes and synthesis of new proteins. The cellular consequence of these changes is an enhancement of the release of transmitter from the terminals of the sensory neurons. According to the Kandelian model, it is this enhancement of neurotransmitter release, referred to as presynaptic facilitation that is responsible for the behavioral enhancement during sensitization.

Some version of the diagram in Figure 1 can be found in practically every introductory textbook in neuroscience. Indeed, the cellular models that have been generated for simple forms of learning and memory, including habituation, sensitization, and classical conditioning, in *Aplysia* are widely regarded as one of the triumphs of modern neuroscience. Several of the mechanisms that have been proposed to explain learning in *Aplysia*—perhaps most prominently, gene transcription triggered by the transcription factor cyclic cAMP response element-binding protein (CREB) (Dash *et al.*, 1990; Kaang *et al.*, 1993)—are not unique to this mollusk, but have been shown to be important for learning several other important model organisms in neuroscience, including *Drosophila* (Yin *et al.*, 1995; Yin *et al.*, 1994), mice (Alarcon *et al.*, 2004; Bozon *et al.*, 2003; Kida *et al.*, 2002; Kogan *et al.*, 1997), and rats (Brightwell *et al.*, 2004; Josselyn *et al.*, 2001).

Despite the impressive advances toward an understanding of the mechanisms of learning and memory made in *Aplysia*, it has become increasingly evident that the scheme depicted in Figure 1 is deficient. In particular, postsynaptic mechanisms, which have until relatively recently have been either completely ignored or given short shrift in cellular models of dishabituation and sensitization in *Aplysia*, are indispensable to these forms of learning. Furthermore, as described below, new work reveals an unexpected and striking similarity between the postsynaptic mechanisms that underlie dishabituation/sensitization in *Aplysia* and those that underlie prominent forms of synaptic plasticity and learning in mammals. This similarity suggests that there has been remarkably little change in the biological machinery of memory during the past 555 million years, the approximate period since the last common protostome-deuterostome ancestor (Erwin and Davidson, 2002). Here I review the recent evidence that necessitates a revision of the presynaptic model of dishabituation/sensitization in *Aplysia*, and propose a new model that integrates pre- and postsynaptic mechanisms.

2. Different Phases of Memory in *Aplysia*

2.1 Behavioral results

To reduce stylistic awkwardness, I will use the term sensitization below to refer to both dishabituation and sensitization, unless otherwise specified. These two cognate forms of nonassociative learning in *Aplysia* are mediated by similar, albeit not entirely identical, mechanisms (see Antonov *et al.*, 1999, 2005; Cohen *et al.*, 1997; Glanzman *et al.*, 1989b; Hochner *et al.*, 1986; Mackey *et al.*, 1987; Marcus *et al.*, 1988; Rankin and Carew, 1988; Wright *et al.*, 1991).

Aplysia exhibits a defensive withdrawal reflex of its gill and siphon that can be sensitized by noxious stimuli, such as electrical shocks applied to the animal's skin (Carew *et al.*, 1971; Pinsker *et al.*, 1970). Repeated application of noxious stimulation can produce sensitization of the withdrawal reflex that persists for days-to-weeks (Frost *et al.*, 1985; Pinsker *et al.*, 1973). Classically, memory for sensitization of the withdrawal reflex has been divided into two phases, short-term memory (STM) and long-term memory (LTM). LTM has been distinguished from the STM by its requirement for protein synthesis and gene transcription (Castellucci *et al.*, 1989; Goelet *et al.*, 1986). In addition, LTM, unlike STM, involves the growth of new synaptic connections between the sensory and motor neurons that mediate the withdrawal reflex (Bailey, 1999; Bailey *et al.*, 1996; Bailey and Chen, 1983; Bailey and Chen, 1988a; Bailey and Chen, 1988b; Bailey and Chen, 1989; O'Leary *et al.*, 1995; Wainwright *et al.*, 2002). Indeed, it seems

likely that the protein synthesis and gene transcription that accompany LTM are involved, in part, in the construction of new sensorimotor synapses (see Bailey *et al.*, 1992; Mayford *et al.*, 1992).

