Published in final edited form as:

Neurobiol Learn Mem. 2015 September ; 123: 35–42. doi:10.1016/j.nlm.2015.04.013.

Latent memory facilitates relearning through molecular signaling mechanisms that are distinct from original learning

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Abstract

A highly conserved feature of memory is that it can exist in a latent, non-expressed state which is revealed during subsequent learning by its ability to significantly facilitate (savings) or inhibit (latent inhibition) subsequent memory formation. Despite the ubiquitous nature of latent memory, the mechanistic nature of the latent memory trace and its ability to influence subsequent learning remains unclear. The model organism *Aplysia californica* provides the unique opportunity to make strong links between behavior and underlying cellular and molecular mechanisms. Using *Aplysia*, we have studied the mechanisms of savings due to latent memory for a prior, forgotten experience. We previously reported savings in the induction of three distinct temporal domains of memory: short-term [10min], intermediate-term [2h] and long-term [24h]. Here we report that savings memory formation utilizes molecular signaling pathways that are distinct from original learning: whereas the induction of both original intermediate- and long-term memory in naïve animals requires mitogen activated protein kinase (MAPK) activation and ongoing protein synthesis, 2h savings memory is not disrupted by inhibitors of MAPK or protein synthesis, and 24h savings memory is not dependent on MAPK activation. Collectively, these findings reveal that during forgetting, latent memory for the original experience can facilitate relearning through molecular signaling mechanisms that are distinct from original learning.

Keywords

forgetting; training pattern; MAPK; ERK; translation

1. Introduction

Memory is typically assessed through the measurement of overt changes in behavior. However, studies of learning and memory have also clearly established that memory can also develop in a latent, non-expressed form following a learning experience and, although

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inaccessible through tests of recall, is revealed through the ability to influence (promote or inhibit) subsequent learning and memory formation in a wide variety of paradigms (e.g., priming (Antzoulatos, Wainwright, Cleary, and Byrne, 2006; Parsons and Davis, 2012; Philips, Tzvetkova, Marinesco, and Carew, 2006), savings (Ebbinghaus, 1885/1913), latent inhibition (Tolman and Honzik, 1930)). In many instances, latent memory can outlast the overt expression of memory for an experience to provide a platform for “savings” (a reduction in the number of study trials or time required for relearning; Ebbinghaus, 1885/1913; Nelson, 1971). While well established as a behavioral feature of memory, the mechanistic nature of the latent memory trace and the means by which savings occurs during relearning remains unclear.

Despite its ubiquitous nature, very few studies of latent memory and savings have been initiated in invertebrate model organisms (Antzoulatos et al., 2006; Matzel, Collin, and Alkon, 1992; Parvez, Stewart, Sangha, and Lukowiak, 2005; Philips et al., 2006; Susswein and Schwarz, 1983), wherein strong links can be made between behavior and the underlying cellular and molecular mechanisms. With its large, readily identifiable neurons and simple nervous system, the invertebrate mollusk *Aplysia californica* has proven to be an advantageous model for elucidating the cellular and molecular mechanisms of memory formation. Moreover, learning in *Aplysia* recapitulates critical features of the human savings phenomenon (Antzoulatos et al., 2006; Philips et al., 2006; Susswein and Schwarz, 1983). We previously showed that a latent memory outlasts the initial forgetting of long-term memory (LTM) for sensitization of the tail-elicited siphon withdrawal reflex (T-SWR) for at least two days, and supports the facilitated induction of three distinct temporal phases of memory during relearning: short-term (10min), intermediate-term (2h) and long-term savings memories (24h) (Philips et al., 2006). Importantly, savings was not observed when retraining was delayed for four days after initial signs of forgetting, identifying a strong parallel to the human learning phenomenon, in which the benefit of prior experience is time-limited (Ebbinghaus, 1885/1913). The demonstration of savings in *Aplysia* has established a unique opportunity to study the cellular and molecular features of the latent memory trace and its facilitation of subsequent memory formation in simple neural circuits.

In the present study, we examined the molecular features of savings in *Aplysia*. First, we replicated our earlier observation of savings for the induction of sensitization memories within the T-SWR (Philips et al., 2006). We then showed that the facilitated induction of 2h and 24h memory requires the interaction between two spaced training trials and is supported by plasticity in the previously described narrow temporal window for two-trial LTM formation in naïve animals (Philips, Tzvetkova, and Carew, 2007). Savings could also be observed when retraining reduced behaving preparations of previously trained *Aplysia*. In molecular studies, we found that the induction of 2h savings memory was unique from the induction requirements for a comparable phase of ITM formation in naïve animals, in that (i) it no longer required activation of the highly conserved mitogen activated protein kinase (MAPK) signaling pathway and (ii) it was not disrupted by the inhibition of ongoing protein synthesis. We additionally showed that 24h savings memory was distinct from the LTM induced in naïve animals, in that its induction was independent of MAPK activation, but still required protein synthesis. Collectively, these findings indicate that latent memory for prior

sensitization facilitates relearning across multiple temporal phases of memory by engaging distinct molecular rules for subsequent memory formation.

