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Behavioral, Cellular, and Molecular Analysis of Memory in *Aplysia* I: Intermediate-Term Memory[1]

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SYNOPSIS. Serotonin (5HT) induces short-term and long-term synaptic facilitation (STF and LTF, respectively) at sensory neuron to motor neuron (SN-MN) synapses in *Aplysia*, and these forms of plasticity are thought to contribute to short-term and long-term memory for behavioral sensitization. Recent evidence in *Aplysia* has identified a third phase of synaptic facilitation—intermediate-term facilitation (ITF)—that is temporally and mechanistically distinct from STF and LTF. Here, we review the findings of recent studies that have examined this unique intermediate-term phase at molecular, cellular, and behavioral levels. The results indicate that, at tail SN-MN synapses, multiple forms of ITF can be distinguished; they are induced via distinct mechanisms and use parallel molecular pathways for their expression. Moreover, we have incorporated the temporal and molecular features of these different forms of ITF at tail SN-MN synapses into behavioral analyses, and found that they accurately predict distinct forms of intermediate-term memory for sensitization of the tail-elicited siphon withdrawal reflex. These findings indicate that different types of experiences engage distinct molecular pathways in the service of memory retention over the same time domain.

Memories can vary remarkably in their persistence, enduring from seconds to minutes, from days to weeks, and in the limit, a lifetime, depending upon the amount and pattern of training (McGaugh, 1966; DeZazzo and Tully, 1995; Hammer and Menzel, 1995; Sutton *et al.*, 2002). Likewise, in several model systems, synaptic plasticity thought to underlie memory has been shown to exist in a variety of temporal domains (Nguyen *et al.*, 1994; Ghirardi *et al.*, 1995; Mauelshagen *et al.*, 1996; Winder *et al.*, 1998; Crow *et al.*, 1999). In one such system, the marine mollusc *Aplysia*, the cellular and molecular mechanisms contributing to different temporal phases of memory have been extensively studied. The best characterized form of learning in *Aplysia* is sensitization, where behavioral responses (elicited by a weak stimulus) become greater in magnitude and duration following the presentation of a strong noxious stimulus (usually a shock applied to the animal's tail). Typically, sensitization in *Aplysia* is studied by assessing the modulation of defensive withdrawal reflexes, where a great deal is known regarding the neural circuitry contributing to the response. For example, the tail-elicited tail withdrawal reflex has an important monosynaptic component in the central nervous system, where tail sensory neurons (SNs) in the pleural ganglion make direct connections with tail motor neurons (MNs) in the pedal ganglion, and these tail MNs in turn innervate the tail (Walters *et al.*, 1983). Another reflex, tail-elicited siphon withdrawal (T-SW), is also initiated by tail SN activation; in this case, the tail SNs connect to siphon MNs in the abdominal ganglion via a polysynaptic pathway involving one or more interneurons (*e.g.*, Cleary and Byrne, 1993).

Memory for sensitization, like other forms of memory, can vary with the amount and/or pattern of training. A single shock enhances defensive withdrawal for a period of minutes, whereas repeated shocks delivered with an intervening rest period produce a form of sensitization that lasts days to weeks (Carew *et al.*, 1971; Pinsker *et al.*, 1973; Frost *et al.*, 1985; Cleary *et al.*, 1998; Sutton *et al.*, 2002). Similarly, the connections between tail SNs and MNs in the central nervous system of *Aplysia* become enhanced for a period of minutes (short-term facilitation; STF) following a single tail shock and for >24 hr (long-term facilitation; LTF) following repeated spaced shocks (Walters *et al.*, 1983; Buonomono and Byrne, 1990; Mercer *et al.*, 1991; Cleary *et al.*, 1998).

Considerable evidence suggests that both behavioral sensitization and SN-MN synaptic facilitation induced by tail shock are mediated by the actions of the biogenic amine serotonin (5HT). 5HT is released in the central nervous system (CNS) of *Aplysia* following tail shock (Marinesco and Carew, 2002), and depletion of 5HT prevents behavioral sensitization (Glanzman *et al.*, 1989). Moreover, heterosynaptic facilitation of SN-MN synapses is produced by intracellular activation of serotonergic interneurons (Mackey *et al.*, 1989), and single and repeated pulses of 5HT mimic the effects of tail shock for STF and LTF, respectively (Walters *et al.*, 1983; Mercer *et al.*, 1991; Emptage and Carew, 1993; Mauelshagen *et al.*, 1996; Zhang *et al.*, 1997). Finally, short-term memory (STM) and long-term memory (LTM) for sensitization can be distinguished in that the former does not require protein synthesis, whereas the latter requires both transcription and translation (Castellucci *et al.*, 1989; Levenson *et al.*, 2000; Sutton *et al.*, 2001*a*), similar to observations in a num-

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ber of other species (Davis and Squire, 1984). 5HT-induced synaptic facilitation at SN-MN synapses shows a similar dissociation in that LTF requires transcription and translation, while STF requires neither (Montarolo *et al.*, 1986; Ghirardi *et al.*, 1995; Martin *et al.*, 1997; Sherff and Carew, 1999; Sutton and Carew, 2000). These results have served to establish 5HT-induced facilitation of SN-MN synapses as a useful cellular model of both short-term and long-term memory for behavioral sensitization.

A Third Phase of Synaptic Enhancement: Intermediate-Term Facilitation

While distinguishing between short-term and long-term phases of memory and synaptic plasticity has been instructive, recent evidence in *Aplysia* has demonstrated that there is more to the story. This changing view began with the demonstration by Ghirardi and colleagues (1995) that an additional phase of synaptic facilitation (intermediate-term facilitation; ITF) could be induced in cultured SN-MN synapses by repeated 5HT pulses. Unlike STF induced by a single pulse of 5HT, which is transient (lasting <30 min), they found that ITF could persist for hours following 5HT washout. Moreover, the induction of ITF required translation but not transcription, which also distinguished this phase from STF and LTF at the mechanistic level, since STF requires neither protein nor RNA synthesis, and LTF requires both (Montarolo *et al.*, 1986; Ghirardi *et al.*, 1995). Together, these results provided definitive evidence for a novel intermediate phase of synaptic facilitation expressed by SN-MN synapses in cell culture.

