Activity-Dependent Presynaptic Facilitation and Hebbian LTP Are Both Required and Interact during Classical Conditioning in *Aplysia*

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Summary

Using a simplified preparation of the *Aplysia* siphon-withdrawal reflex, we previously found that associative plasticity at synapses between sensory neurons and motor neurons contributes importantly to classical conditioning of the reflex. We have now tested the roles in that plasticity of two associative cellular mechanisms: activity-dependent enhancement of presynaptic facilitation and postsynaptically induced long-term potentiation. By perturbing molecular signaling pathways in individual neurons, we have provided the most direct evidence to date that each of these mechanisms contributes to behavioral learning. In addition, our results suggest that the two mechanisms are not independent but rather interact through retrograde signaling.

Introduction

Testing the relationship between mechanisms of synaptic plasticity and learning has been extremely difficult because of the immense complexity of the mammalian brain. For this reason, invertebrate preparations such as *Aplysia* have proven advantageous (reviewed in Carew and Sahley, 1986; Byrne, 1987; Hawkins et al., 1993). The *Aplysia* gill- and siphon-withdrawal reflex undergoes classical conditioning, with many of the behavioral features of conditioning in mammals, including stimulus, response, and temporal specificity, effects of contingency and context, and second-order conditioning, suggesting that conditioning in *Aplysia* and mammals may share common mechanisms (Carew et al., 1981, 1983; Hawkins et al., 1986, 1989, 1998b; Colwill et al., 1988a, 1988b; Walters, 1989). Furthermore, monosynaptic connections between sensory neurons and motor neurons that contribute to the withdrawal reflex exhibit a cellular analog of conditioning with temporal parameters similar to the behavioral conditioning (Hawkins et al., 1983; Walters and Byrne, 1983; Carew et al., 1984; Clark et al., 1994; Murphy and Glanzman, 1996, 1997, 1999). However, these studies of synaptic changes in the isolated nervous system have not been able to address the contribution of plasticity to behavior.

For this reason, we recently developed a simplified preparation for studying the siphon-withdrawal reflex of

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Aplysia, with which it is relatively easy to record the activity of identified neurons and their synaptic connections simultaneously with behavioral learning (Antonov et al., 1999) (Figure 1A). The neural circuit mediating the withdrawal reflex in the simplified preparation has been well characterized (Figure 7A). Monosynaptic connections from LE siphon sensory neurons (Byrne et al., 1974) to LFS siphon motor neurons (Frost and Kandel, 1995) have been estimated to mediate approximately one-third of the reflex response (Antonov et al., 1999). The remainder of the response is mediated by peripheral motor neurons (Perlman, 1979), which also receive monosynaptic input from the LE neurons (Bailey et al., 1979), other unidentified sensory neurons (Frost et al., 1997), and polysynaptic inputs onto the LFS neurons from excitatory and inhibitory interneurons (Frost and Kandel, 1995).

The simplified preparation undergoes classical conditioning that is similar parametrically to conditioning in intact animals (Carew et al., 1981; Antonov et al., 2001), although we do not yet know whether all the other features of conditioning are also similar. During conditioning, synapses from the LE sensory neurons to LFS motor neurons undergo plasticity that is both activity dependent and associative; that is, the excitatory postsynaptic potential (EPSP) is selectively enhanced at synapses from sensory neurons that fire action potentials during the conditioned stimulus (on-field versus off-field) just before the unconditioned stimulus (paired versus unpaired) (Antonov et al., 2001). We have now investigated how the plasticity is generated and to what extent individual cells contribute. More specifically, we have examined the possible roles of two activity-dependent associative cellular mechanisms: enhancement of presynaptic facilitation and Hebbian long-term potentiation (LTP). Activity-dependent enhancement of presynaptic facilitation occurs when presynaptic spike activity is temporally paired with the activity of facilitatory interneurons that stimulate adenylyl cyclase and cAMP-dependent protein kinase (PKA) in the presynaptic neuron (Hawkins et al., 1983, 1993; Walters and Byrne, 1983) (Figure 7B). Spike activity in the presynaptic neuron approximately 0.5 s before the facilitation causes an influx of Ca^{2+} that "primes" the cyclase, leading to enhanced activation of the PKA pathway (Kandel et al., 1983; Ocorr et al., 1985; Abrams et al., 1998). Hebbian long-term potentiation occurs when presynaptic spike activity is temporally paired with depolarization of the postsynaptic neuron (Wigstrom et al., 1986; Kelso et al., 1986). The depolarization is thought to act in part by relieving the Mg^{2+} block of N-methyl-D-aspartate (NMDA)-type glutamate receptor channels in the postsynaptic neuron, leading to enhanced Ca^{2+} influx (Bliss and Collingridge, 1993). Both mechanisms can occur at *Aplysia* sensory neuron-motor neuron synapses in vitro (Lin and Glanzman, 1994a, 1994b, 1997; Eliot et al., 1994; Bao et al., 1997, 1998; Schacher et al., 1997), and both could in principle contribute to behavioral conditioning, because the conditioned stimulus (CS) causes firing of LE sensory neurons and the unconditioned stimulus (US) causes

firing of both facilitatory interneurons (Hawkins and Schacher, 1989; Mackey et al., 1989) and the LFS motor neurons. By perturbing molecular signaling pathways in individual neurons, we find that each of these mechanisms is required for synaptic plasticity and behavioral conditioning, providing what we believe is the strongest evidence to date that either mechanism contributes to learning. In addition, our results suggest that the two mechanisms are not independent but rather interact through retrograde signaling, providing new evidence that pre- and postsynaptic plasticity may occur in a coordinated fashion during learning.