2. Materials and Methods

2.1 Animals

Wild-caught *Aplysia californica* (250-400g; Marinus Scientific, Long Beach, CA and South Coast Bio-Marine, San Pedro, CA) were housed in a 200 gallon tank of artificial seawater (Reef Crystals) at 15°C. To facilitate monitoring of the tail-elicited siphon withdrawal reflex (T-SWR), animals were anesthetized in ice-cooled seawater and the parapodia around the siphon was surgically removed. The ink gland was also removed to permit training in the absence of conspecific signaling through ink release (Stopfer, Chen, and Carew, 1993). Animals recovered for 4-5 days in the home tank before training.

2.2 Behavioral Procedures

The T-SWR was initiated by stimulating the posterior tip of the tail midline with a pulsed water jet (0.4s, 45 psi, Teledyne Water Pik; Philips et al., 2006). The duration of the tail-elicited siphon withdrawal responses was measured from the onset of the stimulus to the initial relaxation of the neck of the siphon. Baseline T-SWR duration was established using the average of three tests (inter-test interval [ITI] = 15 minutes).

After establishing baseline, animals were randomly assigned into either experimental or control groups. The experimental group received *Phase I* sensitization training (four midline tail shocks [TSS; 1 shock: 2s train of 10ms, 15mA DC pulses at 50Hz] inter-shock interval [ISI] = 15 min; Philips et al., 2006). Control animals were not trained, but were tested and housed with trained animals.

Twenty-four hours following training, memory was assessed with the average of two tests of the T-SWR (ITI = 30 min). These posttests at 24h were used to group animals according to previously established criteria (Philips et al., 2006). Trained animals with average responding below 120% pre-training levels were removed from further study (42% of trained animals) because we previously reported that these weakly sensitized animals do not demonstrate long-term savings memory induction with retraining (Philips et al., 2006). Thus, for our studies of short-, intermediate- and long-term savings memory induction mechanisms we only continued with animals whose responses were greater than or equal to 120% pre-training levels at 24h. Importantly, the lack of robust memory expression in a significant fraction of trained animals was by design. *Phase I* training uses weak training stimuli so that long-term memory duration does not persist longer than a week. Animals expressing 120% baseline T-SWR behavior at the initial 24h test demonstrated an average LTM duration of two days.

LTM expressing animals (and matched naïve controls) were subsequently tested every 24h to describe the forgetting curve for each trained animal. Day 1 of “forgetting” was identified as the first day when average responding of trained animals fell below 120% of the baseline average (Philips et al., 2006), and was always confirmed by additional tests 24h later (Day 2 of “forgetting”). Thus, all trained animals demonstrated two consecutive days of apparent

forgetting before they were given *Phase II* training (see Fig. 1A). Whereas *Phase I* training used 4 training trials to establish an original LTM, in *Phase II* we tested for latent memory for the *Phase I* experience by using a savings test of retraining with fewer trials (2 spaced midline tail shocks [1 shock: 2s train of 10ms, 15mA DC pulses at 50Hz], ISI = 15 min; Philips et al., 2006). In *Phase II*, both experimental and control animals received the two training shocks and posttests were conducted at 10 minutes following the first *Phase II* training shock, at 2 hours after the second shock, and at 24 hours (two tests, ITI = 30 min) to assess short, intermediate- and long-term phases of memory induction, respectively.

2.3 Reduced T-SWR Preparation

For the mechanistic analysis of savings memory induction, we administered *Phase II* training in reduced preparations of the tail-elicited siphon withdrawal reflex (T-SWR) of a second cohort of *Aplysia*. In these experiments, *Phase I* training and post-tests were conducted in intact *Aplysia* as described above. However, immediately following tests on Day 2 of forgetting, experimental and matched control animals were anesthetized using isotonic MgCl₂ and reduced behaving preparations of the T-SWR were generated (Fig. 2A) (Sutton, Masters, Bagnall, and Carew, 2001).

