A cellular model of memory reconsolidation involves reactivation-induced destabilization and restabilization at the sensorimotor synapse in Aplysia

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The memory reconsolidation hypothesis suggests that a memory trace becomes labile after retrieval and needs to be reconsolidated before it can be stabilized. However, it is unclear from earlier studies whether the same synapses involved in encoding the memory trace are those that are destabilized and restabilized after the synaptic reactivation that accompanies memory retrieval, or whether new and different synapses are recruited. To address this issue, we studied a simple nonassociative form of memory, long-term sensitization of the gill- and siphon-withdrawal reflex in Aplysia, and its cellular analog, long-term facilitation at the sensory-to-motor neuron synapse. We found that after memory retrieval, behavioral long-term sensitization in Aplysia becomes labile via ubiquitin/proteasome-dependent protein degradation and is reconsolidated by means of de novo protein synthesis. In parallel, we found that on the cellular level, long-term facilitation at the sensory-to-motor neuron synapse that mediates long-term sensitization is also destabilized by protein degradation and is restabilized by protein synthesis after synaptic reactivation, a procedure that parallels memory retrieval or retraining evident on the behavioral level. These results provide direct evidence that the same synapses that store the long-term memory trace encoded by changes in the strength of synaptic connections critical for sensitization are disrupted and reconstructed after signal retrieval.

memory reorganization | memory recall | 5-HT | local protein synthesis | clasto-lactacystin beta-lactone

The processes of memory reactivation (retrieval) have been the focus of several studies over the last decade. Retrieval is thought to return the memory to an unstable (labile) state, in which de novo protein synthesis-dependent reconsolidation is required to continue maintaining the memory over time (1–4). Memory reconsolidation has been reported for a variety of memory paradigms in a number of different animal models (1, 3, 5, 6); however, how memory reconsolidation works remains unclear.

At least two nonmutually exclusive hypotheses have been proposed (7). One hypothesis suggests that reconsolidation is an updating process in which the synapses that encode the preexisting memory are reorganized after memory retrieval so as to recruit new synaptic connections that allow the incorporation of new information (8–10). The second hypothesis suggests a mechanism that is a continuation of the consolidation process at the same set of synaptic connections and that serves to strengthen memory, allowing it to become longer lasting and enduring and thereby preventing forgetting (11). Both of these views of reconsolidation are consistent with retraining or retrieval. In each case, synaptic reactivation could be implicit (e.g., during sleep) or explicit, and both would presumably have the same effect of making the memory stronger, more stable, and more resistant to postretrieval interference.

Both types of reconsolidation hypotheses imply that the stored memory becomes labile after memory retrieval. To address how this occurs, we studied the retrieval of memories and found that they become labile via ubiquitin/proteasome-dependent synaptic protein degradation (9, 12). Moreover, Doyère et al. (13) found that inhibition of reconsolidation is correlated with reduced potentiation at reactivated synapses in the lateral amygdala. The foregoing studies suggest that signal retrieval activates protein degradation in the synaptic connections encoding the initial memory, and that protein synthesis is required for restoring or maintaining the memory. However, it remains unclear whether destabilization and restabilization after memory retrieval occur at the same synaptic connections where potentiation occurs for memory encoding (9), or whether different synaptic connections are involved in the retrieval process (8, 14).

To address this issue, we used the elementary neural circuit that underlies sensitization of the gill- and siphon-withdrawal reflex, a simple form of nonassociative learned fear in Aplysia. A critical component of this reflex that contributes significantly to this behavior is the direct monosynaptic connection from the siphon sensory neuron to gill siphon motor neurons. The sensory-to-motor neuron synapse can be reconstituted in dissociated cell culture, where it is modulated, as in the intact animal, by serotonin (5-HT), a modulatory transmitter released during sensitization training (15). Five applications of 5-HT over a period of 1.5 h—designed to simulate five shocks to the tail that produce long-term behavioral sensitization—produce both a long-term increase in the strength of the sensory-to-motor neuron synaptic connection lasting several days [long-term facilitation (LTF)] (16) and structural remodeling and growth of new sensory-to-motor neuron synapses (17–19). The consolidation of both long-term sensitization and its cellular analog, LTF at the sensory-to-motor neuron synapse, requires de novo protein synthesis (16, 20–23).


