Behavioral, Cellular, and Molecular Analysis of Memory in Aplysia II: Long-Term Facilitation

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SYNOPSIS. Long-term facilitation (LTF) of Aplysia tail sensory neuron–motor neuron (SN–MN) synapses provides a synaptic correlate of memory for long-term behavioral sensitization of the tail-siphon withdrawal reflex. LTF can be induced by repeated exposures of serotonin (5HT) in the isolated pleural-pedal ganglion preparation. In addition, we have shown previously (Sherff and Carew, 1999) that LTF can also be induced by coincident 5HT exposure comprised of a single 25-min exposure of 5HT at the SN cell body and a 5 min pulse of 5HT at the SN-MN synapses. If synaptic 5HT is applied either 15 min before or after somatic 5HT, LTF is significantly reduced or is not induced at all. These results show that two anatomically remote cellular compartments can functionally interact within a surprisingly short time period. In this chapter, we discuss some of the mechanistic implications of this temporal constraint. We also find that coincident LTF and LTF induced by repeated pulses of 5HT differ (1) in whether they induce another temporal phase of facilitation (intermediate-term facilitation, ITF, expressed up to 1.5 hr after 5HT), and (2) in their requirements for protein synthesis. The results described both in this paper and in the preceding companion paper show that there are multiple forms of both ITF and LTF that differ in their induction and expression requirements, and at least in some instances, the different temporal phases of facilitation, and perhaps comparable phases of memory, can be induced independently of each other.

Virtually all animals are endowed with the capacity to encode particular events, store that information, sometimes for extended periods, and then access that information at a later time. In the field of learning and memory, these three feats are described as the induction, storage and retrieval of memory. As described in the previous paper (Sutton and Carew, 2002), memories can exist in a wide range of temporal domains. One major question in the analysis of memory concerns the relationships between these different domains. One possibility is that memories are processed in series: short-term memories are transformed into intermediate-term, which in turn are transformed into long-term memories (Squire, 1987). Another possibility is that memories are processed in parallel, with the original experience initiating the different memory stages independently of one another (Tully et al., 1994; DeZazzo and Tully, 1995; Izquierdo et al., 1998). Of course, combinations of these two alternatives are also possible, with conjoint serial and parallel processing of memories. Experimentally, it can be difficult to distinguish between these possibilities. While important progress has been made on behavioral and pharmacological levels (Izquierdo et al., 1998), a mechanistic analysis of these issues has remained elusive. The marine mollusc Aplysia provides an advantageous system to explore these questions at behavioral, cellular and molecular levels.

As was described in the previous chapter for memory and facilitation in the intermediate time domain in Aplysia, there are also behavioral and synaptic correlates in the long-term time domain. Long-term memory (LTM) and long-term facilitation (LTF), in addition to having similar temporal characteristics (lasting >24 hr), have similar induction requirements. Both need repeated tail shock (that give rise to 5HT release) and activation of protein kinase A (PKA).

Long-term facilitation (LTF, lasting >24 hr) of Aplysia tail sensory neuron-motor neuron (SN-MN) synapses has long been known to be induced by repeated pulses of serotonin (5HT; Montarolo et al., 1986; Mauelshagen et al., 1996; Martin et al., 1997). Repeated 5HT also induces earlier phases of synaptic plasticity, namely, short-term facilitation (STF, lasts about 15 min), which is induced after only a single pulse of 5HT (Emptage and Carew, 1993), and intermediate-term facilitation (ITF, lasts up to 90 min) that requires 5 pulses of 5HT (Mauelshagen et al., 1996). Induction and/or expression of three temporal phases of facilitation all require activation of PKA (Castellucci et al., 1980; Schacher et al., 1988; Manseau et al., 1998; Sutton and Carew, 2000; review by Byrne and Kandel, 1996), which would be consistent with (though not proof of) the possibility that these three phases, which require the same signaling pathway, might be induced in series. In this chapter, we describe our recent findings that there are multiple forms of LTF that differ in their induction requirements and in the combinations of phases of facilitation that they produce. We will discuss two important features of Aplysia synaptic plasticity that are suggested by these results: (1) there is more than one molecular route to synaptic plasticity in the long-term time domain, and (2) that, under some circumstances, ITF and LTF can be induced independently of each other and are therefore likely induced in parallel.