Following a 2h recovery, baseline T-SWR was re-established (RP baseline; 3 tests, ITI = 15min). We tested the same site as was used in the intact, freely behaving animal. Ten minutes after baseline establishment, *Phase II* training was administered. Post-tests were conducted at 10min after the first TS, and at 2h and 24h (3 tests, ITI = 15min) after the second TS. Memory expression was determined by comparison to the baseline T-SWR re-established in the reduced behavioral preparation, since an overall reduction in the T-SWR response duration was observed after surgery (intact vs reduced preparation scores on day of surgery, $p < .05$ paired t test; Fig. 2B).

2.4 Drug Treatments

To test the requirement for protein synthesis in savings learning, we used the translation inhibitor emetine dihydrochloride (Sigma) at a concentration (100 μ M in ASW) that blocks >95% of protein synthesis and disrupts memory formation in the T-SWR reduced behavioral preparations from naïve animals (Sutton et al., 2001). An identical testing and training protocol was used as that described above for savings retraining of reduced T-SWR preparations, except that emetine (100 μ M) was added to the inner CNS chamber 45 min prior to baseline establishment (initial perfusion of 24mL emetine or artificial sea water [460 mM NaCl, 55mM MgCl₂, 11mM CaCl₂, 10mM KCl and 10mM Tris, pH 7.6]) and was maintained in a static bath throughout and for 1 hour after *Phase II* training. Fresh emetine or vehicle (4 mL, 15°C) was perfused into the CNS chamber every 30 minutes for the duration of the treatment. Emetine has no effect on baseline T-SWR responses at this concentration (Sutton et al., 2001). To control for the effectiveness of the emetine treatment, we replicated the finding of Sutton and colleagues (2001), that ITM induction following five spaced tail shocks requires protein synthesis [5 spaced 1.5s 100mA, AC, midline TSs (ISI=15min)]. Post-tests were performed starting 5 minutes after the last shock, and continued every 15 minutes thereafter up through 80 minutes after the last shock. Long-term sensitization was examined in the same preparations with three post-tests 24 hrs later (ITI =

15 minutes). A statistically reliable disruption of ITM and LTM was observed in the presence of emetine and not vehicle ($p < .05$).

To examine the requirement for MAPK signaling during relearning, we used a well-established inhibitor of MEK (the MAPK kinase) to disrupt MAPK activation, and a previously established protocol for the disruption of MAPK-dependent ITM and LTM in the T-SWR with bath application of U0126 (MEK inhibitor) to the CNS chamber (Sharma, Sherff, Shobe, Bagnall, Sutton, and Carew, 2003). Briefly, U0126 (Tocris, 20uM) or the inactive analogue U0124 (20uM) was introduced to the CNS chamber during the establishment of baseline responding (24mL initial drug perfusion after the first two of four pre-tests) at 40min prior to *Phase II* retraining, and treatment was continued (4mL fresh drug every 30min) until 1h post training. Two additional pretests conducted in the presence of drug treatment confirmed no effect of drug or vehicle treatment on baseline T-SWR. This is consistent with previous findings (Sharma et al. 2003). Drug efficacy was confirmed by concurrent studies in which the same batches of drug were shown to significantly disrupt original LTM formation in reduced T-TWR preparations of naïve animals (Philips, Ye, Kopec, and Carew, 2013).

2.5 Data Analysis

Post-training responses of the T-SWR in all animals were characterized as a percentage of the average baseline response. ANOVAs were conducted and followed with planned comparisons corrected for multiple comparisons (Holm-Sidak). Within group significance was explored using single sample paired *t* tests of post-training responses and the average pre-training response. Between group comparisons were made using Student *t* tests. All statistical analyses were performed using Graphpad Prism 6.

3. Results

3.1 Latent memory for a prior sensitizing experience facilitates memory acquisition across two spaced retraining trials

To explore the nature of savings during relearning, we first replicated our principle finding of savings in the formation of multiple temporal phases of memory for sensitization of the T-SWR (Fig. 1B, Philips et al. 2006). The duration of the T-SWR was monitored before and after training. In *Phase I*, baseline T-SWR duration was established across three pre-tests (inter-test interval = 15 min) and experimental animals were trained with four spaced midline tail shocks (*Phase I* training: 2s train, 10ms pulse duration, 50Hz, 15mA direct current; inter-shock interval = 15 min). Control animals did not receive *Phase I* training, but were tested along side experimental animals at all time points. A two-way ANOVA identified significant effects of time ($F(1,14)=20$, $p=0.0006$) and training ($F(1,14)=19$, $p=0.0006$) on *Phase I* post-training responses, as well as a significant interaction ($F(1,14)=11$, $p=0.0046$). Subsequent planned comparisons revealed that *Phase I* training induced a LTM for sensitization in the T-SWR at 24h (trained: mean T-SWR duration expressed as a percentage of baseline duration $\pm$ SEM, $144 \pm 5\%$, $n=8$; untrained: $105 \pm 4\%$, $n=8$, $p < .001$ unpaired *t* test; Fig. 1B). Post-tests every 24h (two tests spaced by 30 min) showed an average LTM expression that lasted 2 days, consistent with our previous report (Philips et