Extending the observations of Ghirardi and colleagues (1995) to tail SN-MN synapses in the intact CNS, Mauelshagen *et al.* (1996) found that 5 spaced pulses of 5HT produces ITF (lasting >90 min) that can be temporally dissociated from both STF and LTF. ITF was easily distinguished from STF by both its induction requirements and its time course: 1 to 4 spaced pulses of 5HT produced only STF (lasting <30 min), whereas 5 pulses of 5HT was required for ITF. Later studies also demonstrated that ITF at tail SN-MN synapses induced by repeated 5HT pulses requires protein synthesis (Sutton and Carew, 2000) as is the case in cultured SN-MN synapses (Ghirardi *et al.*, 1995), which also distinguishes ITF from STF in the intact CNS at the mechanistic level. In addition, Mauelshagen *et al.* (1996) demonstrated that the time course of ITF did not overlap with LTF: ITF decayed completely to baseline within 3 hr, several hours prior to the onset of LTF (10–15 hr). These results demonstrated an intriguing feature regarding the organization of different phases of synaptic facilitation at tail SN-MN synapses: the intermediate-term and long-term phases of facilitation are temporally discontinuous.

Signaling Pathways Engaged During ITF

The identification of ITF (Ghirardi *et al.*, 1995) and its characterization at tail SN-MN synapses (Mauelshagen *et al.*, 1996) set the stage to examine the types of signaling mechanisms that might be involved. As a starting point, it was known that two signaling molecules—cAMP-dependent protein kinase (PKA) and protein kinase C (PKC)—play an important role in both short- and long-term forms of plasticity in SNs and their connections with MNs (Schacher *et al.*, 1988; Byrne and Kandel, 1996; Manseau *et al.*, 1998). In particular, both PKA and PKC contribute to STF induced by a single pulse of 5HT, and the activation of either is sufficient to produce synaptic facilitation (reviewed in Byrne and Kandel, 1996). Since the transient activation of PKA and PKC can produce STF, the persistent activation of one or both of these signaling molecules might contribute to ITF or LTF. Indeed, both PKA and PKC are capable of persistent activation in response to other patterns of 5HT exposure. For example, long-term (24 hr) persistent activation of PKA has been found in SNs after 5HT exposure sufficient for LTF induction; this persistent activation is generated by targeted proteolysis of PKA regulatory subunits through the ubiquitin-proteasome pathway and is required for the induction of LTF (Chain *et al.*, 1995, 1999). Moreover, behavioral training sufficient for LTM for sensitization leads to reduced levels of PKA regulatory subunits in *Aplysia* sensory neurons (Greenberg *et al.*, 1987), suggesting a similar mechanism might be engaged during LTM formation.

Given their role in other phases of synaptic facilitation, do either PKA or PKC exhibit persistent activation in a manner suggestive of a role in ITF? Recent evidence suggests that they do. For example, Sossin and colleagues found that prolonged continuous exposure to 5HT (for 90 min) induces persistent activation of PKC in the isolated CNS measured 2 hr later, and interestingly, this persistent PKC activation required protein but not RNA synthesis (Sossin *et al.*, 1994; Sossin, 1997). Moreover, Müller and Carew (1998) demonstrated that 5HT can induce three distinct phases of PKA activation in tail SNs that bear strong resemblance to the three identified phases of synaptic facilitation at tail SN-MN synapses. A single pulse of 5HT induced transient activation of PKA (lasting minutes), which was insensitive to blockers of protein or RNA synthesis. 5 pulses of 5HT, on the other hand, induced two phases of persistent PKA activation: a long-term phase (evident 20 hr after 5HT) that required both protein and RNA synthesis as described earlier (Chain *et al.*, 1995), as well as an intermediate-term phase (evident 1–1.5 hr after 5HT) that required protein, but not RNA synthesis. Interestingly, similar to comparable phases of SN-MN synaptic facilitation, the intermediate- and long-term phases of persistent PKA activation were temporally discontinuous—PKA activity decayed completely to baseline levels 3 hr after 5HT, prior to the emergence of the long-term phase several hours later (Müller and Carew, 1998). These results demonstrate that 5HT can induce three temporally and mechanistically distinct

phases of PKA activation in tail SNs that bear strong resemblance to the features of STF, ITF, and LTF at tail SN-MN synapses.

The strong correlation between SN PKA activity and SN-MN synaptic facilitation in the intermediate-term domain raised the question of whether these two forms of plasticity are causally related. Since the transient activation of PKA can induce STF, this raised the hypothesis that the intermediate-term persistent activation of PKA observed after 5 pulses of 5HT might be a mechanism contributing to the *expression* of ITF induced in the same fashion. We found that this was the case: the PKA inhibitor KT 5720, which blocks the catalytic activity of PKA downstream of potential activators (*i.e.*, cAMP), completely blocked ITF that had previously been established by 5 pulses of 5HT (Sutton and Carew, 2000). In contrast, the PKC inhibitor chelerythrine (which also targets the catalytic activity of the enzyme downstream of potential activators) had no effect on previously established ITF. Importantly, the blockade of ITF expression was reversible—when the PKA inhibitor was washed out of the bath, synaptic efficacy recovered to a facilitated level comparable with controls. That synaptic facilitation recovered when PKA inhibition was relieved indicated that the processes responsible for ITF induction were intact, demonstrating that the expression of ITF induced by 5 pulses of 5HT requires a persistent activation of PKA, but not PKC. That a persistent activation of PKA with a similar time-course and induction mechanism is observed in the same population of tail SNs further suggests that the expression of ITF derives from plasticity in the SNs themselves, *i.e.*, it is presynaptic. However, an instructive or permissive role for the MN (especially during ITF induction) cannot be ruled out.