The location of the SNs, MNs, and their connections in the Aplysia CNS are particularly advantageous for experimental studies of their specific roles in the induction of synaptic plasticity. The tail SN cell bodies
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are located in the pleural ganglion (Fig. 1A). Their axons travel the length of the pleural-pedal connective (about 3 mm) to the pedal ganglion, where they synapse with tail MNs. We now know that shock of the tail nerve (P9) causes release of 5HT in the vicinities of the tail SN cell bodies and the SN-MN synaptic neuropil (Marinesco and Carew, 2002); thus we can substitute 5HT application for tail shock in the isolated ganglia preparation. The relatively long distance between the SN somata and their MN synapses allows us to construct a barrier between these two cellular compartments (Fig. 1A) and apply 5HT to either the SN somata (somatic compartment) or to the region of the SN-MN synapses (synaptic compartment). Using this preparation, we have found that LTF can be induced by different patterns of 5HT, such as 5HT exposure only to the SN soma or only to the synaptic region (Emptage and Carew, 1993; Sherff and Carew, 2001; and see also Clark and Kandel, 1993). These results invite two questions: First, although 5HT exposure to only one of the synaptic compartments is sufficient to induce LTF, 5HT is released in both compartments during tail shock. Is there an interaction between the 5HT-activated processes induced in the two compartments? Second, do the different patterns of 5HT exposure induce LTF using the same signaling mechanisms, or is there more than one molecular pathway to LTF induction? The compartmental analysis we have developed (Sherff and Carew, 1999) provides a means of directly addressing both of these questions.

INTERACTIONS BETWEEN SN CELL BODIES AND THEIR DISTAL MN SYNAPSES

We have recently examined the temporal and spatial constraints in the induction of long-term facilitation (LTF) in the intact CNS of Aplysia, focusing on interactions between two structurally remote cellular compartments of the CNS: (1) the cell bodies of tail SNs and their proximal synapses onto interneurons, located in the pleural ganglion (somatic compartment), and (2) their distal synapses onto tail MNs (2–3 mm away) in the pedal ganglion (Synaptic compartment) (Fig. 1A). For each compartment we found 5HT exposures that, when applied alone, were insufficient for the induction of LTF, but when they were applied together induced LTF (Sherff and Carew, 1999; and Figs. 1B, 2). These were a 25 min 5HT exposure to the somatic compartment and a 5 minute 5HT exposure to the synapse (which alone induces only short-term facilitation (STF) that lasts <15 min). Alone, neither somatic nor synaptic 5HT induced LTF, but when the synaptic 5HT exposure was given coincident with the final five minutes of somatic 5HT, significant LTF was induced.

An interesting property of this form of LTF (which we call “coincident LTF”) is that there is a limited time within which the two 5HT exposures must occur in order to induce LTF. If synaptic 5HT is presented as little as 15 min before somatic 5HT, LTF is significantly reduced, and if synaptic 5HT is presented 15 min after somatic 5HT, LTF is not induced at all (Fig. 2A; Sherff and Carew, 1999). This narrow time window puts an important constraint on the possible cellular mechanisms that might account for the coincident interaction between the two compartments. One mechanism by which the two compartments could interact could be conceived as “immediate coincidence,” in which a signal at the synapse must communicate with somatic events within 15 min (Fig. 2B). In Aplysia, the calculated rate of retrograde transport is about 1.5
Induction of coincident LTF has a narrow time window. A. LTF was significantly reduced if synaptic 5HT occurred 15 min before somatic 5HT (top traces), and LTF was not induced at all if synaptic 5HT occurred 15 min after (bottom traces) somatic 5HT. Bar graph at right shows summary data for experiments shown here and in Figure 1. B. Two mechanisms by which a synaptic signal might interact with somatic events include “immediate coincidence,” in which a synaptic signal and somatic events interact during or shortly after 5HT exposure, and “delayed coincidence,” in which synaptic events occurring during 5HT exposure cause retrograde transport of a signal to the soma, where it interacts with ongoing somatic processes at some time after 5HT.

This mechanism is too slow to subserve immediate coincidence because, at this rate, it would take over an hour for a synaptic signal to reach the SN soma. Instead, a more rapid mode of transport, such as regenerative Ca\(^{2+}\) waves (Jaffe and Brown, 1994) or an enzymatic chain reaction (Stoop and Poo, 1994; Senger and Campenot, 1997; Bhattacharya et al., 1997), would be more likely. Another mechanism could be thought of as “delayed coincidence” (Fig. 2B), in which a synaptic signal could be transported via retrograde axonal transport to the soma, where it interacts some time later with ongoing events triggered by somatic 5HT. Consistent with this view, as will be described below, translational events in the SN soma are important for LTF induction only 1–3 hr after 5HT exposure. The temporal constraint in this second model is the result of a requirement that the synaptic signal and somatic events must coincide within a brief time window at some time after 5HT. Clearly more studies are required to determine which, if either, of these models is correct, but at the very least they provide a theoretical framework within which to explore this interesting aspect of coincident LTF.