al., 2006). On day 2 of apparent forgetting (two consecutive days with overt memory expression <120% of baseline responding; see Methods), experimental and matched control animals received two spaced training shocks (2s train, 10ms pulse duration, 15mA, 50Hz, direct current) spaced by 15min. These training shocks were designed to be sub threshold for short-term sensitization (10 min) in naïve animals (see Philips et al. 2006; Fig. 1B) and the inter-trial spacing of 15 min was used because this training interval is not permissive to support ITM or LTM induction in naïve animals (Philips et al., 2007; Purcell, Sharma, Bagnall, Sutton, and Carew, 2003; Sutton, Ide, Masters, and Carew, 2002). The use of this weak training paradigm allowed us to test for savings in memory formation at 10min, 2h and 24h post retraining. A two-way repeated measures analysis of variance (ANOVA) identified a significant effect of time ($F(2,28)=3.6$, $p=0.0397$) and *Phase I* training history ($F(1,14)=30$, $p<0.0001$), but no significant interaction ($F(2,28)=1.4$, $p=0.2668$) on *Phase II* responses at 10min, 2h and 24h memory tests. In subsequent planned comparisons, within group analyses revealed significantly enhanced responding at all three post-tests in previously trained animals (10min: $183 \pm 18\%$, 2h: $162 \pm 14\%$, 24h: $130 \pm 12\%$, $p<0.05$ in paired *t* tests corrected for multiple comparisons), but not in untrained controls which received only *Phase II* training (10 min: $113 \pm 8\%$, 2h: $117 \pm 10\%$, 24h: $102 \pm 3\%$, $p>0.1$ in all comparisons; Fig. 1B). There were also significant between group differences at 10min ($p=0.0038$ unpaired *t* test), 2h ($p=0.018$) and 24h ($p=0.037$). These findings replicate our previous report of savings during relearning in this system (Philips et al., 2006).

We next explored whether one or both *Phase II* retraining trials were necessary for the induction of the 2h and 24h savings memories. We found that while animals retrained with a single *Phase II* TS did express the expected 10min short-term memory ($n=6$, $140 \pm 18\%$, $p<0.05$ paired *t* test), they showed no memory at 2h and 24h retention tests ($113 \pm 13\%$ and $105 \pm 7\%$, $p>.5$ both time points; Fig. 1C). Thus, we conclude that both the 2h and 24h savings memories are the result of the interaction between the two spaced *Phase II* retraining trials. Intriguingly, this identifies plasticity in the animal's ability to learn across shorter (15min) training intervals, since this short two-trial training interval is not permissive for ITM and LTM in naïve animals (Philips et al., 2007; Purcell et al., 2003; Sutton et al., 2002).

Finally, we sought to clarify whether the memories formed by *Phase II* retraining were the result of relearning, or whether the daily testing used to monitor memory expression might have supported the formation of an extinction memory that masked expression of the original sensitization memory until *Phase II* training could support the “reinstatement” of the original memory (Delamater, 1997; Rescorla and Heth, 1975). We explored these possibilities by reducing the daily testing schedule to every other day, with the expectation that a reduced testing schedule would slow any extinction learning and result in a longer memory duration (i.e., LTM expressed for an increasing number of days). However, animals in which the 48h tests were eliminated ($n=8$) did not demonstrate a prolonged memory expression when compared to trained conspecifics that received daily testing ($n=5$). Both groups were significantly different from untrained controls ($n=7$) at 24h ($p<.05$ in both comparisons), but not at 72h ($p>.1$ in both comparisons). However, reducing the number of post-tests did not prolong memory expression when compared to conspecifics that received daily testing (Fig. 1D). Reducing the number of post-tests also did not yield a significant

effect on the magnitude of responses at 72h post-tests ($p > .05$, unpaired t test). Additionally, we reasoned that if *Phase II* training served to simply “reinstate” the original memory we would expect that the mechanisms supporting reinstatement of the three temporal phases of memory (10min, 2h, and 24h) would be the same, which they are not (see Fig. 3).

Collectively, these data show that during apparent forgetting, a latent memory for prior sensitization persists to support savings during relearning across short-, intermediate- and long-term temporal domains of memory expression.