Results

Conditioning Involves Both Presynaptic PKA and Postsynaptic Ca^{2+}

As a first approach to distinguishing between possible cellular mechanisms of conditioning, we examined behavior while bathing the abdominal ganglion in either normal seawater (control), the PKA inhibitor KT5720 (2 μM), which blocks activity-dependent presynaptic facilitation at sensory neuron-motor neuron synapses (I. Jin and R.D. Hawkins, 2000, Soc. Neurosci., abstract), or the NMDA receptor antagonist DL-2-amino-5-phosphonovaleric acid (APV, 100 μM), which blocks long-term potentiation (Lin and Glanzman, 1994a). In each case, we compared changes in the withdrawal reflex in two groups that received either paired or unpaired training with a siphon tap CS and tail shock US (Figure 1B). In the control group, paired training produced a significant increase in the response to the CS compared to either the pretest or unpaired training (Figures 1C and 1D). In previous experiments, paired training also produced a greater increase in the withdrawal response than did training with either the CS alone or the US alone (Antonov et al., 2001). These results demonstrate classical conditioning of the siphon-withdrawal reflex in the simplified preparation.

KT5720 selectively reduced the response of the paired group to approximately the level of the unpaired group on each test, and APV had a similar effect on the last two of the four tests (Figure 1D). Surprisingly, APV also increased the response of the unpaired group to approximately the level of the paired group on the first test. Because APV produced a similar increase during training with the US alone (data not shown), this result could be due to a transient enhancement of US effectiveness that might be caused, for example, by blocking competing inhibitory effects of the US (Mackey et al., 1987). However, by the final post-test, KT5720 and APV each blocked the conditioning, suggesting that both cellular mechanisms may contribute.

Although KT5720 and APV were restricted to the abdominal ganglion, they might act at sites other than the LE sensory neurons and LFS motor neurons. In particular, it is not clear to what extent the synapses between those neurons have NMDA-like glutamate receptors. The sensory neuron-motor neuron PSPs are thought to be glutamatergic (Dale and Kandel, 1993; Trudeau and Castellucci, 1993) and can be mimicked by exogenous application of L-glutamate (Figure 2A). Studies on neurons in culture suggest that the PSPs have NMDA-like properties (Dale and Kandel, 1993), with an early non-

NMDA component and a late NMDA component like many vertebrate glutamatergic PSPs (Glanzman, 1994; Conrad et al., 1999). To test whether that is also true in vivo, we examined the effects of the NMDA antagonist APV (200 uM) and the non-NMDA antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 50 uM) and 6,7-dinitroquinoxaline-2,3-dione (DNQX, 50 uM) on the peak and late (50–75 ms after the peak) parts of the LE-LFS PSP under the same conditions as our behavioral experiments (Figures 2B–2D). APV significantly decreased the late part of the PSP ($F[1,24] = 53.00$; $p < 0.01$) but not the peak, whereas CNQX significantly decreased the peak of the PSP ($F[1,24] = 9.18$; $p < 0.01$) but not the late part. DNQX produced a significant decrease in both parts ($p < 0.01$ in each case), indicating that it is a more potent and perhaps less selective antagonist at *Aplysia* synapses. These results are consistent with the idea that the sensory neuron-motor neuron PSPs have an NMDA component in vivo, although it is not yet known to what extent that component has voltage and Mg^{2+} dependencies similar to those of vertebrate NMDA receptors. In addition, these results indicate that the sensory neuron-motor neuron PSPs are a possible site of action of APV in the experiments shown in Figure 1.