**Repeated 5HT and Coincident 5HT Differ in Their Ability to Induce ITF**

Preliminary results indicate that repeated 5HT and coincident 5HT, in addition to differentially inducing LTF, also differ in their ability to induce ITF (expressed 30–60 min after 5HT; Ghirardi et al., 1995; Mauelshagen et al., 1996; Sutton and Carew, 2000). We found that repeated, temporally spaced 5HT pulses applied to both soma and synapse induce both ITF and LTF. In contrast, coincident 5HT induces LTF but not ITF. Both procedures induce comparable STF. These data show that the different temporal phases of facilitation can, under some circumstances, be induced independently, and raise the hypothesis that these two patterns of 5HT exposure recruit different cellular signaling pathways, or at least differ in the combination of signaling pathways they require for induction of LTF.

**Protein and RNA Synthesis Requirements Differ for LTF Induction by Coincident 5HT and Repeated 5HT**

Previous studies of *Aplysia* SN-MN synapses in cell culture show that induction of LTF by repeated somatic and synaptic 5HT requires translation of new protein and transcription of new mRNA (Montarolo et al., 1986; Ghirardi et al., 1995). Furthermore, the protein synthesis requirement for LTF induction by repeated 5HT at the synapse alone is solely presynaptic (Martin et al., 1997; see also Trudeau and Castellucci, 1995). Thus far we have established that LTF induced by repeated 5HT and coincident 5HT differ in both the combination of phases of facilitation that they induce (ITF and LTF) and in their sites of induction for LTF (LTF induced by repeated 5HT requires 5HT at either
the soma alone, the synapse alone, or both, while coincident LTF requires both somatic and synaptic 5HT exposure). Given these differences, we next examined the protein and RNA synthesis requirements for coincident LTF to see whether they, too, might differ from those necessary for LTF induced by repeated 5HT. We found that protein synthesis is required in both compartments for the induction of coincident LTF; however, translation of new protein was required at different times at the two sites (Sherff and Carew, 1999). Specifically, the protein synthesis blocker emetine prevented the induction of coincident LTF (1) when perfused into the synaptic region during 5HT exposure (emetine was perfused into the bath beginning 30 min prior to 5HT exposure and was washed out of the bath 30 min after 5HT exposure), and (2) when perfused to the somatic region between 1 and 3 hr after 5HT (but not during or 3–6 hr after 5HT) (Fig. 3A). These results show that there is a requirement for immediate protein synthesis in the synaptic compartment and a requirement for delayed protein synthesis at the SN soma. Because the synaptic region includes both the presynaptic terminals and the postsynaptic cells, the local protein synthesis requirement at the synapse could reside in the SN, the MN or both. While the molecular components of LTF that have been described so far have been predominantly presynaptic in origin, there is growing evidence that 5HT has effects at the MN as well. For example, the MN response to glutamate increases after 5HT exposure (Trudeau and Castellucci, 1995), which may be the result of an increase in AMPA receptor insertion (Chitwood et al., 2001). To determine whether there is a postsynaptic component to the translational requirement, we injected the postsynaptic MN with the translational blocker gelonin prior to coincident 5HT exposure and found that the induction of coincident LTF was blocked under these conditions (Fig. 3B1).

Our results examining coincident LTF differ from those obtained in experiments using repeated pulses of 5HT at *Aplysia* SN-MN synapses in culture in that we found a postsynaptic protein synthesis component that contributes to coincident LTF induction, whereas in culture, gelonin only blocked LTF induction when injected into the SN, not the MN (Martin et al., 1997). To examine the similarities and differences between LTF induced by repeated 5HT and by coincident 5HT, we repeated our gelonin experiments using repeated 5HT pulses to the soma and the synapse, and found that gelonin in the MN did not block LTF induction (Fig. 3B2), confirming the previous observations for cultured synapses (Martin et al., 1997). These data...
show that the requirement for postsynaptic protein synthesis is dependent on the pattern and site of 5HT exposure. We hypothesize that coincident 5HT may be a “minimal” induction protocol for LTF; an idea that is based on the finding that, with coincident 5HT, neither somatic nor synaptic 5HT exposures alone can induce LTF, while with repeated 5HT, exposure to either region alone is capable of inducing LTF. One explanation for the differences we observe between repeated 5HT and coincident 5HT is that there might always be a modest contribution of postsynaptic protein synthesis to LTF, and in the minimal form induced by coincident 5HT, this postsynaptic component plays a significant role in LTF induction. In contrast, with LTF induced by repeated 5HT, the postsynaptic component of protein synthesis is overshadowed by a strong presynaptic component, which is engaged by the repeated presynaptic exposures to 5HT.