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During sensitization training, stimulating the tail activates interneurons that release 5-HT onto the mechanoreceptor sensory neurons that innervate the siphon skin (24), resulting in a strengthening of the sensory-to-motor neuron synapses that control the siphon-withdrawal reflex (SWR) (25–27). Furthermore, the molecular mechanisms that govern behavioral long-term sensitization also govern the learning-related synaptic plasticity exhibited by the sensory-to-motor neuron synapses (16, 21, 28, 29). Therefore, the long-term memory (LTM) for sensitization in Aplysia and its sensory-to-motor neuron synapses are useful tools for studying fundamental properties of synapses, such as destabilization and restabilization after memory retrieval.

Here we first examined whether reconsolidation is required for maintaining behavioral LTM after retrieval or retraining of sensitization in Aplysia. We then investigated whether synaptic disruption and reconstruction are also necessary, at the same set of synaptic connections between the sensory and motor neurons that initially stored the memory, for maintaining LTF (the cellular correlate of sensitization) after synaptic reactivation protocols that mimic retrieval or retraining of the behavioral modification in the intact animal.

**Results**

Consistent with previous results, repetitive tail shocks induced behavioral long-term sensitization in Aplysia (Fig. S1A) (21, 25, 26, 30). The duration of the SWR was increased significantly at 24 h after training; however, in the group that was injected with the protein synthesis inhibitor emetine, LTM was specifically impaired, whereas short-term memory (STM) remained intact (Fig. S1A). Two-way ANOVA with time points and drug groups as factors revealed significant effects of time points \( F_{(2,30)} = 65.73, P < 0.01 \), drug groups \( F_{(2,15)} = 4.67, P < 0.05 \), and the interaction between them \( F_{(4,30)} = 9.64, P < 0.01 \).

A growing body of evidence supports the idea that when a memory is recalled and thereby reactivated, it undergoes a labile state, and that a new, protein synthesis-dependent reconsolidation process is required to maintain that memory (1–4). To examine whether new protein synthesis is also required to maintain the behavioral LTM for sensitization in Aplysia after memory retrieval, we injected emetine immediately after retrieval of the sensitized response (Fig. S1B). We found that emetine injection immediately after the first reactivation test (retrieval by brushing the siphon) significantly impaired LTM in the second reactivation test performed 24 h later, whereas vehicle injection had no effect on the second test (Fig. S1B).

Moreover, vehicle or emetine injection in the absence of a first reactivation test did not affect LTM after the second test (Fig. S1B). Two-way ANOVA with time points (for pretest and second LTM test) and drug groups as factors revealed significant effects of time points \( F_{(1,51)} = 188.2, P < 0.01 \), drug groups \( F_{(2,51)} = 13.17, P < 0.01 \), and the interaction between them \( F_{(2,51)} = 12.49, P < 0.01 \). These results suggest that storage of the behavioral LTM for sensitization in the animal becomes labile after memory retrieval, and that de novo protein synthesis-dependent reconsolidation is required for its long-term maintenance.

Our recently reported data in mice demonstrated that a reactivated fear memory becomes labile via ubiquitin/proteasome-dependent protein degradation (9). In addition, it is well known that ubiquitin-proteasome system is critical for long-term changes, such as LTF and long-term depression in Aplysia (31–34). We thus tested whether the behavioral LTM for sensitization in Aplysia is also destabilized via ubiquitin/proteasome-dependent protein degradation after memory retrieval. To do so, we first determined the concentration of the ubiquitin/proteasome inhibitor clasto-lactacystin β-lactone (βlac) needed to examine the effect of ubiquitin/proteasome inhibitor βlac on the LTM for sensitization of the SWR in Aplysia. We found that 0.5 μM βlac did not affect the duration of behavioral sensitization, whereas higher concentrations (≥1.0 μM) increased this duration (25). Consistent with previous results regarding fear memory (9), we found that inhibiting ubiquitin/proteasome with the injection of 0.5 μM βlac after the first reactivation test (retrieval by brushing the siphon) prevented the memory disruption induced by emetine injection (Fig. 1). βlac injection alone after the first reactivation test did not affect long-term sensitization (Fig. 1). Two-way ANOVA with time points (for pretest and second LTM test) and drug groups as factors revealed significant effects of time points \( F_{(1,40)} = 296.9, P < 0.01 \), drug groups \( F_{(3,40)} = 13.08, P < 0.01 \), and the interaction between them \( F_{(3,40)} = 10.68, P < 0.01 \). In addition, two-way ANOVA with the application of emetine and βlac as factors at the second LTM test showed a significant effect of the interaction between the two factors \( F_{(1,40)} = 11.24, P < 0.01 \). These results indicate that inhibition of ubiquitin/proteasome prevents LTM from becoming labile after retrieval, and that ubiquitin/proteasome-dependent protein degradation is critical for retrieval-dependent disruption of long-term sensitization in Aplysia.