ITF Induction by 5 Pulses of 5HT—A Role for Local Protein Synthesis?

Since ITF requires translation but not transcription, the new protein synthesis necessary for ITF induction must derive from the translation of pre-existing mRNA. This mechanistic feature of ITF, coupled with the physical distance (~2–3 mm) between tail SN cell bodies and their distal synapses onto tail MNs raises a critical issue: where is the protein synthesis necessary for ITF occurring? Since the induction of ITF by repeated 5HT pulses occurs only over about a 1 hr interval, synthesis at the SN cell body coupled with anterograde transport seems unlikely, given that the rate of fast axonal transport in *Aplysia* neurons is ~1.5 mm/hr (Ambron *et al.*, 1992; Gunstream *et al.*, 1995). Thus, an attractive hypothesis is that ITF is induced by protein synthesis of pre-existing mRNA localized near the synapse. Consistent with this idea, isolated SN neurites in culture are capable of protein synthesis and this local protein synthesis is necessary for LTF induced by focal 5HT application to the synapse (Martin *et al.*, 1997). Preliminary evidence from our laboratory (Sherff and Carew, 2001) has demonstrated that the induction of ITF at tail SN-MN synapses occurs independently of the SN cell body, further suggesting that local protein synthesis at or near the synapse may be involved in the induction of ITF.

Multiple Forms of ITF at Tail SN-MN Synapses

Although the preceding discussion describes one mechanism contributing to ITF, it does not speak to whether alternative pathways can be engaged for the induction and/or expression of ITF. In other words, is the mechanism outlined above the only mechanism for ITF, or might other signaling pathways be capable of inducing and sustaining synaptic facilitation in the intermediate-term domain? To begin to address this question, we examined whether SN activation coincident with 5HT could enhance the induction of ITF. While STF, ITF, and LTF can all be induced in the absence of intrinsic synaptic activity, it is well established that SN activity coincident with 5HT can enhance both the magnitude of STF (Hawkins *et al.*, 1983; Walters and Byrne, 1983) as well as the induction of LTF (Schacher *et al.*, 1997). Moreover, other studies (Murphy and Glanzman, 1997; Bao *et al.*, 1998) have shown that SN activity can interact with 5HT or tail nerve shock to enhance facilitation in the intermediate-term time domain, raising the possibility that SN activity and 5HT can also cooperate in the induction of ITF.

Since tail MNs are innervated by multiple tail SNs, the activity-dependent and activity-independent components of 5HT-induced synaptic facilitation can be examined in the same postsynaptic tail MN receiving connections from two different tail SNs. We used this experimental arrangement to ask whether coincident SN activity can enhance the induction of ITF, and whether this enhancement was selective to synapses made by activated SNs. We found that a single pulse of 5HT, which normally induces only STF (lasting >20 min) in inactive SNs, induced ITF (lasting >80 min) in SNs that were activated coincident with 5HT exposure (Sutton and Carew, 2000). Importantly, the same pattern of SN activation in the absence of 5HT did not induce any facilitation in either the short- or intermediate-term range. Thus, SN activity and a single pulse of 5HT, when each of which are alone insufficient for ITF induction, can interact to induce synaptic facilitation that extends well into the intermediate-term domain. We should emphasize that we chose a pattern of SN activation (10 hz) that did not produce synaptic facilitation in the absence of 5HT. However, SN activation at higher frequencies can itself lead to persistent facilitation of SN-MN synapses (Eliot *et al.*, 1994; Lin and Glanzman, 1994; Bao *et al.*, 1998; Schaffhausen *et al.*, 2001). Taken together, the results described above demonstrate that ITF at tail SN-MN synapses can be induced in at least two ways: 1) by 5 spaced pulses of 5HT in the absence of SN activity (activity-independent ITF), and 2) with a single pulse of 5HT in the presence of coincident SN activity (activity-dependent ITF).