3.2 Savings is observed in reduced behaving preparations of previously trained *Aplysia*

To characterize the molecular basis of the facilitated memory acquisition, we examined whether savings could be observed during retraining of reduced behaving preparations of *Aplysia*, that permit the pharmacological manipulation of discrete components of the animal's central reflex circuit while inducing and monitoring memory formation (Sharma, Sherff, Stough, Hsuan, and Carew, 2006; Shobe, Zhao, Stough, Ye, Hsuan, Martin, and Carew, 2009; Sutton et al., 2001). Using the strategy outlined in Figure 1A, intact naïve *Aplysia* received *Phase I* training or they were left untrained and memory expression was monitored with daily testing. On day two of forgetting, previously trained and matched naïve animals underwent surgery to produce reduced preparations of the *Aplysia* in which the essential peripheral and central components for the T-SWR remained intact (Fig. 2A). After a two hour recovery period, the baseline T-SWR for each animal was re-established (RP Baseline, avg. of three pre-tests, ITI=15min; Fig. 2B) and *Phase II* training was administered (2 shocks, ISI=15min). Although stable across three tests, the average T-SWR duration for each animal was moderately, but significantly decreased following surgery to $81 \pm 5\%$ of pre-surgery levels ($p < 0.01$, paired t test). Subsequent responses were normalized to this new reduced baseline (Fig. 2B). A significant effect of *Phase I* training history was observed on responses in the reduced preparations following *Phase II* training ($F_{1,9} = 27$, $p = 0.0373$). In subsequent planned comparisons, significant memory induction was observed at 10 min (exp: $170 \pm 20\%$; con: $98 \pm 3\%$, $p < 0.01$ unpaired t test), 2h (exp: $144 \pm 12\%$; con: $101 \pm 6\%$, $p < 0.01$) and at 24h (exp: $128 \pm 4\%$; con: $91 \pm 3\%$, $p < 0.0001$). These findings demonstrate that the molecular mechanisms of savings during relearning are accessible for analysis in the reduced behaving T-SWR preparation.

3.3 Latent memory facilitates the induction of a unique protein synthesis-independent 2h memory

Although the expression of short-, intermediate- and long-term temporal phases of memory are expressed following retraining, it is not clear whether these memories share the same induction requirements as have been reported for original memory formation in naïve animals. Therefore, we next tested the necessity of ongoing protein synthesis for savings memory induction, which is a requirement for original ITM and LTM, but not STM (Sutton et al., 2001). An irreversible inhibitor of protein synthesis, emetine, was introduced to the CNS chamber of reduced T-SWR preparations before, during and for 1h after training. When retraining occurred in the presence of emetine, 10min savings memory induction was not disrupted (eme: $n=5$, $151 \pm 16\%$; veh: $n=4$, $131 \pm 12\%$, $p > 0.4$; Fig. 3) and 24h savings memory induction was significantly disrupted (eme: $106 \pm 10\%$; veh: $169 \pm 25\%$, $p < 0.01$). Thus, the 10min and 24h temporal domains of savings memory share mechanistic similarity

to original STM and LTM, respectively. However, 2h savings memory induction was not disrupted by emetine treatment (eme: $136 \pm 6\%$; veh: $151 \pm 13\%$, $p>0.4$), which is distinct from original ITM (Sutton et al., 2001). These findings support two conclusions: (i) the latent memory trace does not require ongoing protein synthesis for its endurance and interaction with relearning, and (ii) the relearning of intermediate-term memory for sensitization relies on mechanisms which are at least partially distinct from those recruited for original memory formation.

3.4 The induction of intermediate-term and long-term savings memories is MAPK-independent

MAPK activation in the tail SNs of the T-SWR reflex circuit is essential for the induction of ITM and LTM for sensitization of naïve animals (Philips et al., 2013; Sharma et al., 2003) and is upstream of some of the translational requirements for LTM (Kelleher, Govindarajan, Jung, Kang, and Tonegawa, 2004). Thus, we next tested the requirement for MAPK during relearning by retraining reduced preparations from previously trained animals in the presence of the selective MEK (MAPK kinase) inhibitor, U0126 ($n=7$), or its inactive analogue, U0124 ($n=5$) according to the protocol of Sharma et al. (2003). An ANOVA revealed no significant difference between the two drug treatment groups in memory expression following *Phase II* training ($F_{2,20}=0.77$, $p=0.4752$; Fig. 4). The induction of 10min (U0126: $218 \pm 35\%$, U0124: $188 \pm 31\%$, $p>0.4$), 2h (U0126: $158 \pm 13\%$, U0124: 188 ± 47 , $p>0.4$) and 24h (U0126: $145 \pm 17\%$, U0124: $156 \pm 16\%$, $p>0.5$) savings memory was not disrupted by CNS exposure to the MEK inhibitor. Importantly, to control for drug efficacy we showed that the same MEK inhibitor used in the above experiments could effectively block original LTM formation in reduced behaving preparations (Philips et al., 2013), consistent with previous findings (Sharma et al., 2003). Thus, although original memory formation requires MAPK signaling for its induction, relearning appears to be uncoupled from the requirement for additional MAPK signaling.