We also observed similar results when weak behavioral training (retraining, involving two electrical shocks to the tail) was applied as a reactivation signal instead of the first reactivation test (retrieval by brushing the siphon) used in the previous experiments. Emetine injection immediately after retraining significantly impaired post-retraining (PR)-LTM 24 h later, whereas vehicle or βlac injection alone injection had no effect (Fig. 2). In addition, emetine or vehicle injection without retraining did not impair storage of the PR-LTM trace. Moreover, βlac injection prevented the impairment of PR-LTM storage induced by emetine injection (Fig. 2).

![Fig. 1. Protein degradation-dependent destabilization of LTM for behavioral sensitization.](Image)

## Fig. 1. Protein degradation-dependent destabilization of LTM for behavioral sensitization. (Upper) Schematic of the experimental procedure used to evaluate the effect of βlac on LTM after retrieval. (Lower) Bar graph showing mean ± SEM of SWR duration on the pretest, first LTM test, and second LTM test. Compared with the vehicle-injected group (veh; \( n = 10 \)), βlac injection alone after the first LTM test (βlac; \( n = 11 \)) had no effect on LTM on the second LTM test, whereas the emetine-injected group (emetine; \( n = 13 \)) exhibited impaired LTM on the second LTM test. However, concurrent injection of βlac with emetine (emetine + βlac; \( n = 10 \)) prevented impairment of LTM on the second LTM test induced by emetine injection \( F_{(1,40)} = 13.28, P < 0.01 \), one-way ANOVA. **P < 0.01, Newman–Keuls multiple-comparison test.**
We next asked whether the same sensory-to-motor neuron synapse that was facilitated by five pulses of 5-HT also undergoes destabilization and restabilization after synaptic reactivation. To address this question, we first measured the basal strength of the sensory-to-motor neuron synapse (first recording), and then induced LTF with five pulses of 5-HT. At 24 h after the 5-HT treatment, we retested synaptic strength (second recording) and found that it was significantly greater than the basal level (all groups, $P < 0.01$, one-sample $t$ test compared with basal level of 0).

After the second recording, we applied homosynaptic activation (HA) (21) as a reactivation (retrieval) signal by generating four action potentials (at 1-min intervals) in the sensory neuron of the sensory-to-motor neuron synapse. For the third recording (at 24 h after the second recording), the facilitated synaptic strength of the reactivated group (HA + vehicle group) was maintained and was significantly greater than the basal level ($P < 0.01$, one-sample $t$ test compared with basal level), although the synaptic strength at the third recording showed a tendency toward a decrease compared with the strength at the second recording (Fig. 3A). However, combining HA with emetine after the second recording (HA + emetine group) disrupted the strength of the facilitated synapse, causing a reversion to its basal strength at the third recording ($P > 0.43$, one-sample $t$ test compared with basal level) (Fig. 3A).

Vehicle application without HA (vehicle group) or emetine treatment without HA (emetine group) at the second recording did not disrupt the facilitated synaptic strength, and so the synaptic strength was significantly different from the basal level ($P < 0.05$, one-sample $t$ test compared with basal level) (Fig. S2). The emetine-only group, in which only emetine was applied without the second recording at 24 h after the first recording, also maintained synaptic strength at the third recording (Fig. S2). Although the group to which emetine was applied 24 h after the first recording exhibited some reduction in synaptic strength, this reduction was not statistically significantly different from that in either the vehicle or emetine-only group. These results suggest that LTF at the sensory-to-motor neuron synapse reactivated by HA undergoes a reconsolidation phase after synaptic reactivation.