Within the last decade a third phase of memory for sensitization has become recognized. This phase, termed intermediate-phase memory (ITM), can be differentiated both temporally and mechanistically from the other two phases of memory. ITM, like LTM, can be induced by multiple tail shocks. Sutton and colleagues (2002; 2001) reported that a single shock produces sensitization of the siphon-withdrawal reflex that decays within ~ 30 min. By contrast, 5 individual shocks at 15 min intervals produce sensitization that declines to zero within 3 hr after training, but then reappears by 24 hr after training. Therefore, training with multiple shocks produces a biphasic pattern of sensitization. The first phase contains two components, an early component (STM) and a later component (ITM); the second phase consists of a single component, LTM. Mechanistically, ITM can be distinguished from STM by a lack of dependence on protein synthesis (but see below); ITM can be distinguished from LTM in not depending on gene transcription (Sutton *et al.*, 2001). In addition, ITM induced by multiple tail shocks requires the persistent activation of protein kinase A (PKA) for its maintenance (Sutton *et al.*, 2001). More recently, a second form of ITM has been identified. This form, rather than being induced by multiple shocks, is revealed by testing the site on the tail to which a single tail shock has been delivered (Sutton *et al.*, 2004). This type of learning is referred to as site-specific sensitization; it is believed to result from the conjunction of the firing of sensory neurons that innervate the shocked site on the tail and a neuromodulatory transmitter released from heterosynaptic facilitatory neurons activated by the tail shock (see Bao *et al.*, 1998; Eliot *et al.*, 1994; Walters, 1987). Site-specific ITM differs from ITM induced by multiple shocks (repeated-trial ITM) in a lack of dependence on protein synthesis for its induction (Sutton *et al.*, 2004). Site-specific ITM can be further distinguished from repeated-trial ITM in its dependence on persistent activation of protein kinase C (PKC), rather than PKA. Interestingly, results from experiments using inhibitors differentially selective for different isoforms of PKC indicate that maintenance of site-specific ITM is selectively dependent on the activity of a PKM-like isoform (Sutton *et al.*, 2004). In vertebrates PKM can be generated either through the proteolytic cleavage of atypical PKC, or through *de novo* synthesis (Hernandez *et al.*, 2003). When generated by proteolytic cleavage, PKM remains persistently active because it lacks autoinhibition from the PKC regulatory domain. However, the activity of one isoform of PKM, PKM ζ , appears to be maintained via ongoing protein synthesis (Hernandez *et al.*, 2003). Recent evidence indicates that PKM ζ plays a key role in the persistence of learning-related synaptic plasticity in mammals, as well as in memory maintenance (Pastalkova *et al.*, 2006; Sacktor *et al.*, 1993; Serrano *et al.*, 2005; Shema *et al.*, 2007). A PKM isoform has recently been identified in *Aplysia*, although unlike PKM ζ , it appears to be formed by calpain-dependent proteolytic cleavage from atypical PKC (Bougie *et al.*, 2006; Bougie *et al.*, 2007).

2.2 Synaptic correlates of different memory phases

Each of the three memory phases for sensitization—STM, ITM, and LTM—has a synaptic correlate. These are forms of facilitation of the synaptic connections between the central sensory and motor neurons that participate in the withdrawal reflex (Byrne *et al.*, 1974; Byrne *et al.*, 1978a; Byrne *et al.*, 1978b; Dubuc and Castellucci, 1991; Frost *et al.*, 1988; Walters *et al.*, 1983a). In addition to producing enhancement of the withdrawal reflex, sensitizing stimuli such as tail shock facilitate the sensorimotor synapse (Antonov *et al.*, 1999; Castellucci and Kandel, 1976; Walters *et al.*, 1983b). Significant evidence indicates that this facilitation is mediated, at least in part, by serotonin (5-HT). Serotonin (5-HT) is an endogenous monoamine (Hawkins, 1989; Kistler *et al.*, 1985) that is released within the central nervous system (CNS) of *Aplysia* after sensitizing stimulation (Marinesco, 2002 #1142, also see Zhang *et al.*, 2003). Application of 5-HT facilitates sensorimotor connections (Brunelli *et al.*, 1976; Rayport and

Schacher, 1986), as does stimulation of an identified serotonergic interneuron, CB1 (Mackey *et al.*, 1989). Depletion of 5-HT within the nervous system impairs sensitization (Glanzman *et al.*, 1989b). Therefore, 5-HT is likely to play a central role in sensitization in *Aplysia*, although other facilitatory transmitters may also participate (Abrams *et al.*, 1984; Hawkins *et al.*, 1981; Ocorr and Byrne, 1985).