4. Discussion

We have examined the behavioral and molecular features of memory formation during relearning that is superimposed on the platform of a latent memory for prior experience. Whereas the facilitated induction of short-term (10min) memory required a single retraining trial, the induction of longer-lasting intermediate-term (2h) and long-term (24h) memories required two trials spaced at a 15min interval. In reduced behaving preparations, we showed that intermediate-term and long-term temporal domains of savings memories have distinct molecular requirements: intermediate-term memory expressed out at least 2h following training was independent of both protein synthesis and MEK/MAPK signaling, whereas long-term memory at 24h required protein synthesis, but was independent of MEK/MAPK signaling. Collectively, these studies show that the molecular mechanisms for savings memory formation are distinct from original memory requirements.

4.1 Enhanced encoding of individual experiences and an increased learning rate are features of “savings” in *Aplysia*

It has long been appreciated that latent memory for a previously acquired memory can establish savings during relearning by reducing the amount of “work” (fewer trials to criterion) subsequently required for lasting memory formation (Ebbinghaus, 1885/1913). However, how prior experience supports memory formation from fewer trials during relearning has remained unclear. One can imagine at least two possible mechanisms for savings: differences in the rate of learning (e.g., the number of required trials is reduced from 4 to 2), and differences in the initial strength of the memory (4 trials are still required, but relearning only requires 2 trials because residual memory strength is equivalent to that contributed by the first 2 training trials). There is compelling evidence supporting the participation of both mechanisms (Mawase, Shmuelof, Bar-Haim, and Karniel, 2014; Medina, Garcia, and Mauk, 2001; Rescorla, 2002), which may be influenced by a number of factors (e.g., task, relearning after forgetting vs extinction of the original memory). Thus, a unified mechanism for savings has not been identified.

Our work has produced supporting evidence for two savings mechanisms in *Aplysia*: an enhanced, or “primed” response to individual retraining trials and an increased learning rate. Considering the first mechanism, the present study has confirmed prior observations of a “primed” behavioral response to individual retraining trials (Antzoulatos et al., 2006; Philips et al., 2006). Each *Phase II* retraining trial was designed to be sub threshold for short-term memory formation in naïve *Aplysia*, yet animals with latent memory consistently develop an immediate (10min) sensitization memory following retraining trial 1 (Figs. 1, 2; Philips et al., 2006). This primed response requires the presence of latent memory for prior sensitization, since we previously showed that savings is lost when retraining is delayed until after 4 days of forgetting (Philips et al., 2006). Intriguingly, Byrne and colleagues (Antzoulatos et al., 2006; Wainwright, Byrne, and Cleary, 2004; Wainwright, Zhang, Byrne, and Cleary, 2002) have shown a correlation between a similar priming of sensitization and learning-related changes in structural elements of sensory neurons, including increased number of varicosities and increased neurite length. Alternatively, the enhanced response to retraining could be mediated through an increased excitability of sensory and/or motor neurons in the T-SWR reflex circuit following the original training (Cleary, Lee, and Byrne, 1998; Walters, Byrne, Carew, and Kandel, 1983) that may persist during forgetting as part of the latent memory trace. Future studies will be directed at exploring the structural and cellular features of the latent memory trace.

A second savings mechanism supported by our data is an increased learning rate. We show that the facilitated induction of 2h and 24h savings memories required two spaced retraining trials and developed across a 15min inter-trial interval (Fig. 1B,C). Interestingly, our prior work has established a narrow temporal window that can support LTM formation across two spaced training trials (Two-Trial learning): two trials spaced by 45min, but not by 15min or 1h can induce LTM in naïve animals (Philips et al., 2007; Philips et al., 2013; Sutton et al., 2002). We have observed a similar temporal window for ITM induction in naïve *Aplysia* (unpublished data). Thus, we hypothesize that the ability of animals to acquire intermediate-term and long-term memory across the short 15min two-trial training interval is indicative of

a transient state of “behavioral metaplasticity” (Schmidt, Abraham, Maroun, Stork, and Richter-Levin, 2013) in the two-trial learning window that permits the more rapid encoding of information during relearning. Characterization of the range of two-trial training intervals that are permissive for relearning should provide additional insight into the mechanisms that underlie the facilitated reacquisition of 2h and 24h memories. For instance, it will be important to distinguish whether the permissive retraining window is shifted earlier (i.e., 15min, but not 45min or 1h training intervals are permissive) or is broadened (i.e., both 15min and 45min intervals are permissive) to support savings.