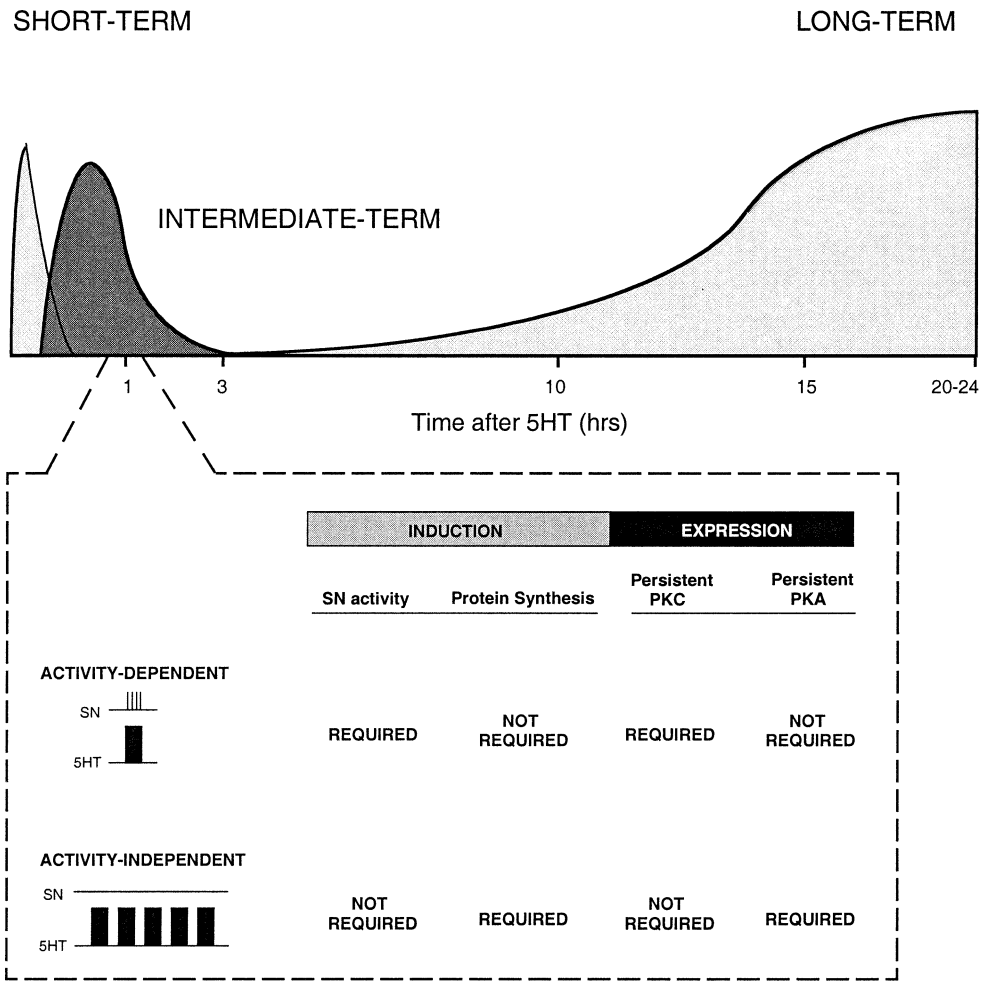


FIG. 1. Two mechanistically distinct forms of intermediate-term facilitation at tail SN-MN synapses. *Top:* Schematic representation of the time-course of three distinct temporal phases of synaptic facilitation produced by 5HT at *Aplysia* SN-MN synapses in the intact CNS (Mauelshagen *et al.*, 1996). *Inset:* Two forms of ITF differ in their requirements for SN activity and protein synthesis during induction, and for persistent activation of PKA and PKC during expression.

While the ITF induced via activity-dependent and activity-independent means appeared phenotypically similar, closer examination revealed that these procedures actually give rise to two mechanistically-distinct forms of ITF (Fig. 1). In contrast to the activity-independent form of ITF induced by 5 pulses of 5HT, we found that the induction of activity-dependent ITF did not require protein synthesis (Sutton and Carew, 2000). Interestingly, a recent study (Bailey *et al.*, 2000) has demonstrated that the activity-dependent induction of LTF in cultured SN-MN synapses (first described by Schacher *et al.*, 1997) is also independent of new protein synthesis, suggesting that this particular mechanistic distinction between activity-dependent and activity-independent forms of plasticity extends into the long-term domain as well (see Sherff and Carew, 2002). While the induction of activity-dependent and activity-independent forms of ITF are clearly different, the possibility remained that they could still engage the same molecular mechanism for their expression, simply via different signaling routes. However, we found that the molecular mechanisms underlying the expression of each form of ITF were also distinct. Whereas expression of the activity-independent form of ITF was blocked by inhibitors of PKA but not PKC, the activity-dependent form had the opposite requirements (Sutton and Carew, 2000). In this case, blocking PKA activity had no affect on the expression of activity-dependent ITF once it had been established. However, two PKC inhibitors (H7 and chelerythrine) both were effective in abolishing previously-established activity-dependent ITF, demonstrating that the expression of this form of ITF requires persistent activation of PKC, but not PKA (Fig. 1).

BEHAVIORAL ANALYSIS OF SENSITIZATION: IS THERE AN INTERMEDIATE PHASE OF MEMORY?

Cellular models of memory, such as SN-MN synaptic facilitation in *Aplysia* and long-term potentiation in mammalian systems, have been extremely powerful in shaping our thinking of how memories are processed in the nervous system. For example, the obser-

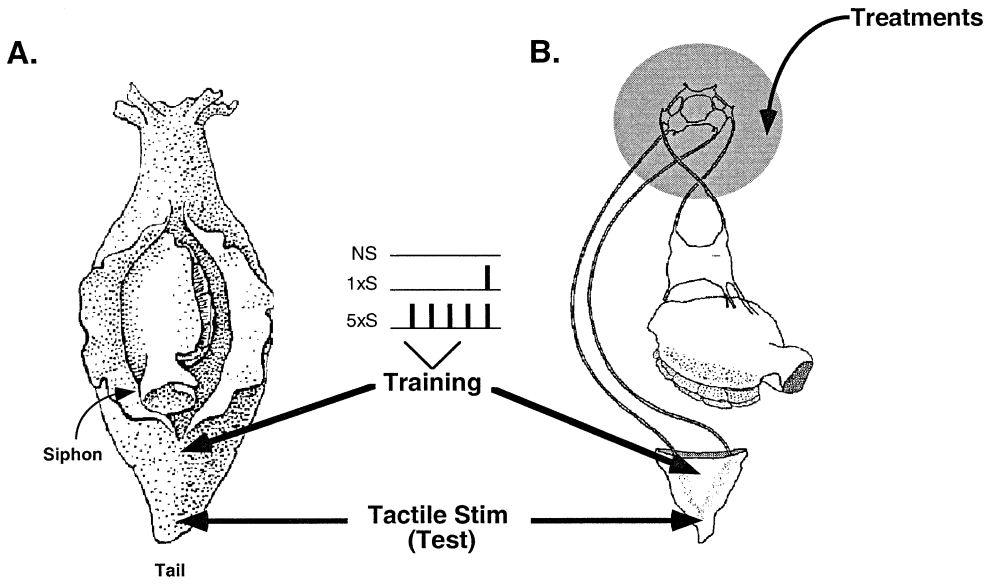


FIG. 2. Preparations for behavioral analysis of memory for sensitization. Identical training and testing procedures can be applied both to intact animals (A) and reduced behavioral preparations (B), consisting of the surgically-excised tail and mantle attached to the CNS. In the reduced preparation, specific regions of the CNS (ring ganglia) are functionally isolated and perfused independently of the rest of the preparation, allowing for rapid drug delivery and removal selectively to the ring ganglia [For more details, see Sutton *et al.* (2001*a*)].

vation that LTF can be induced in the absence of STF in the CNS of *Aplysia* (Emptage and Carew, 1993) suggested that at least some short-term and long-term memories can be processed in parallel, a prediction that has been borne out in more recent behavioral studies (*e.g.*, Izquierdo *et al.*, 1998). In this framework, the features of ITF determined from cellular studies of SN-MN synapses in *Aplysia* are not only suggestive of a novel intermediate phase of memory for sensitization, they also provide the tools with which one can dissect this putative phase from STM and LTM. Specifically, one can ask whether the temporal and mechanistic features of ITF predict one or more forms of intermediate-term memory (ITM) for sensitization.