To examine the role and mechanisms of plasticity at the sensory neuron-motor neuron synapses more directly, we performed experiments similar to those shown in Figure 1 in which we either injected LE sensory neurons with a peptide inhibitor of PKA (PKAi) that blocks activity-dependent presynaptic facilitation (Bao et al., 1998) or we injected LFS motor neurons with the Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), which blocks Hebbian long-term potentiation (Lin and Glanzman, 1994a; Bao et al., 1997). In the interleaved control experiments, we injected LE or LFS neurons with vehicle. Because the response measured in this preparation is thought to be mediated by approximately five to eight LE sensory neurons (Byrne et al., 1974; Hickie et al., 1997) and two to three LFS motor neurons (Antonov et al., 1999), with the neurons in each class acting in parallel, blocking plasticity in a single neuron would not be expected to block behavioral conditioning. Therefore, we injected either three to six LE sensory neurons with PKAi or two to three LFS motor neurons with BAPTA and examined behavioral conditioning (Figure 3). All of the neurons that were injected in these experiments were first shown to contribute to mediating the conditioned responses; that is, the LE sensory neurons were activated by the siphon tap CS (Figure 3A2), and stimulation of the LFS motor neurons produced measurable siphon withdrawal (Figure 3B2). Both types of injections reduced conditioning of siphon withdrawal, particularly for the latter test trials, as evidenced by a significant injection × pairing interaction on the final post-test ($F[2, 42] = 3.95$; $p < 0.05$). Injecting several LE neurons with PKAi significantly reduced the effect of pairing, suggesting the involvement of activity-dependent presynaptic facilitation. In addition, injecting several LFS neurons with BAPTA also significantly reduced the effect of pairing, supporting the involvement of long-term potentiation. These results indicate that both mechanisms are required for behavioral conditioning of the siphon-withdrawal reflex.
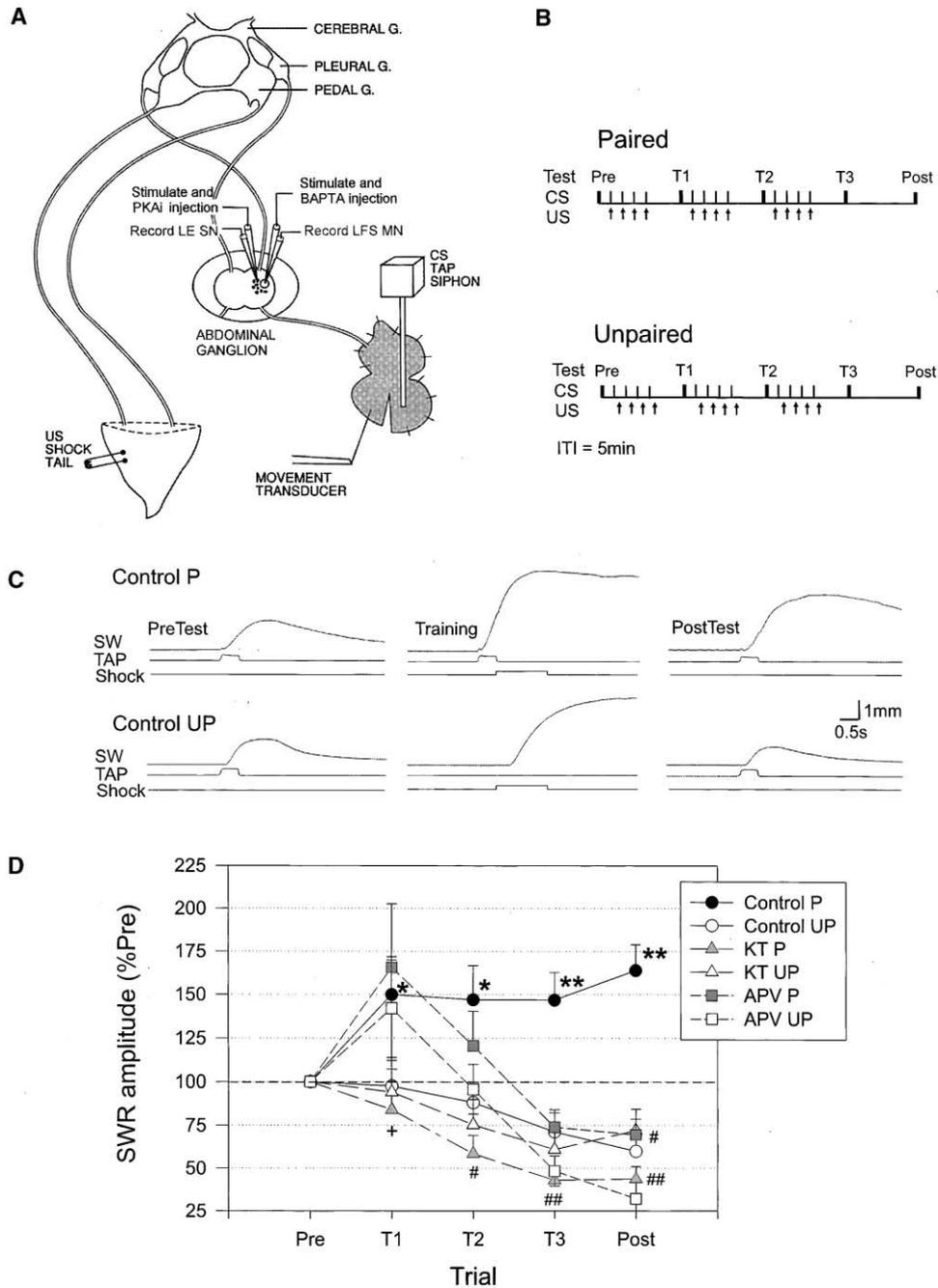
Figure 1. Pharmacological Studies of Classical Conditioning of the *Aplysia* Siphon-Withdrawal Reflex in the Simplified Preparation

(A) Experimental preparation.

(B) Training protocols. There were three blocks of four training trials, with either paired or unpaired stimulation with a siphon tap CS and tail shock US on each trial. The intertrial interval (ITI) was 5 min. The response to the CS was tested before training (Pre), 15 min after each block (T1–T3), and again 45 min after the last block (Post). See Experimental Procedures for details.

(C) Examples of the siphon withdrawal produced by the CS on the pretest and final post-test after either paired (P) or unpaired (UP) training with the abdominal ganglion bathed in normal seawater (control).