4.2 Savings during relearning establishes multiple phases of memory using induction requirements that are distinct from original learning

The present study also explores a poorly understood feature of savings: the ability to facilitate relearning of multiple temporal phases of memory formation. Most studies of savings have focused on the reacquisition of LTM. However, studies of learning and memory in invertebrates, including *Aplysia*, *C. elegans*, *Drosophila*, and *Lymnaea*, have revealed multiple mechanistically distinct temporal phases of memory, for which corresponding temporal phases have been identified in vertebrates (Barco, Bailey, and Kandel, 2006; Davis, 2011; Steidl, Rose, and Rankin, 2003; Stough, Shobe, and Carew, 2006). The present study has advanced our understanding of savings across multiple temporal domains of memory in two ways. First, we found that the three temporal phases of savings memories in *Aplysia* (10min, 2h and 24h) do not share the same induction mechanisms: 24h, but not 2h or 10min savings memory induction was disrupted by the translational inhibitor, emetine (Fig. 3). Second, we identified that the molecular mechanisms for savings memory induction are distinct from the induction requirements for naïve animals: original ITM and LTM induction requires protein synthesis and MAPK activation (Sharma et al., 2003; Sutton et al., 2001), whereas 2h savings memory induction did not require ongoing protein synthesis or MAPK activation, and 24h savings memory induction required protein synthesis, but not MAPK.

How the molecular signaling requirements for relearning are modified is unclear. The MAPK signaling and new protein synthesis recruited during original LTM induction are widely acknowledged requirements for the establishment of long-term synaptic plasticity and the structural changes important for memory maintenance (Kelleher et al., 2004; Sweatt, 2004). A reasonable hypothesis is that these structural changes (e.g., increased synapse size or synapse number) are maintained in some stages of forgetting to remove the requirement for further induction of MAPK and protein synthesis signaling in savings memory formation. Indeed, this is consistent with the finding that long-lasting structural changes in sensory neurons are correlated with savings in the ability to subsequently induce STM in *Aplysia* (Antzoulatos et al., 2006). Continued investigation of the plasticity and structural changes that develop and remain during forgetting will help to clarify the unique mechanistic basis that underlies savings in memory formation during relearning.

Additional work is also required to identify the extent to which relearning is mechanistically distinct from original learning. While our present analysis has focused on two major signaling requirements for original intermediate-term and long-term memory formation

(MAPK and translation), it will be important to explore the relative contribution of other signaling pathways that are required for original memory induction (e.g., PKA, PKC and transcription). Intriguing questions include: In the absence of a requirement for MAPK activation, what signaling pathways are required for relearning? How do these mechanisms interact with the latent memory trace that persists from the original learning episode?

Finally, the distinct savings learning mechanisms identified in the present study are also consistent with the influence of prior experience on subsequent learning in other model systems and learning paradigms. For example, MAPK signaling is required for the acquisition of conditioned taste aversion memory in rats when trained using an unfamiliar taste (Berman, Hazvi, Rosenblum, Seger, and Dudai, 1998), but MAPK is not recruited or required for conditioned taste aversion using a familiar taste (Berman and Dudai, 2001). Similarly, although NMDAR signaling is required for original LTM formation, the subsequent learning of spatial and contextual LTMs has been shown to be insensitive to NMDAR blockers (Archbold, Bouton, and Nader, 2010; Bannerman, Good, Butcher, Ramsay, and Morris, 1995; Li and Richardson, 2013; Sanders and Fanselow, 2003; Tayler, Lowry, Tanaka, Levy, Reijmers, Mayford, and Wiltgen, 2011), and may be independent of additional original learning requirements such as *arc* (Tayler et al., 2011). NMDAR-independent learning is observed after extinction of LTM (Archbold et al., 2010), when the original LTM is still overtly expressed (Bannerman et al., 1995) and following forgetting (Li and Richardson, 2013), in agreement with the present study. Thus, prior experience in a wide variety of learning paradigms contributes to behavioral metaplasticity during subsequent learning and appears to do so through a change in the molecular signaling requirements for memory formation.