We initially explored this issue by taking advantage of the fact that, in the absence of intrinsic SN activity, ITF at tail SN-MN synapses has a number of distinguishing characteristics: 1) it requires repeated pulses of 5HT (*e.g.*, 5), whereas STF can be induced with a single pulse; 2) it persists far longer than STF (<30 min), yet completely decays (by about 3 hr) several hours prior to the emergence of LTF (10–15 hr; Mauelshagen *et al.*, 1996); 3) it requires translation of new protein but not transcription of mRNA; and 4) its expression can be reversibly blocked by transient inhibition of the catalytic activity of PKA. Moreover, the cellular protocol for inducing this form of ITF (5 spaced pulses of 5HT in the absence of SN activity) translates relatively easily into behavioral parameters (5 spaced tail shocks applied to a site sufficiently separated from the test site to activate non-overlapping populations of tail SNs during training and testing; see Fig. 2). Thus, to examine the behavioral relevance of activity-independent ITF, we turned to a behavioral analysis of tail-elicited siphon withdrawal (T-SW) and its modulation by sensitization training.

TEMPORAL DYNAMICS OF MEMORY FOR SENSITIZATION: DISCONTINUITY BETWEEN MEMORY PHASES

Do different amounts of training reveal distinct temporal phases of memory in *Aplysia*? In freely moving animals, a single tail shock induced STM for sensitization that decayed within 25 min. In contrast, 5 tail shocks (each spaced by 15 min) induced memory for sensitization that endured far longer, >90 min, but still decayed by 3 hr after training (Sutton *et al.*, 2001*a*). When we re-tested the same animals the following day (20–24 hr after training), we noticed that a subset of animals trained with 5 shocks also showed LTM for sensitization, whereas none of the animals trained with a single shock did. Interestingly, in that subset of animals that demonstrated LTM, the retention curve was clearly biphasic: these animals demonstrated ITM (lasting >90 min), but this memory decayed completely by 3 hr, despite the fact that memory for sensitization was again apparent the following day. Further experiments revealed that this ''dip'' in memory for sensitization extended to at least 6 hr after training, and that the passage of time alone could account for the temporal discontinuity between ITM and LTM for sensitization (Sutton *et al.*, 2001*a*).

That memory retention might exhibit such a striking biphasic profile seems somewhat counter-intuitive. However, a similar U-shaped curve for memory retention as a function of time has been demonstrated in a number of species including rats (Kamin 1957, 1963), mice (Robustelli *et al.*, 1970), goldfish (Riege and Cherkin, 1971), octopus (Sanders and Barlow, 1971),

chicks (Rosenzweig *et al.*, 1993), honeybee (Gerber and Menzel, 2000) and humans (Tallarico, 1973). This conserved feature of memory processing was first identified by Kamin (1957) examining memory for shuttle-box avoidance in rats, and the general phenomenon (where periods of high memory retention are intercalated with periods of lesser retention) is often referred to as the ''Kamin effect.'' Thus, the temporal discontinuity observed for ITM and LTM for sensitization in *Aplysia* is consistent with a substantial behavioral literature.

An important question concerning the dynamics of memory we uncovered centers on the features of memory processing or behavioral testing that the biphasic profile reflects? Kamin (1957, 1963) originally suggested that the biphasic profile could reflect an incomplete overlap of two independent memory processes, but others have suggested that it might reflect a deficit in memory retrieval (Klein and Spear, 1970) or a deficit in behavioral performance (*e.g.,* Barrett *et al.*, 1971). It is important to note here that the Kamin effect is observed in both appetitively-motivated (Gerber and Menzel, 2000) and aversively-motivated (Kamin, 1957, 1963; Rosenzweig *et al.*, 1993; Sutton *et al.*, 2001*a*) behaviors, and in memory based on non-associative learning (Sutton *et al.*, 2001*a*), classical conditioning (*e.g.,* Gerber and Menzel, 2000), and instrumental conditioning (Kamin, 1957, 1963). Thus, it is unlikely that the biphasic profile of memory retention observed across all of these studies owes to a specific feature of the task or species examined.

There are two broad classes of hypotheses that could account for the temporal discontinuity we observe between ITM and LTM. One is that the ''dip'' reflects the complete decay of ITM several hours before the behavioral expression of LTM. Another possibility is the two phases of memory overlap, but behavioral inhibition contributes to sculpting the retention profile. The potential contribution of behavioral inhibition is a real possibility given that transient inhibition of defensive withdrawal is often observed immediately after tail shock in studies of memory for sensitization in *Aplysia* (Mackey *et al.*, 1987; Rankin and Carew, 1988; Marcus *et al.*, 1988; Hawkins *et al.*, 1998). In this case, the transient inhibition following a single tail shock is thought to over-ride STM for sensitization for the first few minutes following training, such that overt behavioral sensitization has a delayed onset (*e.g.,* Marcus *et al.*, 1988; Hawkins *et al.*, 1998). Thus, it is possible that memory for senstization is actually continuous, but performance is sculpted by behavioral inhibition from 3–6 hr after training. However, if the ''dip'' in behavioral sensitization from 3–6 hr after training with 5 tail shocks reflects behavioral inhibition rather than a decay of memory for sensitization, one would predict that training delivered within this time period would be less effective (or in the limit, completely ineffective) in producing sensitization. Contrary to this hypothesis, we found that animals previously trained with 5 tail shocks still demonstrated STM for sensitization during the ''dip,'' with a magnitude and time-course that was indistinguishable from animals without a previous history of training with 5 shocks (Sutton and Carew, in preparation). These results argue against the hypothesis that behavioral inhibition is responsible for the lack of sensitization from 3–6 hr after 5-shock training.