(D) Average siphon withdrawal on each test in groups that received paired or unpaired training with the abdominal ganglion bathed in either normal seawater, the PKA inhibitor KT5720, or the NMDA receptor antagonist APV (n = 15 per group). There were significant overall effects of pairing ($F_{[1,84]}$ = 10.08; $p < 0.01$), drug ($F_{[2,84]}$ = 10.78; $p < 0.001$), and the drug × pairing interaction ($F_{[2,84]}$ = 9.23; $p < 0.001$). Planned comparisons showed that paired training produced a greater increase in the amplitude of siphon withdrawal than unpaired training on each test in the control group and that this effect was blocked on the post-test by either KT5720 or APV. Responses have been normalized to the average values on the pretest, which were 2.8 mm (Con, P), 2.9 mm (Con, UP), 3.8 mm (KT, P), 2.7 mm (KT, UP), 3.2 mm (APV, P), and 2.6 mm (APV, UP), not significantly different by a one-way ANOVA. The average response to the first tail shock US was 7.6, 6.7, 8.1, 5.8, 7.6, and 7.3 mm, not significantly different. In this and subsequent conditioning figures, $**p < 0.01$, $*p < 0.05$ for the difference between the paired and unpaired groups, and $\#\#p < 0.01$, $\#p < 0.05$, $+p < 0.05$ one-tail for the reduction of that difference by each drug (the drug × pairing interaction) in planned comparisons at each test.
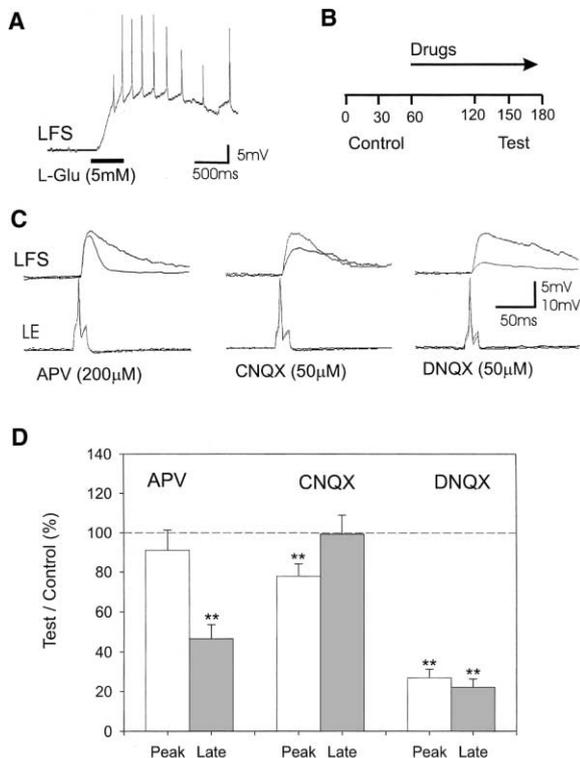
Figure 2. The Sensory Neuron-Motor Neuron PSPs Have an APV-Sensitive Component

(A) Local application of L-glutamate mimics the sensory neuron-motor neuron PSP. L-glutamate was applied by pressure ejection from a pipette positioned close to the LFS cell body.

(B) Protocol for examining the effects of glutamate antagonists on the PSP. The monosynaptic PSP from an LE sensory neuron to an LFS motor neuron was tested three times at 30 min intervals (control) followed by a 60 min rest, and then the PSP was tested three more times (test). The ganglion was perfused with normal seawater containing APV, CNQX, or DNQX from the start of the rest period to the end of the experiment. This protocol minimizes homosynaptic depression of the PSP in normal seawater (data not shown).

(C) Superimposed examples of the LE-LFS PSP during the control period and in the presence of APV, CNQX, or DNQX.

(D) Average change in the peak and late (50–75 ms after the peak) parts of the PSP in experiments like those shown in (C) (n = 5 per group). There was a significant drug × peak/late interaction (F [2,12] = 20.82; p < 0.01). The average of the three test values has been normalized to the average of the three control values for each neuron. The average control values were 8.1mV (APV, peak), 9.7mV (CNQX, peak), and 10.6mV (DNXQ, peak), not significantly different by a one-way ANOVA, and 4.6mV (APV, late), 4.9mV (CNQX, late), and 5.7mV (DNQX, late), not significantly different. **p < 0.01 in planned comparisons of the change in each measure from the control level.

Sites of Cellular and Synaptic Plasticity Contributing to Conditioning

To analyze the cellular mechanisms contributing to conditioning in more detail, we recorded intracellularly from an identified LFS siphon motor neuron and an LE siphon sensory neuron simultaneously with the behavior. On each test trial, we recorded siphon withdrawal, evoked firing of the LFS motor neuron and LE sensory neuron, the membrane resistance of each neuron, and the monosynaptic EPSP produced in the LFS neuron by direct stimulation of the LE neuron. In addition, we either in-

jected the LE sensory neuron with PKAi or we injected the LFS motor neuron with BAPTA. In the interleaved control experiments, we injected a LE or LFS neuron with vehicle.

The control experiments (Figure 4) identified sites of cellular and synaptic plasticity contributing to the conditioning, confirming some of the major findings of Antonov et al. (2001). Briefly, paired training produced a significantly greater increase in siphon withdrawal than unpaired training (F[1,46] = 21.36; p < 0.001), replicating behavioral conditioning. Evoked LFS firing changed roughly in parallel with the changes in siphon withdrawal, with paired training producing a significantly greater enhancement of firing than unpaired training (F[1,46] = 25.66; p < 0.001). Moreover, the increase in evoked LFS firing correlated significantly overall with the increase in siphon withdrawal (r = 0.694; p < 0.001), and pairing did not have any significant additional effect on withdrawal when this correlation was factored out in an analysis of covariance. These results suggest that pairing-specific changes in evoked firing of the LFS motor neurons make an important contribution to conditioning of the siphon-withdrawal reflex in this preparation.

There was no significant change in the membrane resistance of the LFS motor neuron, suggesting that the changes in evoked firing of the LFS neuron during conditioning are probably due to changes in the synaptic input to those neurons, including input from the LE neurons. The siphon tap was positioned within the receptive fields of the LE sensory neurons that were recorded in these experiments so that they all fired and contributed to firing of the LFS motor neuron and to siphon withdrawal. Evoked firing of the LE neurons increased following either paired or unpaired training, but increased more following paired training (F[1,46] = 4.87; p < 0.05). Furthermore, the increase in LE firing correlated significantly overall with the increases in siphon withdrawal (r = 0.427; p < 0.01) and LFS firing (r = 0.440; p < 0.01), but pairing still had highly significant additional effects when these correlations were factored out in analysis of covariance. These results suggest that the increase in evoked firing of the LE sensory neurons contributes to the increases in firing of the LFS motor neurons and siphon withdrawal, but that other mechanisms also contribute.