A brief (<5 min) application of 5-HT produces short-term facilitation (STF) of the sensorimotor synapse, i.e., facilitation that lasts < 30 min (Brunelli *et al.*, 1976; Mauelshagen *et al.*, 1996; Rayport and Schacher, 1986), as does a single tail shock (Antonov *et al.*, 1999; Walters *et al.*, 1983b). Five spaced pulses (typically, a 5–6 min-long pulse is used) of 5-HT yields synaptic facilitation that can persist for ≥ 24 hr, or long-term facilitation (LTF) (Mauelshagen *et al.*, 1996; Montarolo *et al.*, 1986). Similarly, multiple spaced shocks to the animal's tail or body wall can also produce LTF of the sensorimotor synapse (Cleary *et al.*, 1998; Frost *et al.*, 1985). Just as training with multiple, spaced sensitizing stimuli recruits not only LTM, but also ITM (above), multiple, spaced applications of 5-HT recruit intermediate-term facilitation (ITF), as well as LTF, of the sensorimotor synapse (Ghirardi *et al.*, 1995; Mauelshagen *et al.*, 1996). ITF can also be induced by pairing a brief bout of activity in the sensory neuron with a single pulse of 5-HT (Bao *et al.*, 1998; Sutton and Carew, 2000). This activity-dependent ITF parallels activity-dependent ITM (above).

3. Mechanisms of Learning-Related Synaptic Facilitation in *Aplysia*

3.1. Presynaptic mechanisms

3.1.1 Short-term facilitation—The mechanisms of STF have been extensively reviewed previously (Byrne and Kandel, 1996; Kandel, 2001). STF appears to be mediated by changes in the presynaptic sensory neurons (but see below). Briefly, 5-HT activates a presynaptic adenylyl cyclase, leading to the synthesis of cyclic AMP (cAMP) and activation of PKA. Activation of PKA closes presynaptic K⁺ channels, which results in a longer action potential and greater presynaptic influx of Ca²⁺ during an action potential. In addition to modulation of action potential duration, 5-HT can recruit a second facilitatory mechanism; this mechanism is particularly potent when the sensorimotor synapse is depressed (Gingrich and Byrne, 1985; Hochner *et al.*, 1986). At depressed synapses 5-HT causes a spike duration-independent facilitation of release via a process that is poorly understood but that appears to involve mobilization of presynaptic vesicles. This second process depends predominately on PKC activity (Ghirardi *et al.*, 1992; Manseau *et al.*, 2001).

3.1.2 Intermediate-term facilitation—As outlined above, activity-independent ITF, which results from multiple, spaced exposures of the sensorimotor synapse to 5-HT, depends on protein synthesis and persistent activation of PKA. Until recently, it was unclear whether the necessary protein synthesis occurs in the sensory or motor neuron. However, the locus of persistent PKA activity appears to be presynaptic. Müller and Carew (1998) found that 5 spaced pulses of 5-HT produced persistent phosphorylation of PKA in sensory neurons; the early phase of this persistent change, which decayed by 3 hr, depended on protein, but not RNA, synthesis, and therefore corresponds to ITM. Activity-dependent ITF, which does not require protein synthesis, depends on persistent PKC activity (Sutton and Carew, 2000). A recent study by Zhao *et al.* (2006) has demonstrated that the sensory neuron is one site for the persistent activity of PKC. Zhao *et al.* specifically expressed fluorescently tagged isoforms of PKC in sensory neurons. They found that sensory neuron firing paired with a pulse of 5-HT caused the Ca²⁺-dependent isoform of PKC in *Aplysia*, Apl I (Kruger *et al.*, 1991), to translocate from the cytoplasm to the cell membrane, thereby indicating its activation (see also Sossin *et al.*, 1994). Zhao *et al.* also found that overexpressing a dominant-negative form of Apl I in sensory neurons blocks activity-dependent ITF.