Although not definitive, the results described above support the idea that the biphasic profile of senistization may reflect a temporal discontinuity between intermediate-term and long-term memory phases. This hypothesis is further suggested by two other lines of evidence. First, the biphasic profile is not only observed at the behavioral level, but also over the same temporal domains at the cellular level (Mauelshagen *et al.*, 1996) and the molecular level (Müller and Carew, 1998) (see Fig. 3). Second, in both of these latter cases, the intermediate- and long-term phases separated by the ''dip'' also have distinct mechanisms—the intermediate-term phase requires protein but not RNA synthesis, whereas the long-term phase requires both. Thus, if the biphasic retention profile reflects temporally discontinuous memory phases, memory processing in the two time domains might be subserved by distinct molecular mechanisms (see below).

ITM has a Unique Molecular Signature

The mechanisms underlying memory for sensitization of T-SW can be studied in a reduced behavioral preparation consisting of the surgically excised tail and mantle (which contains the siphon) along with the intervening circuitry that mediates T-SW (see Fig. 2B). In this preparation, distinct regions of the CNS can be functionally isolated to allow for selective drug administration to the ring ganglia (containing tail SN-MN synapses and tail SN-interneuron synapses) and not the abdominal ganglion (containing the siphon MNs). This experimental arrangement is very useful, since the primary components of the circuitry underlying siphon withdrawal are contained within the abdominal ganglion, allowing us to selectively manipulate the function of SNs and interneurons that are upstream of this essential circuit. Moreover, since this functional isolation is achieved by way of a perfusion system, drugs can be rapidly removed from the ring ganglia allowing for an examination of mechanisms underlying memory expression.

With the use of the reduced preparation described above, one can now ask whether STM, ITM, and LTM have distinct macromolecular synthesis requirements for their induction. In fact, they do. Application of the protein synthesis inhibitor emetine to the ring ganglia had no effect on STM induced by a single tail shock, but completely blocked the induction of ITM after 5 spaced tail shocks (Sutton *et al.*, 2001*a*). However, the RNA synthesis inhibitor actinomycin D had no effect on the induction of ITM, but did block the induction of LTM measured the next day (Sutton *et al.*, 2001*a*). These results, together with two previous studies, indicate that three phases of memory for sensitization,