One other mechanism that could contribute to an increase in withdrawal is an increase in the strength of the synaptic connections from the sensory neurons to the motor neurons. To investigate this possibility, we examined the amplitude of the unitary monosynaptic EPSP from the LE sensory neuron to the LFS motor neuron and found that it changed roughly in parallel with the changes in siphon withdrawal and evoked firing of the LFS neurons during conditioning, with paired training producing significantly greater facilitation than unpaired training (F[1,46] = 11.37; p < 0.01). Furthermore, the increase in the amplitude of the EPSP correlated significantly overall with the increases in siphon withdrawal (r = 0.355; p <0.01) and evoked LFS firing (r = 0.420; p < 0.01). When we examined the area of the EPSP, we obtained similar results (data not shown). In other experiments (Antonov et al., 2001), we found that the pairing-specific increase in monosynaptic PSPs is restricted to LE neurons that fire during the siphon tap
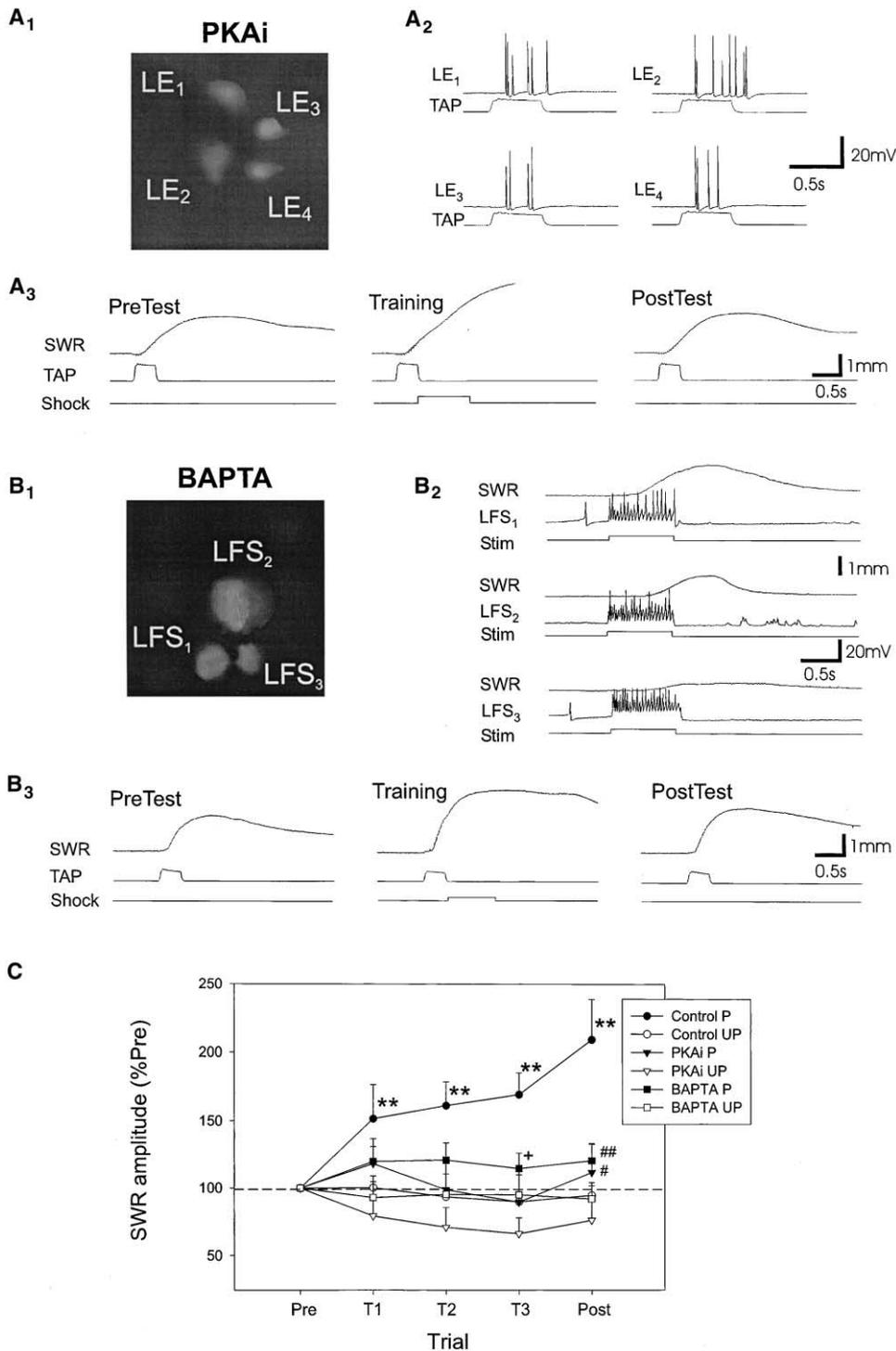
Figure 3. Contributions of Presynaptic PKA and Postsynaptic Ca²⁺ to Behavioral Conditioning

(A) Example of the block of conditioning by injecting PKAi into four LE sensory neurons. (A1) Fluorescence image taken at the end of the experiment showing that the four neurons had been successfully injected. (A2) Response of each of the neurons to the siphon tap CS. (A3) Siphon withdrawal produced by the tap before (pretest) and after (posttest) paired training.

(B) Example of the block of conditioning by injecting BAPTA into three LFS motor neurons. (B1) Fluorescence image showing that the three neurons had been successfully injected. (B2) Siphon withdrawal produced by intracellular stimulation of each of the neurons. (B3) Siphon withdrawal produced by the tap before and after paired training.