3.1.3 Long-term facilitation—Most of the work on the cellular mechanisms of LTF has been performed using sensorimotor synapses in dissociated cell culture. LTF is induced by repeated, spaced pulses of 5-HT. In the most common protocol sensorimotor cocultures are treated with five 5-min pulses of 5-HT, with 15 min between the pulses (Montarolo *et al.*, 1986). The results from studies of LTF have been extensively reviewed (Bailey *et al.*, 2004; Hawkins *et al.*, 2006, and Fig. 1; Kandel, 2001), so I will summarize only the major mechanisms of LTF here. LTF depends on both protein and RNA synthesis. LTF is commonly believed to result from the prolonged activation of presynaptic PKA due to the repeated application of 5-HT. The prolonged activation of PKA causes it to translocate from the cytoplasm of the sensory neuron to the nucleus (Bacskai *et al.*, 1993). In the nucleus PKA phosphorylates CREB-1 (Bartsch *et al.*, 1998; Dash *et al.*, 1990; Kaang *et al.*, 1993). CREB-1 is a transcription factor, and its phosphorylation stimulates RNA synthesis. Downstream from CREB-1 are the immediate response genes, including the CAAT box enhancer binding protein (C/EBP) (Alberini *et al.*, 1994). Activation of the immediate response genes, in turn, stimulates the transcription of downstream genes that trigger long-term structural changes in the sensory neurons, including the growth of new presynaptic varicosities and new neurites (Glanzman *et al.*, 1990). These presynaptic structural changes contribute to the formation of new synaptic connections between the sensory and motor neurons (Bailey *et al.*, 1992; Mayford *et al.*, 1992). In addition to the growth of new synapses, LTF is mediated by more subtle morphological changes, including the activation of previously “silent” presynaptic terminals, which lack presynaptic vesicles. During LTF these empty terminals become filled with synaptic vesicles and thereby functional (Kim *et al.*, 2003). (Note that presynaptic activation of silent synapses also contributes to ITF.)

One presynaptic mechanism recently recognized to be crucial to LTF is the release from the sensory neurons of the sensory neuron-specific neuropeptide sensorin (Brunet *et al.*, 1991). In a series of elegant studies Schacher and colleagues have shown that spaced, repeated pulses of 5-HT cause sensorin to be released from presynaptic terminals, and that the release of sensorin is required for LTF (Hu *et al.*, 2007; Hu *et al.*, 2004a; Hu *et al.*, 2004b; Hu *et al.*, 2006). Release of sensorin is triggered by activation of PKA as well as PKC, depending on the stimulation protocol (see Hu *et al.*, 2007). The released sensorin binds to autoreceptors on the sensory neuron, and thereby stimulates the activity of mitogen-activated protein kinase (MAPK), which, in turn, is believed to play a key role in stimulation of CREB-1 activity (Martin *et al.*, 1997b).

3.2. Postsynaptic mechanisms

STF requires only presynaptic changes (Jin *et al.*, 2005; Li *et al.*, 2005), although, as I discuss below, postsynaptic changes may normally contribute to STF.

3.2.1 ITF and modulation of AMPA receptor trafficking—Chitwood *et al.* (2001) provided evidence that postsynaptic mechanisms make a critical contribution to activity-independent ITF. Their experiments used isolated siphon motor neurons in dissociated cell culture. Chitwood stimulated the motor neurons individually with brief pulses (“puffs”) of glutamate, the sensory neuron transmitter (Dale and Kandel, 1993; Levenson *et al.*, 2000) (although see Trudeau and Castellucci, 1993). They electrophysiologically recorded the evoked responses to the glutamate puffs (Glu-EPs) with a sharp microelectrode. A 10-min application of 5-HT to the motor neurons produced enhancement of the Glu-EP that persisted for > 40 min after washout of the monamine. (In recent experiments we have found that the enhancement can persist for ≥ 2 hr after 5-HT washout [G. Villareal and D. L. Glanzman, unpublished data].) The persistent enhancement of the Glu-EP by 5-HT depended on elevated intracellular Ca^{2+} , because it was blocked by prior injection of the rapid Ca^{2+} chelator BAPTA (Adler *et al.*, 1991) into the motor neuron. Tests with glutamate receptor antagonists indicated

that the modulatory effect of 5-HT was specific for AMPA-type receptors. This result suggested that 5-HT modulates the functional expression of AMPA receptors in the motor neuron, possibly by causing exocytotic insertion of new AMPA receptors into the plasma membrane. To test this possibility, Chitwood et al. injected an inhibitor of exocytosis, botulinum toxin, into the motor neuron prior to application of 5-HT. The presence of the toxin blocked the enhancement of the glutamate response.

Li et al. (2005) extended the results of Chitwood et al. (2001) to synaptic facilitation and learning. Using sensorimotor cocultures, Li et al. showed that postsynaptic injection of BAPTA blocked ITF produced by a 10-min application of 5-HT. This result indicates that ITF requires elevated postsynaptic Ca^{2+} . In other experiments Li et al. identified release from intracellular stores as the source of the critical rise in postsynaptic Ca^{2+} . Both inositol 1,4,5-trisphosphate (IP3) receptor-mediated and ryanodine receptor-mediated Ca^{2+} stores appear to mediate 5-HT's facilitatory actions. Additional support for the idea that ITF is mediated by modulation of postsynaptic AMPA receptor trafficking was provided by the finding that postsynaptic injection of botulinum toxin disrupted ITF.