To determine whether ubiquitin/proteasome-dependent synaptic destabilization and protein synthesis-dependent synaptic restabilization occur at the same sensory-to-motor neuron synapse, we applied 0.1 M β-lac, which has no effect on basal synaptic strength or on consolidation of LTF, to the reactivated synapse using HA (Fig. 3 and Fig. S3). Consistent with the behavioral results, β-lac treatment prevented the disruption of LTF induced by emetine (HA + β-lac + emetine), whereas β-lac treatment alone (HA + β-lac) had no effect (Fig. 3A). One-way ANOVA with HA groups at the third recording revealed a significant effect of drug treatment, and application of the Newman–Keuls multiple-comparison post hoc test showed significantly lower synaptic strength in the HA + emetine group at the third recording compared with the HA + vehicle, HA + β-lac, HA + β-lac + emetine, and HA + β-lac + groups (Fig. 3A). Synaptic strength in the HA + β-lac + emetine and HA + β-lac groups was not significantly different from that in the HA + vehicle group at the third recording. Two-way ANOVA with time points (for the first and the third recordings) and drug groups as factors revealed significant effects of time points [$F_{(1,31)} = 40.96$, $P < 0.01$], drug groups [$F_{(3,31)} = 4.38$, $P < 0.05$], and the interaction between them [$F_{(3,31)} = 4.38$, $P < 0.05$].

These results suggest that consolidated LTF at the sensory-to-motor neuron synapse is destabilized via ubiquitin/proteasome-dependent protein degradation after synaptic reactivation (retrieval), and that synaptic destabilization and restabilization after synaptic reactivation occur at the same synaptic connections. Possible cellular signaling cascades, such as alterations in intracellular Ca$^{2+}$ levels, that may be recruited by HA of sensory neurons also might be involved in synapse destabilization.

Because reactivation of the behavioral LTM for sensitization by retraining (two electrical shocks to the tail) also demonstrated labile and reconsolidation phases similar to reactivation of LTM by retrieval (brushing the siphon), we investigated another reactivation method in sensory-to-motor neuron cocultures. We retreated the synaptic connection with five pulses of 5-HT to mimic the retraining protocol of electrical shocks used in the behavioral experiments. We found that five pulses of 5-HT increased the synaptic strength of sensory-to-motor neuron synapses at the second recording (Fig. 4). After the second recording 24 h later, we again applied five pulses of 5-HT as a reactivation (retraining) signal, then retested synaptic strength. Emetine treatment during and after 5-HT retrieval (5 × 5-HT + emetine) significantly disrupted the facilitation of the synapse, whereas retrieval with vehicle and 5-HT (5 × 5-HT + vehicle) did not affect maintenance of synaptic strength (Fig. 4). Moreover, β-lac treatment combined with emetine (5 × 5-HT + β-lac + emetine group) prevented emetine-induced disruption of LTF; whereas β-lac treatment (5 × 5-HT + β-lac) alone had no effect (Fig. 4). Results for the 5 × 5-HT + β-lac + emetine group and the 5 × 5-HT + β-lac group were not significantly different from those for the 5 × 5-HT + vehicle group at the third recording. Two-way ANOVA with time points (for the first and the third recordings) and drug groups as factors revealed significant effects of time points [$F_{(1,47)} = 21.98$, $P < 0.01$], drug groups [$F_{(5,47)} = 4.85$, $P < 0.01$], and the interaction between them [$F_{(5,47)} = 4.85$, $P < 0.01$]. Although we cannot exclude the possibility that a single stimulation of the sensory neuron used to evoke
the excitatory postsynaptic potential (EPSP) during the second recording provides sufficient activity to account for a partial effect of emetine (Fig. S2), our results do suggest that emetine has a stronger effect when applied in association with a multiple stimulation paradigm (HA) or 5-HT retraining that likely mimics stronger retrieval stimuli. Thus, once reactivated, LTF at the sensory-to-motor neuron synapse undergoes labile and reconsolidation phases.

Taken together, our data suggest that at both the cellular and behavioral levels, LTM becomes labile after retrieval or retraining, and that de novo protein synthesis-dependent reconsolidation is required to maintain memory storage both in the reflex of the animal and in the underlying synaptic mechanism in the neural circuit of the reflex.