(C) Average siphon withdrawal on each test in groups that received either paired or unpaired training following injection of either vehicle (control), PKAi, or BAPTA. Behavioral conditioning was blocked by injecting either 3–6 LE neurons with PKAi or 2–3 LFS neurons with BAPTA. The average amplitude of siphon withdrawal on the pretest was 1.8 mm (con, P; n = 6), 2.0 mm (con, UP; n = 6), 2.0 mm (PKAi, P; n = 8), 2.0 mm (PKAi, UP; n = 8), 2.1 mm (BAPTA, P; n = 10), and 2.5 mm (BAPTA, UP; n = 10), not significantly different by a one-way ANOVA. The average response to the first tail shock US was 4.3, 4.6, 4.0, 4.0, 4.4, and 4.1 mm, not significantly different. The average number of LE cells injected was 4.3 (con, P; n = 3), 3.7 (con, UP; n = 3), 4.0 (PKAi, P), and 3.9 (PKAi, UP), not significantly different. The average number of LFS cells injected was 2.0 (con, P; n = 3), 2.0 (con, UP; n = 3), 2.3 (BAPTA, P), and 2.2 (BAPTA, UP), not significantly different.
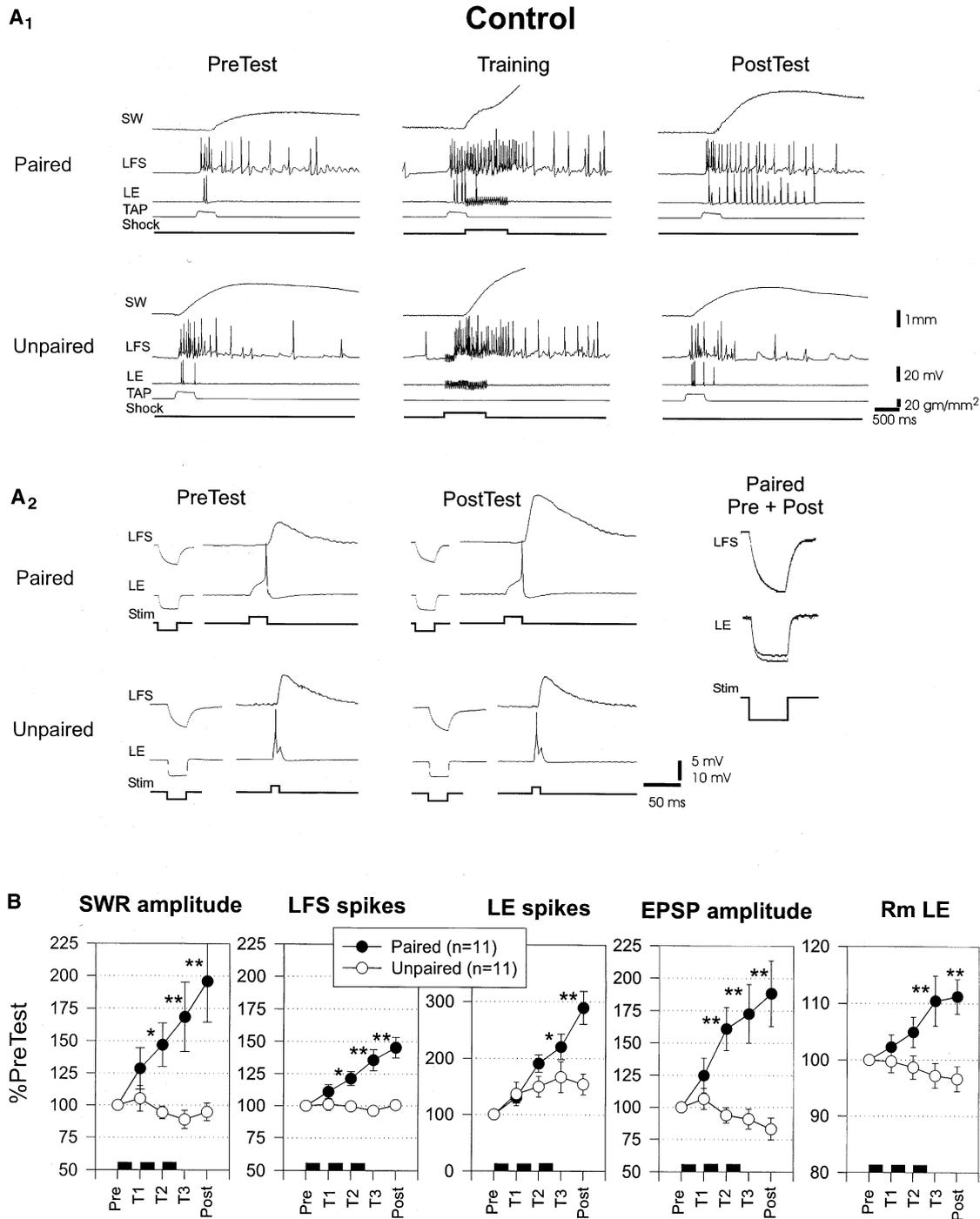
A₁

Control

A₂

B

Figure 4. Electrophysiological Studies of Classical Conditioning in Control Experiments in which Either Vehicle or Nothing Had Been Injected into the LE or LFS Neuron

(A) Examples of siphon withdrawal (SW) and evoked firing of an LFS siphon motor neuron and an LE siphon sensory neuron (A₁), as well as the monosynaptic EPSP from the LE neuron to the LFS neuron and the membrane resistance of the LFS and LE neurons (A₂) on the pretest and the final post-test following paired or unpaired training.