Li et al. (2005) also tested whether facilitation of siphon sensorimotor connections due to sensitizing stimulation—in this case, stimulation of the tail nerves—involves enhancement of the functional expression of AMPA receptors. Tail nerve shock induced persistent facilitation of siphon sensorimotor connections in the abdominal ganglion. Importantly, the nerve shocks produced greater facilitation of the AMPA receptor-mediated component of the excitatory postsynaptic potential (EPSP), then of the NMDA receptor-mediated component. This result provides strong support for the notion that learning-related synaptic facilitation in *Aplysia* involves modulation of AMPA receptor trafficking. Furthermore, the result is inconsistent with the idea that the nerve shock-induced synaptic facilitation is mediated exclusively or predominately by presynaptic facilitation. If this were the case, one would expect the AMPA receptor- and NMDA receptor-mediated components of the EPSP to have been equally facilitated. Li et al. also found that injecting either BAPTA or heparin into the siphon motor neuron prior to tail shock blocked the synaptic facilitation. These results indicate that elevated postsynaptic Ca^{2+} , due to release from intracellular stores, is critical for nerve shock-induced synaptic facilitation, as well as for 5-HT-dependent facilitation. Taken together, the results from the experiments in the abdominal ganglion reinforce those from the *in vitro* experiments. Both sets of experiments point to a central role for modulation of the functional expression of AMPA receptors—possibly through exocytotic insertion of additional receptors—as well as release of Ca^{2+} from postsynaptic intracellular stores, in mediating persistent synaptic enhancement.

To show that these postsynaptic processes play a role in behavior, Li et al. made use of a reduced preparation, consisting of the CNS, together with the tail and siphon, as well as the peripheral nerves that connect the tail and siphon to the CNS. They tested the effect of loading siphon motor neurons with botulinum toxin on dishabituation of the siphon-withdrawal reflex due to tail shock. Li et al. showed that injecting botulinum toxin into just two siphon motor neurons prior to the start of testing blocked dishabituation of the reflex. This result demonstrates that postsynaptic exocytosis is critical not only for synaptic facilitation, but also for learning in *Aplysia*.

The results of Chitwood et al. (2001) and Li et al. (2005) are reminiscent of the results from recent studies of synaptic plasticity and learning in mammals. In particular, studies of long-term potentiation (LTP) in the hippocampus have shown that modulation of AMPA receptor trafficking plays a critical role in LTP (Malinow, 2003). Furthermore, an elegant study by Rumpel et al. (2005) has demonstrated that this mechanism is also important in learning in rats. These investigators used an acute gene delivery technique to transfect neurons in the lateral

amygdala—a structure critical for fear conditioning—with a construct that blocked the synaptic incorporation of AMPA receptors. This molecular manipulation subsequently impaired fear conditioning to a tone and foot shock. Thus, current evidence indicates that, in both the snail and rat, glutamate receptor trafficking is key to learning and memory.

3.2.2 ITF and rapid, local postsynaptic protein synthesis—Although protein synthesis has been shown to be necessary for activity-independent ITF (Ghirardi *et al.*, 1995; Sutton and Carew, 2000), it has not been shown whether the critical site for protein synthesis is pre- or postsynaptic (or both). We have recently discovered that ITF involves local postsynaptic protein synthesis. Using isolated motor neurons in culture, Villareal *et al.* (2004) tested whether the enhancement of the glutamate response produced by a 10-min application of 5-HT required protein synthesis. In their initial experiments the irreversible protein synthesis inhibitor emetine was applied to the bath prior to the start of testing. Unexpectedly, the presence of emetine eliminated all enhancement of the Glu-EP due to 5-HT. This result implied a rapid requirement for protein synthesis in the enhancement. It was possible, however, that the increase in the Glu-EP required merely that certain small proteins be present prior to the onset of 5-HT, and that these proteins have a rapid turnover. If so, prior application of emetine might impair the enhancement of the glutamate response in the absence of a requirement for *de novo* protein synthesis. To test this idea, we carried out experiments in which emetine and 5-HT were applied coincidentally. In this case there was some facilitation of the glutamate response while 5-HT was present in the bath; but when 5-HT was washed out, the facilitation declined to zero within 15 min. This result indicates that the latest time at which *de novo* protein synthesis is necessary for facilitation of the Glu-EP is 15 min after washout of 5-HT. We obtained similar results with another protein synthesis inhibitory, cycloheximide (G. Villareal and D. L. Glanzman, unpublished data).