A previous study found that the persistence of LTF in culture still requires local protein synthesis at the sensory-to-motor neuron synapse at 24 h and 48 h after treatment with five pulses of 5-HT, suggesting that the time window for consolidation is a natural consequence of long-term training (35). Consequently, we tested whether de novo protein synthesis-dependent reconsolidation is also required for maintaining LTF at 72 h after the synaptic training—a time point at which inhibitors of local protein synthesis no longer disrupt the 5-HT–induced newly formed sensory neuron varicosities or LTF. The facilitated synapse was reactivated by five pulses of 5-HT at 72 h (retraining) after the first 5-HT treatment (training), and the strength was retested 48 h later (120 h after the training; Fig. S4), because the effect of emetine on both LTF and growth is more pronounced at this time point than at 24 h after its application. FMRFamide-induced depression (34). The ubiquitin/proteasome system is known to have a critical role in the consolidation of long-term sensitization in Aplysia (32, 36) and FMRFamide-induced depression (34). The ubiquitin/proteasome system is known to have a critical role in the consolidation of long-term sensitization in Aplysia (32, 36) and FMRFamide-induced depression (34). The ubiquitin/proteasome system is known to have a critical role in the consolidation of long-term sensitization in Aplysia (32, 36) and FMRFamide-induced depression (34). The ubiquitin/proteasome system is known to have a critical role in the consolidation of long-term sensitization in Aplysia (32, 36) and FMRFamide-induced depression (34). The ubiquitin/proteasome system is known to have a critical role in the consolidation of long-term sensitization in Aplysia (32, 36) and FMRFamide-induced depression (34). The ubiquitin/proteasome system is known to have a critical role in the consolidation of long-term sensitization in Aplysia (32, 36) and FMRFamide-induced depression (34). The ubiquitin/proteasome system is known to have a critical role in the consolidation of long-term sensitization in Aplysia (32, 36) and FMRFamide-induced depression (34). The ubiquitin/proteasome system is known to have a critical role in the consolidation of long-term sensitization in Aplysia (32, 36) and FMRFamide-induced depression (34). The ubiquitin/proteasome system is known to have a critical role in the consolidation of long-term sensitization in Aplysia (32, 36) and FMRFamide-induced depression (34). The ubiquitin/proteasome system is known to have a critical role in the consolidation of long-term sensitization in Aplysia (32, 36) and FMRFamide-induced depression (34). The ubiquitin/proteasome system is known to have a critical role in the consolidation of long-term sensitization in Aplysia (32, 36) and FMRFamide-induced depression (34). The ubiquitin/proteasome system is known to have a critical role in the consolidation of long-term sensitization in Aplysia (32, 36) and FMRFamide-induced depression (34). The ubiquitin/proteasome system is known to have a critical role in the consolidation of long-term sensitization in Aplysia (32, 36) and FMRFamide-induced depression (34). The ubiquitin/proteasome system is known to have a critical role in the consolidation of long-term sensitization in Aplysia (32, 36) and FMRFamide-induced depression (34). The ubiquitin/proteasome system is known to have a critical role in the consolidation of long-term sensitization in Aplysia (32, 36) and FMRFamide-induced depression (34). The ubiquitin/proteasome system is known to have a critical role in the consolidation of long-term sensitization in Aplysia (32, 36) and FMRFamide-induced depression (34). The ubiquitin/proteasome system is known to have a critical role in the consolidation of long-term sensitization in Aplysia (32, 36) and FMRFamide-induced depression (34). The ubiquitin/proteasome system is known to have a critical role in the consolidation of long-term sensitization in Aplysia (32, 36) and FMRFamide-induced depression (34). The ubiquitin/proteasome system is known to have a critical role in the consolidation of long-term sensitization in Aplysia (32, 36) and FMRFamide-induced depression (34). The ubiquitin/proteasome system is known to have a critical role in the consolidation of long-term sensitization in Aplysia (32, 36) and FMRFamide-induced depression (34).
system also functions as an inhibitory constraint on synaptic strength and growth in a translation-dependent, but not a transcription-dependent, manner (37). Indeed, consistent with results of Zhao et al. (37), we also observed an increase of both basal synaptic transmission at sensory-to-motor neuron synapses in culture and reactivation with 5-HT treatment. (Upper) Schematic of the experimental procedure used to evaluate the effect of emetine on LTF after synaptic reactivation with 5-HT treatment. (Lower) Bar graphs showing mean percentage change ± SEM in EPSP amplitudes. Lower, Left: On the second recording, the changes in EPSP amplitudes were significantly different from the basal level recorded at the first recording, and were similar across groups. Lower, Right: Concurrent application of emetine with five pulses of 5-HT (S × 5-HT + emetine; n = 12) after the second recording impaired LTF at the third recording, whereas 5-HT (S × 5-HT + veh; n = 12) or β-lac treatment (S × 5-HT + β-lac; n = 14) alone after the second recording had no effect on LTF. β-lac treatment combined with emetine (S × 5-HT + β-lac + emetine; n = 13) prevented LTF impairment on the third recording. *P < 0.05, **P < 0.01, one-sample t test compared with basal level [F(0.47) = 4.85, P < 0.01; one-way ANOVA]. *P < 0.05, **P < 0.01, Newman-Keuls multiple-comparison test).