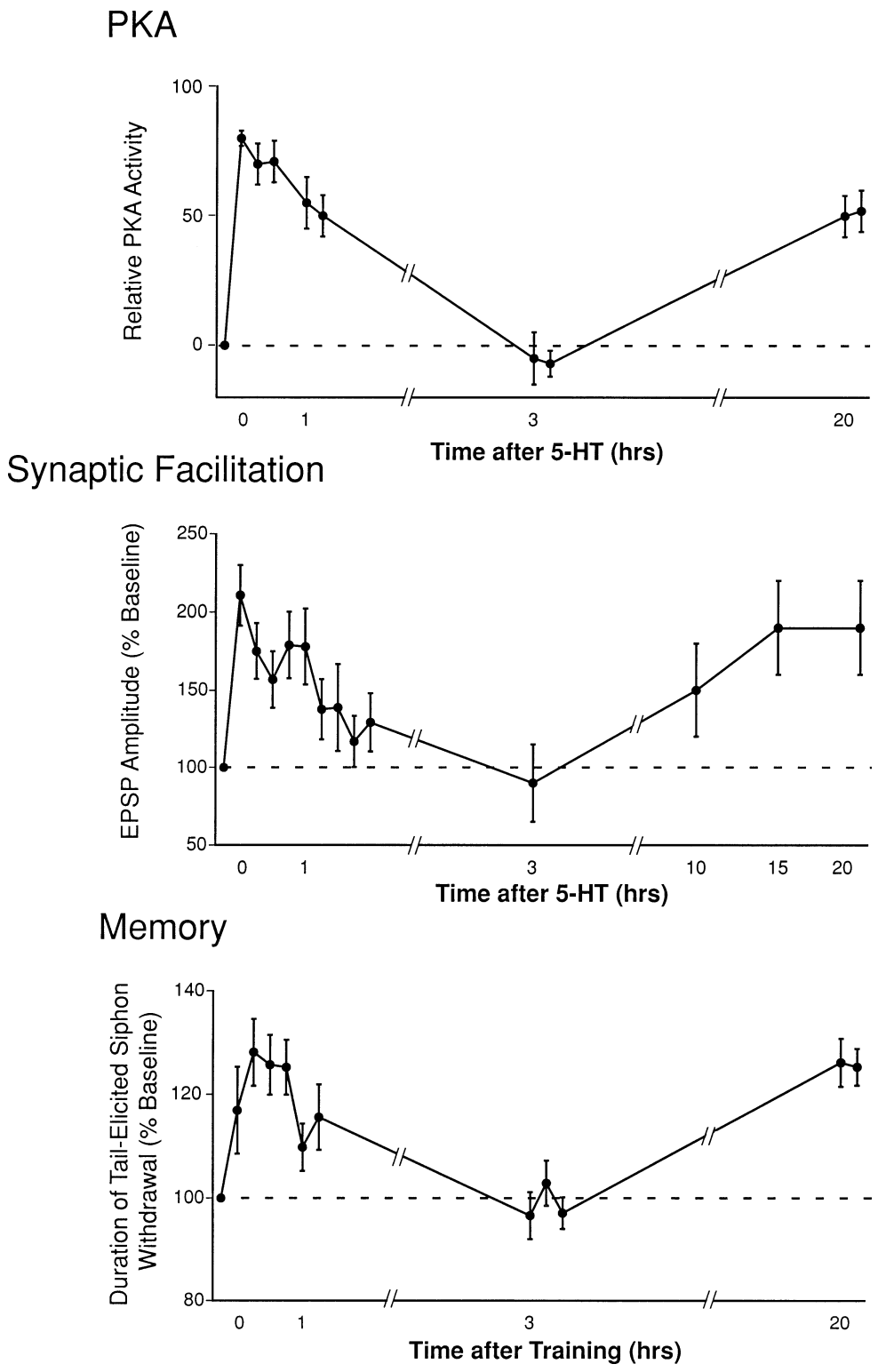


FIG. 3. Temporal dynamics of intermediate-term and long-term synaptic facilitation, SN PKA activation, and memory for sensitization. Mean (±SEM) PKA activity relative to untreated control in tail SNs (Top panel, from Müller and Carew, 1998) and mean (±SEM) EPSP amplitude relative to baseline of tail SN-MN synapses (Middle panel, from Mauelshagen *et al.*, 1996) after 5 pulses of 5-HT. Mean (±SEM) duration of T-SW relative to baseline after 5 spaced tail shocks (Bottom Panel, from Sutton *et al.*, 2001*a*). In each case, the intermediate phase (<3 hr) of plasticity/memory completely decays prior to the onset of the long-term phase (>20 hr).

like their synaptic counterparts, each have unique molecular signatures: STM requires neither protein nor RNA synthesis, ITM requires translation but not transcription, and LTM requires both translation and transcription (Castellucci *et al.*, 1989; Levenson *et al.*, 2000; Sutton *et al.*, 2001*a*). Thus, the ''dip'' in memory for sensitization, like that observed for cellular and molecular plasticity in tail SNs, is associated with a transition in macromolecular synthesis requirements suggesting that the temporal discontinuity reflects distinct non-overlapping memory phases.

Given the close temporal and mechanistic correspondence between tail SN PKA activity, tail SN-MN synaptic facilitation, and memory for sensitization, it next became of interest to determine the extent to which the expression of ITM for sensitization depends on the activity of PKA. We examined this issue by blocking PKA activity (with KT 5720) 30 min *following* training with 5 tail shocks, during ITM expression. As was the case for the expression of activity-independent ITF, blockade of PKA activity reversibly disrupted the expression of ITM (Sutton *et al.*, 2001*a*). These results demonstrate that the expression of ITM after 5 tail shocks requires a persistent activation of PKA. Thus, the same temporal and molecular characteristics that distinguish ITF from other phases of synaptic plasticity distinguish ITM from other phases of memory for sensitization.

SITE-SPECIFIC MEMORY FOR SENSITIZATION REVEALS A DISTINCT FORM OF ITM

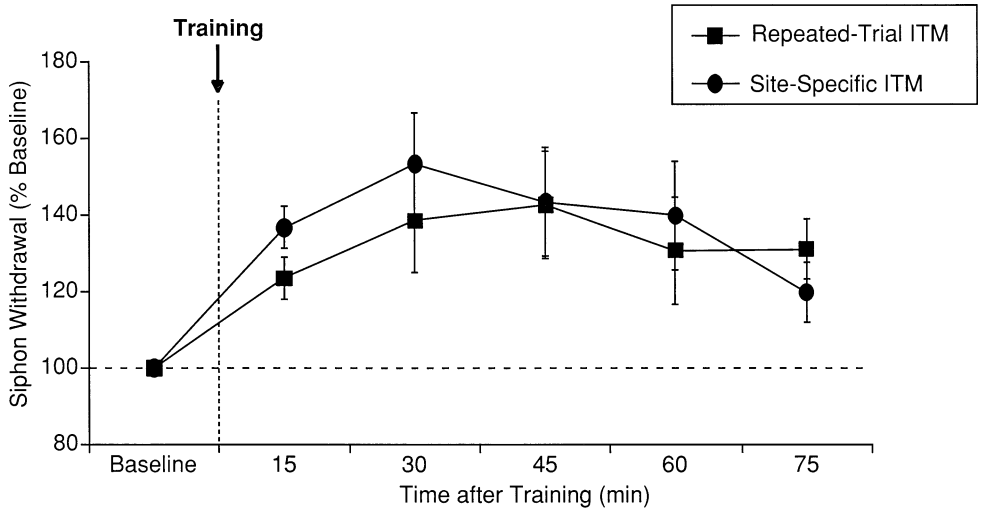
The preceding discussion indicates that the properties of ITF at tail SN-MN synapses induced by 5 pulses of 5HT accurately predicted an intermediate phase of memory for sensitization induced by 5 tail shocks. That multiple forms of ITF can be expressed at tail SN-MN synapses (Sutton and Carew, 2000) would further predict that other forms of ITM for sensitization of T-SW may also exist. In particular, the features of activity-dependent ITF would suggest that 5HT (released by tail shock) could interact with SN activity to induce a form of ITM that was protein synthesis-independent and required persistent activation of PKC, but not PKA, for its expression. But, how does one apply the stimuli that induce activity-dependent ITF (5HT and coincident SN activation) in a behaviorally-relevant manner? To accomplish this, we took advantage of the fact that tail shock induces both 5HT release (Marinesco and Carew, 2002) and activation of a sub-population of tail SNs that innervate the site of the shock (Walters *et al.*, 1983). Thus, testing T-SW at the site of shock might reveal an activity-dependent enhancement of sensitization, since this test should recruit tail SNs that are exposed to 5HT coincident with their own activation. Indeed, Walters (1987) demonstrated that both the magnitude and duration of sensitization was enhanced when testing shocked sites, relative to non-shocked sites. Moreover, the magnitude and duration of tail SN-MN synaptic facilitation following training was enhanced in SNs that were activated by training compared to those that were not activated, and facilitation in activated SNs persisted well into the intermediate-term domain (>120 min).