We have also investigated the transcriptional requirement for LTF induction by coincident 5HT, using two different blockers of mRNA synthesis, actinomycin-D (preincubation for 1 hr prior to 5HT) and DRB (5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole; preincubation 1 hr prior to and during 5HT). We found that transcription is necessary in both somatic (presynaptic) and the synaptic regions (Fig. 3C; Sherff and Carew, 1999) (recall that the synaptic compartment includes the MN cell body), indicating that new RNA synthesis is required in both SNs and MNs.

**Summary and Conclusions**

In the current paper, and in the preceding companion paper, we have described two different temporal phases of synaptic facilitation: ITF and LTF. Both of these phases have behavioral correlates, namely, ITM and LTM for sensitization of the tail-siphon withdrawal reflex, which are similar in both time course and mechanism. Because an animal in its natural surroundings would likely benefit from modulation of its behavior (such as the T-SW reflex) in response to a wide range of patterns and modalities of stimulation, it is not surprising that we are able to induce synaptic changes associated with memory processes through a variety of different experimental procedures. The interesting feature of this system is that the different procedures are able to induce memories or phases of facilitation in the same temporal domains, although they require the activation of different molecular pathways. For example, Sutton and Carew (2000) described activity-dependent and activity-independent forms of ITF, and in the present paper, we described repeated and coincident forms of LTF. These forms of facilitation differ in their induction and expression requirements. Activity-dependent ITF requires persistent activation of PKC (but not PKA) for expression, and does not require protein synthesis for induction, while activity-independent ITF requires persistent activation of PKA (not PKC) as well as protein synthesis. Considering long-term processes, the induction of LTF by repeated 5HT requires only presynaptic protein synthesis, while coincident LTF requires both presynaptic and postsynaptic protein synthesis. Yet another form of LTF that is activity-dependent has been described by Bailey et al. (2000). It is induced at synapses between cultured Aplysia SNs and MNs by pairing a single train of action potentials in the SN with 5HT puffed onto the synapse, a procedure first used by Eliot et al. to induce LTF (1994). Bailey and colleagues find that this form of LTF is unique in that it does not require protein synthesis for its induction. In addition to providing another example of a different form of LTF, these results also demonstrate that LTF, like ITF (Sutton and Carew, 2000), can be induced by activity-dependent processes.

At the outset, we discussed the fact that memories might be formed by serial processes (Fig. 4A), parallel processes (Fig. 4B), or a combination of both. The ability of coincident somatic and synaptic 5HT exposure to induce LTF without first inducing ITF indicates that facilitation in these two time domains can be induced independently, that is, in parallel. Taken together, our results demonstrating: (1) that there are several molecular pathways that can be activated to give rise to a specific temporal phase of facilitation or memory, and (2) that the different temporal phases can be induced in parallel, lead us to suggest the model for memory formation in the T-SW reflex in Aplysia shown in Figure 4B. This model posits that different temporal domains of memory can be differentially and independently engaged by different experiences in the animal’s environment, which could dramatically increase both the flexibility and complexity of memory processing. If this view proves correct, it will be of considerable interest to determine the adaptive significance of such a strategy for memory formation. We should point out that the fact that both ITF (Sutton and Carew, 2000) and LTF (Bailey et al., 2000) can be induced in an activity-dependent manner suggests the possibility that activity-dependent ITF and LTF could be seral processes; in which case, the different temporal memory phases in the Aplysia tail-siphon withdrawal reflex could be induced by either serial or parallel processes.

One of the more powerful features of the Aplysia SN-MN synapse as a model system for the study of the cellular and molecular processes underlying memory formation and maintenance is that this system can provide important mechanistic clues at the molecular, cellular and behavioral levels. In the intermediate time domain, Sutton and Carew (2000) have been able to correlate two forms of facilitation, activity-dependent and activity-independent ITF with two forms of memory, site-specific ITM and repeated trial ITM. Furthermore, they have begun to dissect out the different molecular pathways that are required for each form. This raises the challenge for future studies of identifying behavioral correlates for the different forms of LTF that have been mechanistically dissociated. For example, it will be interesting to know whether there is a behavioral correlate for memory induced using behavioral training patterns that incorporate the principle
features of coincident 5HT. We now know that 5HT is released both in the region of the SN soma as well as at the synapse in response to shock of the tail nerve P9 (Marinesco and Carew, 2002), but we do not yet know whether it can be released differentially in response to behaviorally relevant stimuli. In addition, it will be interesting to identify the circumstances under which activity can substitute for (or interact with) 5HT exposure. An activity-dependent form of LTM has been demonstrated in Aplysia by Walters (1987). In principle, an activity-dependent coincident LTF in which SN soma and synapse received temporally coordinated 5HT could contribute to this form of LTM. Thus it will be an intriguing challenge to explore the degree to which parallel and/or serial processing at the cellular and molecular levels contribute to different forms of long-term memory in Aplysia.

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REFERENCES