(B) Average results from experiments like the ones shown in (A). Paired training produced significantly greater increases in siphon withdrawal, LFS firing, LE firing, the monosynaptic EPSP, and the membrane resistance (Rm) of the LE neurons. There were no significant differences between experiments with injections of either vehicle or nothing (see Experimental Procedures), and the results have been pooled. The data have been normalized to the average values on the pretest. The average amplitude of siphon withdrawal on the pretest was 1.8 mm (Con, P), 2.5 mm (Con, UP), 2.5 mm (PKAi, P), 2.3 mm (PKAi, UP), 1.9 mm (BAPTA, P), and 2.4 mm (BAPTA, UP), not significantly different by a one-way ANOVA. The average LFS firing was 13.6, 14.5, 12.4, 10.9, 13.6, and 13.3 Hz, not significantly different. The average LE firing was 2.9, 2.6, 3.4, 2.6, 3.8, and 3.6 Hz, not significantly different. The average amplitude of the monosynaptic EPSP was 7.9, 7.8, 9.9, 11.9, 13.1, and 10.0mV, not significantly different. The average response to the first tail shock US was 4.1, 4.2, 4.8, 4.9, 4.5, and 3.9 mm, not significantly different.

(on-field) and does not occur for PSPs from LE neurons that do not fire (off-field). Because the monosynaptic PSPs from LE sensory neurons to LFS motor neurons make a substantial contribution to the withdrawal reflex in this preparation (Antonov et al., 1999), our results indicate that associative, activity-dependent increases in those PSPs make an important contribution to the increases in LFS firing and behavior during conditioning.

Mechanisms of the Synaptic Plasticity Contributing to Behavioral Conditioning

The associative increases in synaptic strength in these experiments might be due to either activity-dependent enhancement of presynaptic facilitation or Hebbian long-term potentiation. Activity-dependent presynaptic facilitation involves changes in the membrane properties and consequent broadening of action potentials in the LE neurons (Hawkins et al., 1983; Eliot et al., 1994; Clark et al., 1994). These changes are thought to be due to a reduction in K^+ current, which results in an increase in membrane resistance that could also contribute to the observed increase in evoked firing of the LE neurons. We were not able to measure action potential width accurately in these experiments, but we were able to measure the membrane resistance of the LE neurons and found that it changed roughly in parallel with changes in the monosynaptic PSP during conditioning, with paired training producing significantly greater enhancement of membrane resistance than unpaired training ($F[1,46] = 5.85$; $p < 0.05$). Like the pairing-specific increase in the monosynaptic PSP, the pairing-specific increase in membrane resistance is restricted to LE neurons that fire during the siphon tap (Antonov et al., 2001). Furthermore, the increase in membrane resistance of the LE neurons correlated significantly overall with each of the other measures including PSP amplitude ($r = 0.450$; $p < 0.001$), suggesting that changes in the membrane properties of the LE neurons contribute to changes in the monosynaptic PSPs during conditioning.

Although an associative increase in LE membrane resistance is consistent with activity-dependent presynaptic facilitation, it is also not incompatible with long-term potentiation. We therefore tested the possible contributions of these two mechanisms to the enhancement of the monosynaptic PSP during conditioning by either injecting a specific peptide inhibitor of PKA into the LE sensory neuron (Figure 5) or injecting the Ca^{2+} chelator BAPTA into the LFS motor neuron (Figure 6). Both of these procedures reduced the effect of pairing on PSP amplitude, particularly for the latter test trials, as evidenced by a significant injection $\times$ pairing $\times$ test interaction ($F[6,138] = 3.79$; $p < 0.01$). Injecting the LE neuron with PKAi significantly reduced the effect of pairing on the final post-test, supporting the involvement of activity-dependent presynaptic facilitation. However, injecting the LFS neuron with BAPTA also significantly reduced the effect of pairing on PSP amplitude, supporting the involvement of long-term potentiation. In addition, there were tendencies for postsynaptic BAPTA to produce a nonassociative decrease in the unpaired PSP and also to reduce the effect of pairing on LFS firing, but these trends were not significantly different from the vehicle control data. Results for PSP area were similar

to those for amplitude (data not shown). Because we injected only a single pre- or postsynaptic neuron, behavioral conditioning was normal in these experiments, indicating that the preparations were otherwise healthy. These results suggest that activity-dependent presynaptic facilitation and long-term potentiation both make important contributions to associative facilitation of the monosynaptic PSPs during conditioning.

The injections also reduced the effect of pairing on the membrane properties of the LE sensory neuron, as evidenced by significant injection $\times$ pairing $\times$ test interactions for both evoked LE firing ($F[6,138] = 2.43$, $p < 0.05$) and LE membrane resistance ($F[6,138] = 2.34$, $p < 0.05$). Injecting the LE neuron with PKAi significantly reduced the effects of pairing on evoked LE firing and membrane resistance on the final post-test, consistent with the idea that PKA mediates most of the changes in sensory neuron membrane properties during activity-dependent presynaptic facilitation (Eliot et al., 1994; Bao et al., 1998; Byrne and Kandel, 1996). Surprisingly, however, injecting the LFS neuron with BAPTA also significantly reduced the effects of pairing on LE firing and membrane resistance. Because there are no known synaptic connections from the LFS motor neurons to other neurons, and postsynaptic BAPTA is not thought to leak to the presynaptic terminals (Bao et al., 1997), the simplest interpretation of these results is that during training, a rise in Ca^{2+} in the motor neuron stimulates production of a retrograde messenger that interacts with the PKA pathway in the LE sensory neuron (Figure 7B).

Discussion

Activity-Dependent Presynaptic Facilitation and Hebbian LTP Both Contribute to Behavioral Conditioning

Our results lead to two major conclusions. First, they show that activity-dependent presynaptic facilitation and Hebbian LTP both contribute to behavioral conditioning. We have found: (1) that conditioning of the siphon-withdrawal reflex is blocked by bath application of either the PKA inhibitor KT5720 or the NMDA receptor antagonist APV; (2) that behavioral conditioning is also blocked by injection of either PKAi into 3–6 sensory neurons or BAPTA into 2–3 motor neurons; (3) that associative plasticity at sensory neuron-motor neuron synapses contributes importantly to the conditioning; and (4) that the synaptic plasticity during conditioning is blocked by injection of either PKAi into the sensory neuron or BAPTA into the motor neuron.