The rapidity of the requirement for protein synthesis suggested that the critical site for the protein synthesis is the neurites of the motor neuron, rather than the cell body. To test this possibility, we performed experiments on isolated neurites of motor neurons in cell culture. The giant gill motor neuron L7 (Glanzman *et al.*, 1989a; Koester and Kandel, 1977) was used in these experiments. An L7 neuron was dissociated from the CNS, and placed into cell culture. Twenty-four hr later, the major neurite of the motor neuron was severed close to the cell body, and the cell body removed. Experiments were performed on the neurite 24–48 hr later. The neurite was impaled with a sharp microelectrode, and the neurite was stimulated with puffs of glutamate, as in the experiments on the whole motor neuron. A 10-min pulse of 5-HT produced persistent enhancement of the glutamate response, and this enhancement was blocked when emetine was present in the bath (Li *et al.*, 2006). The results for the isolated neurite were like those for the whole motor neuron. Thus, the critical site for the protein synthesis required for the enhancement of the glutamate response is the processes of the motor neuron.

We have extended these results to ITF with experiments on sensorimotor synapses in cell culture using the general protocol of Li *et al.* (2005). Injecting a cell membrane-impermeant protein synthesis inhibitor, gelonin, into the motor neuron prior to the start of testing disrupted ITF (Q. Li and D. L. Glanzman, unpublished data). Interestingly, the postsynaptic gelonin had no effect on facilitation while 5-HT was present. This result might appear to support the idea that STF does not depend on postsynaptic processes. However, because STF is commonly regarded as lasting < 30 min (Sutton and Carew, 2002), our results for the isolated motor neuron/neurite raise the possibility that rapid, local postsynaptic protein synthesis normally contributes to STF, as well as to ITF.

We do not yet know the identity of the postsynaptic proteins whose local synthesis is stimulated by 5-HT. Recent work indicates that application of dopamine triggers dendritic synthesis of AMPA receptors in hippocampal neurons (Smith *et al.*, 2005). The possibility that a 10-min

pulse of 5-HT causes AMPA receptors to be locally synthesized in motor neurons is attractive, particularly in light of our earlier results (Chitwood *et al.*, 2001; Li *et al.*, 2005). We also do not know the signaling pathway that mediates the local protein synthesis in motor neurons. One likely candidate, however, is PKC (Villareal *et al.*, 2003). An intriguing possibility is that the local protein synthesis supports the ongoing activity at the synapse of a PKM ζ -like kinase (Hernandez *et al.*, 2003). But at present we have no evidence for this idea.

3.2.3 The role of postsynaptic Ca²⁺ and postsynaptic protein synthesis in LTF

—Despite the exclusively presynaptic nature of the model of LTF presented in Figure 1, it has long been apparent that LTF must depend, at least to some extent, on postsynaptic mechanisms. Thus, Glanzman *et al.* (1990) reported that the presynaptic structural changes that accompany LTF do not occur in the absence of the motor neuron. These investigators treated isolated sensory neurons in culture to the standard five spaced applications of 5-HT. They found that the 5-HT treatment did not produce any structural changes in the sensory neurons when they were not in contact with a motor neuron. More recently, Hu *et al.* (2007) have found that so-called associative LTF, which is induced by pairing a bout of tetanic stimulation of the sensorimotor synapse with a single pulse of 5-HT, depends, like nonassociative LTF, on a rapid increase in sensorin expression and secretion. The increase in sensorin expression during associative LTF involves local synthesis of the neuropeptide, and this synthesis requires the presence of the postsynaptic motor neuron.

In addition, several studies have found that LTF is accompanied by an increase in the number of functional AMPA receptors at the sensorimotor synapse (Trudeau and Castellucci, 1995; Zhao *et al.*, 2003; Zhu *et al.*, 1997). This suggests that modulation of postsynaptic AMPA receptor trafficking may contribute to LTF (see also Li *et al.*, 2004). But it remains to be proved that the enhancement of the AMPA receptor-mediated synaptic component shown to accompany LTF is actually *necessary* for LTF. Trudeau and Castellucci (1995) found that LTF of sensorimotor connections in the abdominal ganglion was accompanied by a long-term enhancement of the current evoked by a glutamate receptor agonist, homocysteic acid (HCA), in L7 motor neuron. However, whereas prior injection of the protein synthesis inhibitor gelonin into the postsynaptic L7 neuron blocked the enhancement of the HCA-evoked current in L7, it did not block LTF of the sensorimotor synapse. This result appears to argue against a functional role for modulation of glutamate receptor trafficking in LTF. Nonetheless, a serious methodological problem with the Trudeau and Castellucci (1995) study is that the investigators measured the response to HCA by voltage-clamp recordings in the soma of the L7 neuron. As our experiments using the isolated neurite of L7 (above) show, 5-HT can produce local enhancement of the motor neurite's sensitivity to glutamate. It is unclear whether the electrophysiological assessments of glutamate receptor function performed by Trudeau and Castellucci in the cell body of L7 were sufficiently sensitive to measure changes in the number of AMPA receptors *in the postsynaptic membrane*. Notice that the majority, if not all, of sites of sensorimotor contact occur on the neurites of L7, rather than on its soma (Bailey and Chen, 1988a; Winlow and Kandel, 1976). For this reason the assertion by Trudeau and Castellucci that their voltage-clamp measurements of the response of the somal membrane of L7 to applied HCA reflected the number of functional glutamate receptors at sensorimotor synapses is problematic, particularly given the enormous size of the L7 cell body (see below).