Methods

Aplysia Behavior Task. In all experiments, behavioral results were videotaped, and the duration of the SWR was measured by a blinded observer. Before the sensitization training, SWR duration was measured by briefly (~1 s) brushing the siphon (pretest). At 35 min after the pretest, 10 electrical tail shocks (100 mA for the training and retraining used in Fig. 2, and 50 mA for other experiments, 60 Hz AC, for 1.5 s, with a 10-min interval between shocks) were given to the freely moving Aplysia (training). For retraining, two electrical shocks (100 mA for 1.5 s, with 10-min interval between shocks) were used. SWR duration was measured again at 1 h (STM test), 24 h (LTM or first LTM test), or 48 h (second LTM test or post-retraining [PR-LTM] after the training).

Electrophysiology. At 4 d after sensory-to-motor coculture, the first EPSP was evoked in an LFS motor cell by stimulating the sensory neurons with a brief depolarizing stimulus using an extracellular electrode (first recording). During EPSP measurement, the motor cell was impaled intracellularly with a glass microelectrode filled with 2 M K-acetate, 0.5 M KCl, and 10 mM K-Hepes (10–15 MΩ), and the membrane potential was held at ~40 mV below its resting value. Then the sensory-to-motor neuron synapses received five pulses of 5-HT (10 μM, 5 min/pulse, with a 15-min interval between pulses) to induce LTF. To reactivate the facilitated synapse, HA or five pulses of 5-HT treatment was performed. HA represents activation induced by four action potentials in the sensory neuron of the sensory-to-motor synapse with a 1-min interval. We used this multiple recording paradigm to mimic stronger retrieval stimuli. To avoid providing long-term potentiation (39) or long-term depression (40) stimuli to sensory cells, a 1-min interval was applied. When the synaptic facilitation was examined at 120 h after the training (Fig. 53), half of the synapses were also tested at 24 h after the training to confirm the successful initiation of LTF; these synapses were no different from the other synapses at later testing time points (Fig. 55). To examine the effect of protein synthesis inhibition or proteasome inhibition after the reactivation, we applied emetine (100 μM; Sigma-Aldrich) or β-lac (0.1 μM; Calbiochem) in the bath for 2.5 h immediately after the second recording. Bath application affects both the cell body and synapses simultaneously, which can serve to mimic our behavioral experimental conditions (i.p. injection). To examine the effect of proteasome inhibition on basal synaptic transmission, we applied β-lac (0.1 μM) or vehicle (0.1% DMSO) for 2.5 h immediately after the first recording, and performed the second recording 24 h later. When emetine or β-lac was applied with 5-HT treatment, the inhibitor (emetine or β-lac) was bath-applied at 30 min before the first application of 5-HT and remained in the bath throughout the 5-HT treatment and for 30 min after the 5-HT treatment. For the reactivation experiments, we excluded sensory-to-motor neuron synapses showing less than a 20% increase in synaptic strength on the second recording compared with the first recording.

Information on general procedures and drug application for the behavioral experiments, cell culture, and statistical analysis is provided in SI Methods.
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