4.3 The nature of the latent memory trace

The present study has focused on the mechanisms for the induction of savings memory, that must interact with a “platform” for relearning that is provided by the maintained latent memory trace for the original experience. However, our findings have also provided some initial insights into the mechanistic nature of the platform (latent memory) itself. Savings in short-term and intermediate-term memory induction was observed during the exposure of the central nervous system to well-established inhibitors of protein synthesis (Fig. 3) and MAPK (Fig. 4). This implies that these treatments (i) did not disrupt the maintenance of the latent memory trace, or (ii) its ability to facilitate the acquisition of short- and intermediate-term savings memories. This is consistent with the notion that the latent memory is, at least in part, the maintenance of MAPK-dependent and protein synthesis-dependent long-term structural changes at the synapse and/or cellular excitability that were established during original learning. Intriguingly, the formation and maintenance of long-term plasticity and structural changes at sensory neuron synaptic varicosities have been shown to depend on the *Aplysia* CPEB prion protein (Miniaci, Kim, Puthanveetil, Si, Zhu, Kandel, and Bailey, 2008; Si, Choi, White-Grindley, Majumdar, and Kandel, 2010), which has a long half-life and would be expected to be resistant to the inhibition of new protein synthesis.

In conclusion, we have shown that, although an original LTM for sensitization is no longer behaviorally expressed, a latent memory trace persists and can facilitate subsequent memory

formation through molecular signaling that is distinct from memory formation requirements in naïve animals. These findings provide a deeper understanding of the widely observed phenomenon of savings and insight into what it means to “forget.” Our observations may also be important for the design of cognitive enhancing strategies, since the molecular signaling targets to facilitate relearning may be distinct from those required for original memory formation.

Acknowledgments

We thank Dr. Thomas Carew, Dr. Cristina Alberini and Dr. Ashley Kopec for helpful comments on an earlier version of this manuscript. This work was supported by funding from Achievement Rewards for College Scientists to GTP and NIH grant MH 094792 to Thomas J. Carew. Funding sources had no involvement in the study design, data collection or interpretation, the writing of the report, or decision to submit the article for publication.

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Highlights

- Latent memory supports savings in multiple temporal domains of memory formation
- Savings learning can be studied in a reduced behavioral preparation of *Aplysia*
- Savings learning mechanisms are distinct from original learning requirements

Figure 1. Savings during relearning in *Aplysia*

(A) Overall training and testing schedule to monitor original learning (Phase I), forgetting and relearning (Phase II). Savings is observed in the induction of three temporal phases of memory during relearning in (B) intact *Aplysia* (vertical arrows represent individual training trials, inter-trial interval [ITI]= 15 minutes). (C) Two retraining trials are necessary for 2h and 24h savings memory induction: a single retraining trial (arrow below x-axis) is sufficient for short-term (10min), but not 2h or 24h savings memory induction. (D) No differences are observed in Phase I LTM duration between conditions of daily memory tests versus testing every other day.

Figure 2. Savings during relearning can be studied in reduced behaving preparations of previously trained *Aplysia*

(A) Schematic of the reduced preparation (RP) for monitoring T-SWR behavior with the ability to manipulate the molecular environment of the central nervous system. (B) Intact behaving *Aplysia* receive Phase I training, or no training (Phase I naïve), and memory expression is monitored with daily tests (identical procedures for Phase I training/testing in Figure 1). On Day 2 of forgetting, experimental and naïve animals undergo a surgical procedure to produce the reduced preparation, and baseline T-SWR duration is re-established (RP baseline). Phase II training is administered to the reduced preparations (Phase II [RP]) and subsequent tests of the T-SWR are normalized to the RP baseline. Savings is observed in the induction of the same three temporal phases of memory that are evident following relearning in intact *Aplysia*.

Figure 3. 24h, but not 2h savings memory induction requires protein synthesis

Reduced preparations were prepared of previously trained animals on day2 of forgetting an original LTM for sensitization and were retrained during exposure of the isolated central nervous system to the irreversible inhibitor of protein synthesis, emetine (100uM) prior to and throughout retraining, as diagrammed. Tests at 10min, 2h and 24h identified that only 24h savings memory induction required new protein synthesis.

Figure 4. Savings memory induction does not require MAPK

Reduced preparations were prepared of previously trained animals on day2 of forgetting an original LTM for sensitization and were retrained during exposure of the isolated central nervous system to the active MEK inhibitor (U0126, 20uM) or its inactive analogue (U0124, 20uM), as diagrammed. No significant disruption of savings memory formation was observed in either drug condition.