We have recently reexamined the issue of whether sensory neuron autonomous processes are sufficient to support LTF. All of our experiments were performed using sensorimotor cocultures comprising pleural sensory neurons and small siphon (LFS) motor neurons (Lin and Glanzman, 1994). LTF was induced using the original method of Montarolo *et al.* (1986), with five spaced pulses of 5-HT. We first asked whether LTF depends on postsynaptic Ca²⁺. Accordingly, in some experiments the Ca²⁺ chelator BAPTA was injected into the motor neuron before testing the synapse on the first day and prior to 5-HT treatment. Cocultures that

received the 5-HT treatment, but not the postsynaptic injection of BAPTA, showed significant LTF compared to control cocultures that received neither 5-HT nor the postsynaptic injection of BAPTA. By contrast, synapses that received the 5-HT treatment plus postsynaptic BAPTA did not exhibit LTF (Cai and Glanzman, 2006). Thus, LTF, like ITF, requires elevation of postsynaptic intracellular Ca^{2+} .

Next we asked whether LTF, like ITF, involves postsynaptic protein synthesis. To test this possibility motor neurons in some cocultures received a prior injection of one of two cell membrane-impermeant inhibitors of protein synthesis, gelonin or the cap analog m⁷ GpppG (Huber *et al.*, 2000). Both inhibitors of protein synthesis blocked LTF when injected postsynaptically (D. Cai and D. L. Glanzman, unpublished data). This result is somewhat surprising. There have been two prior reports that postsynaptic blockade of protein synthesis did not affect LTF. One (Trudeau and Castellucci, 1995) was performed in the CNS, whereas the other was performed using sensorimotor cocultures (Martin *et al.*, 1997a). In the former study 5-HT was applied continuously to the abdominal ganglion for 60 min (note that this procedure produces significant LTF of sensorimotor synapses in the ganglion); in the latter study, 5-HT was iontophoresed onto the synapse in culture, using five spaced applications. A third study that tested the involvement of postsynaptic protein synthesis in LTF got mixed results. This study, by Sherff and Carew (2004), was performed using the pleural-pedal ganglia. Here, the pleural sensory neurons are physically separated from their postsynaptic targets, which lie in the pedal ganglion. Two separate methods were used to induce LTF. One involved the traditional five spaced applications of 5-HT, which were applied to the pleural-pedal ganglia. The second method of 5-HT treatment was the so-called “asymmetric” method. In this method 5-HT is applied to the pleural ganglion, where the somas of the sensory neurons are located, for 25 min, and to the pedal ganglion, where the sensorimotor synapses (as well as the somas of the motor neurons and other neurons) are located for the 5 min period corresponding to the end of the 5-HT pulse in the pleural ganglion. This treatment also produces robust LTF. Sherff and Carew found that prior postsynaptic injection of gelonin blocked LTF to the spaced training protocol, but not to the asymmetric protocol.

What can one make of these apparently conflicting results? One possible answer arises from consideration of the postsynaptic target that was used in the Trudeau and Castellucci (1995) and Martin *et al.* (1997a), the giant motor neuron L7. If, as the results from our studies of ITF (above) indicate, it is local postsynaptic protein synthesis that is critical, then the intrasomal injections used in these two studies may not have delivered sufficient quantity of gelonin to the critical postsynaptic sites due to the huge volume of the L7 cell. By contrast, because we used the small siphon motor neurons in our experiments, it might have been easier for us to affect local postsynaptic protein synthesis via intrasomal injections of protein synthesis inhibitors. Of course, this explanation does not account for the different results obtained by Sherff and Carew from their postsynaptic gelonin injections. Presumably, the same target neurons were used for the spaced and asymmetric 5-HT treatments. At present, therefore, the question of whether postsynaptic protein synthesis is necessary for LTF must be regarded as unresolved.