Collectively, the findings discussed above suggested that activity-dependent ITF might contribute to an intermediate phase of memory for site-specific sensitization. To explore this hypothesis, we examined whether a single tail shock could induce ITM for sensitization selectively at the shocked site in the reduced preparation described above. In this experiment, T-SW was tested on one side of the tail, and different groups of animals received a single shock either to the test site itself or to another site on the same side of the tail. Consistent with our previous observations, a single shock induced STM (lasting <30 min) when test stimuli were delivered outside of the shocked area. In contrast, when testing the shocked site itself, ITM for sensitization (lasting >90 min) was observed (Sutton *et al.*, 2001*b*). Further analysis revealed that this effect required plasticity in the CNS, since reversible CNS inactivation during training completely prevented site-specific ITM. Thus, ITM for sensitization of T-SW can be induced in two ways: 1) by repeated tail shocks to a non-test site or, 2) by a single tail shock applied to the test site.

Is ITM for sensitization induced by these different training procedures the same? Close examination of this issue revealed that site-specific ITM, while phenotypically similar to ITM induced by repeated tail shocks, actually represents a distinct form of memory for sensitization. Whereas ITM induced by repeated tail shocks was dependent on protein synthesis for induction and persistent PKA activation for expression, blocking protein synthesis or PKA activity had no affect on either the induction or expression of site-specific ITM (Sutton *et al.*, 2001*b*). In this regard, activity-dependent ITF at tail SN-MN synapses can be distinguished from activity-independent ITF by the same lack of dependence on protein synthesis and PKA (Sutton and Carew, 2000). Moreover, similar to activity-dependent ITF, the expression of site-specific ITM was reversibly blocked by the PKC inhibitor chelerythrine (Sutton *et al.*, 2001*b*). Again, the fact that memory recovered following washout of the drug indicates that the expression of site-specific ITM requires a persistent activation of PKC. Thus, the data provide a striking correlation: the temporal and mechanistic features of activity-dependent ITF are recapitulated in its behavioral counterpart, site-specific ITM.

THE FEATURES OF ITM EXPRESSION REVEAL UNIQUE PROCESSES RESPONSIBLE FOR INDUCTION

Since site-specific ITM is induced rapidly, is protein synthesis-independent, and is activity-dependent, one possible mechanism for generating the persistent PKC activation required for expression involves limited proteolysis of PKC by the Ca^{2+}-activated protease calpain, generating a persistently-active catalytic fragment known as a PKM (Inoue *et al.*, 1977; Suzuki *et al.*, 1992). How might this proteolytic generation of PKM



	Induction	Expression
Repeated-Trial ITM	Protein synthesis-dependent; mRNA synthesis-independent	Persistent PKA activation
Site-Specific ITM	Protein synthesis-independent; Proteolysis-dependent	Persistent PKC/PKM activation

FIG. 4. Two distinct forms of intermediate-term memory for sensitization have unique induction and expression requirements.

contribute to site-specific ITM? A sub-population of tail SNs that innervate the shocked site should experience the modulatory effects of 5HT coincident with their own activation during tail shock, and testing the site of shock should recruit that same population of SNs. Therefore, in these SNs, the interaction of activity with the modulatory effects of tail shock may lead to activation of calpain (driven by activity) coincident with PKC translocation (driven by 5HT), resulting in proteolytic generation of a PKM. Importantly, the proteolytic induction mechanism in those activated SNs would be rapid and independent of protein synthesis, which are the core features of site-specific ITM. Is the persistent activation of PKC associated with site-specific ITM derived from a PKM? We have investigated this question using two parallel lines of inquiry: 1) by examining the effects of a PKC inhibitor that interacts specifically with the regulatory domain of PKC, and 2) by examining the effects of blocking calpain function directly.

While chelerythrine blocks PKC activity by interacting with the catalytic domain of the enzyme, another PKC inhibitor, calphostin C, blocks PKC activity by interacting with the regulatory domain. Thus, the hypothesized role of a proteolytically-generated PKM generates two clear predictions regarding the effects of calphostin C on the induction and expression of site-specific ITM. First, calphostin C should block the *induction* of site-specific ITM when applied prior to training, because PKC translocation (via the regulatory domain) to the membrane is thought to be required for calpain-dependent cleavage (Kishimoto *et al.*, 1983). Second, once induced, the *expression* of site-specific ITM should be resistant to inhibition by calphostin C, since the regulatory domain would no longer be associated with a PKM. These predictions were borne out. Application of calphostin C to the ring ganglia blocked the induction, but not expression, of site-specific ITM (Sutton *et al.*, 2001*b*). We next examined the effect of blocking calpain activity in the ring ganglia on the induction and expression of site-specific ITM. Similar to the effects of calphostin C, two structurally-distinct calpain inhibitors, ALLM and MDL 28170, each blocked the induction, but not expression, of site-specific ITM (Sutton *et al.*, 2001*b*). Together, these studies provide parallel lines of evidence suggesting that the induction of site-specific sensitization entails the proteolytic processing of PKC, and in turn, the catalytic fragments generated by such processing are responsible for maintaining the memory in the intermediate-term range (Fig. 4).

SUMMARY AND CONCLUSIONS

The analysis of ITM in *Aplysia* has yielded several insights into the underlying cellular and molecular mechanisms that contribute to memory processing. For example, as is the case in a number of systems, different temporal phases of memory for sensitization in *Aplysia* can be distinguished not only by their persistence, but also by their underlying molecular requirements. Thus, when training and testing stimuli are applied to distinct sites on the tail, 3 phases of memory

for sensitization can be identified: STM (<30 min) that requires neither protein nor RNA synthesis, ITM (>90 min) that requires protein but not RNA synthesis, and LTM (>24 hr) that requires both. These phases, however, are not unitary: within a temporal domain, distinct forms of memory with unique molecular mechanisms can also be distinguished. Thus, ITM induced by repeated tail shocks to a non-test site requires protein synthesis and persistent activation of PKA for its expression, whereas site-specific ITM induced by a single shock to the test site is protein synthesis-independent and requires persistent PKC activation for its expression. These results indicate that, depending on the unique features of a given learning experience, very different classes of mechanisms can be engaged to subserve memory in a particular time domain. Thus, while qualitatively different types of experiences can be remembered over similar temporal intervals, the nervous system may accomplish this using distinct molecular strategies.

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