3.2.4 The role of retrograde signaling in ITF and LTF—We and others (Hu *et al.*, 2007; Jin *et al.*, 2004; Sherff and Carew, 2004) have shown that both ITF and LTF depend on postsynaptic mechanisms, including elevated postsynaptic Ca^{2+} and postsynaptic protein synthesis (see also Jin and Hawkins, 2003). Furthermore, it is clear that LTF, at least, and possibly ITF as well (Jin *et al.*, 2006), is expressed, in part, through persistent presynaptic changes. How are the pre- and postsynaptic changes coordinated? This coordination would appear to require some form of transsynaptic communication. I propose that the persistent presynaptic changes produced by 5-HT do not result from *direct* actions of this monoamine on the sensory neuron; instead, I suggest that 5-HT’s presynaptic effects are *indirect*, and are

mediated by a retrograde signal. It is logical to suppose, based on our results, that the retrograde signal is triggered by elevated intracellular Ca^{2+} within the motor neuron (Fig. 2). What might this retrograde signal be? At present, we do not know. However, recent evidence from Eric Kandel's laboratory indicates that a transsynaptic interaction between neuroligin and neuroligin may subserve retrograde signaling during LTF (Choi-Y.-B. *et al.*, 2007). It seems likely, however, that there will be other retrograde signals involved in both ITF and LTF. This is an important and challenging area for the field of learning-related synaptic plasticity in *Aplysia*.

6. Conclusions

As the present review indicates, knowledge of postsynaptic mechanisms is crucial for understanding learning-related synaptic plasticity in *Aplysia*. It is, moreover, intriguing that the postsynaptic mechanisms—such as modulation of AMPA receptor trafficking and rapid, local postsynaptic protein synthesis—that are beginning to be recognized as essential to ITF and LTF have previously been implicated in learning-related synaptic plasticity in vertebrates (see, e.g., Huber *et al.*, 2000; Malinow, 2003; Tsokas *et al.*, 2005). This is unlikely to be the result of coincidence. Instead, it is more plausible that the basic cellular and molecular mechanisms of memory arose early in evolution and were maintained.

Although some may find this conclusion surprising, it would not have surprised Charles Darwin. In his final book, *The Formation of Vegetable Mould Through the Action of Worms, with Observations on Their Habits*, published in 1881, Darwin observed that earthworms show "some degree of intelligence." We can be confident that the great biologist was not using the word "intelligence" metaphorically. If as Darwin was convinced, worms and other higher invertebrates do indeed exhibit intelligence—or, as we would say today, cognition—then the complex neuronal machinery of cognition must reside within these relatively humble creatures as well (see Giurfa, 2007; Kristan and Gillette, 2007; Rankin and Dubnau, 2007).

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Fig. 1. Presynaptic cellular model for short- and long-term sensitization in *Aplysia*. See the text for details. From (Kandel, 2001).

Fig. 2.

Revised cellular models for different phases of sensitization-related synaptic facilitation in *Aplysia*. The *dashed lines* depict pathways for which experimental evidence is currently lacking. (A) Short-term facilitation (STF). This phase lasts < 30 min. STF can be both induced and expressed presynaptically. (B) Intermediate-term facilitation (ITF). This phase lasts 90 min-to-3 hr. According to the model, ITF is induced postsynaptically, by release of Ca^{2+} from intracellular stores in the motor neuron, and expressed both pre- and postsynaptically. The presynaptic expression results from one or more retrograde signals, activated by postsynaptic Ca^{2+} , which stimulate both PKA and PKC within the sensory neuron. (C) Long-term facilitation (LTF). LTF persists for ≥ 24 hr, and involves gene transcription, as well as both

pre- and postsynaptic protein synthesis. Like ITF, LTF is induced postsynaptically, and expressed pre- and postsynaptically. LTF is triggered by repeated, spaced applications of 5-HT/sensitizing stimuli. A novel feature of the model in *C* is that it assumes that prolonged activation of presynaptic PKA, which leads to sensorin release and the translocation of PKA to the presynaptic nucleus, is triggered by elevated postsynaptic Ca^{2+} , via retrograde signaling. In addition, for at least some forms of both LTF and ITF the retrograde signal may activate presynaptic PKC (Hu *et al.*, 2007; Jin *et al.*, 2004) as well as presynaptic PKA. From (Glanzman, 2007).