LTP has long been a popular candidate for a cellular mechanism of learning. However, testing that idea has been difficult in more complex systems, and our results provide the most direct demonstration to date that LTP contributes to and is required for behavioral learning. Previous studies on vertebrate systems have provided support for the idea that LTP participates in learning (e.g., Tsien et al., 1996; Mayford et al., 1996; Rogan et al., 1997; Oda et al., 1998; Tsvetkov et al., 2002), but the evidence has been less direct, and in a number of cases, LTP and learning are dissociated (e.g., Zamanillo et al., 1999). One possible reason for such dissociations is that learning and neural plasticity are not unitary phenom-
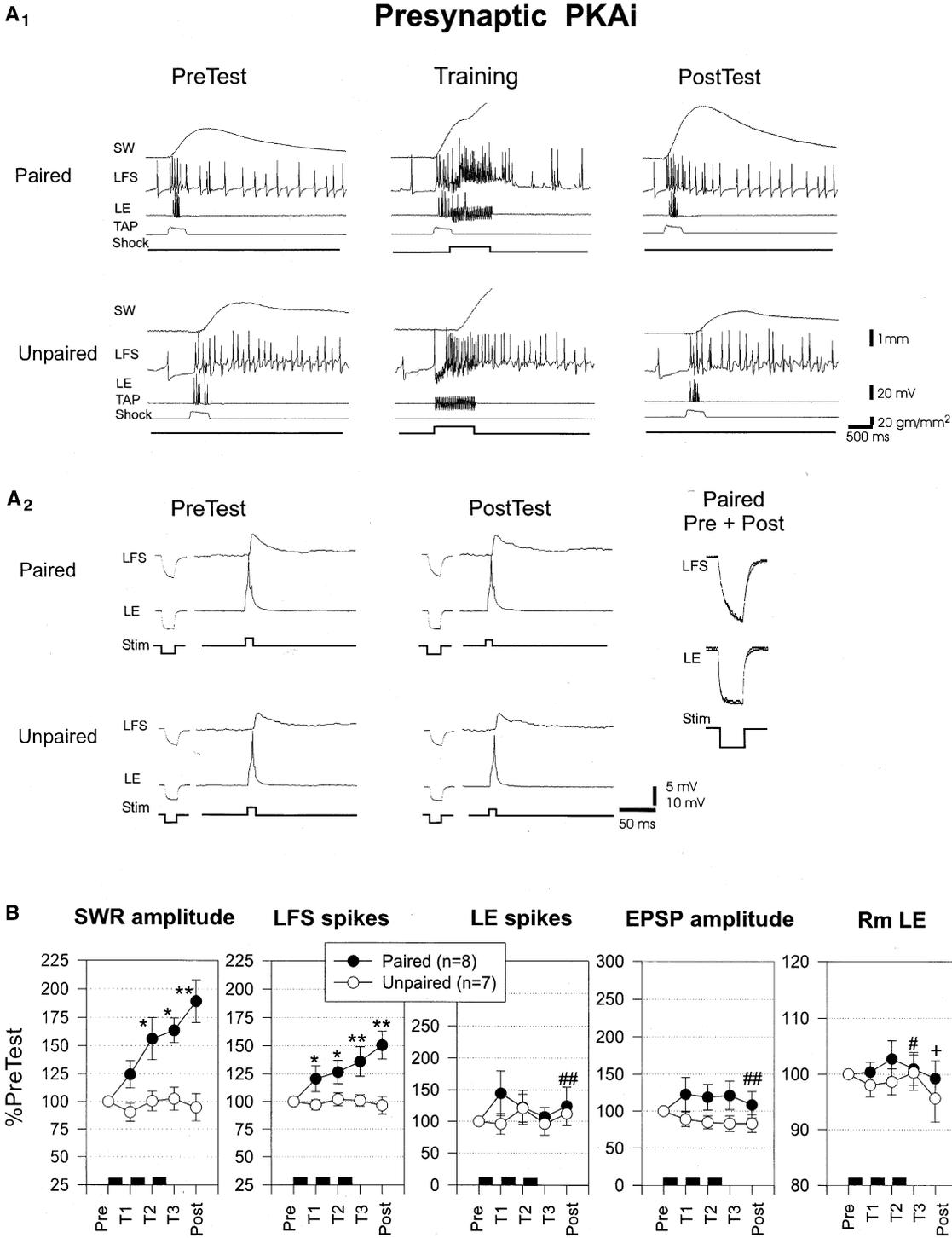
Presynaptic PKAi

Figure 5. Electrophysiological Studies of Classical Conditioning in Experiments in which a Peptide Inhibitor of PKA (PKAi) Had Been Injected into the LE Neuron

Presynaptic PKAi blocked the pairing-specific increases in the monosynaptic EPSP, the membrane resistance of the LE neuron, and evoked firing of the LE neuron (see Figure 4).

ena, but rather, both are multifaceted, so that it is necessary to compare the corresponding facets of each (Hawkins, 1997). By using the simplified *Aplysia* preparation, we have been able to match the behavioral results with the corresponding neural results in two ways.

First, learning generally has several components at the behavioral level and several substrates at the neural level, and those may not all undergo plasticity in parallel. For example, even for a behavior as simple as the *Aplysia* gill- and siphon-withdrawal reflex, the gill and siphon
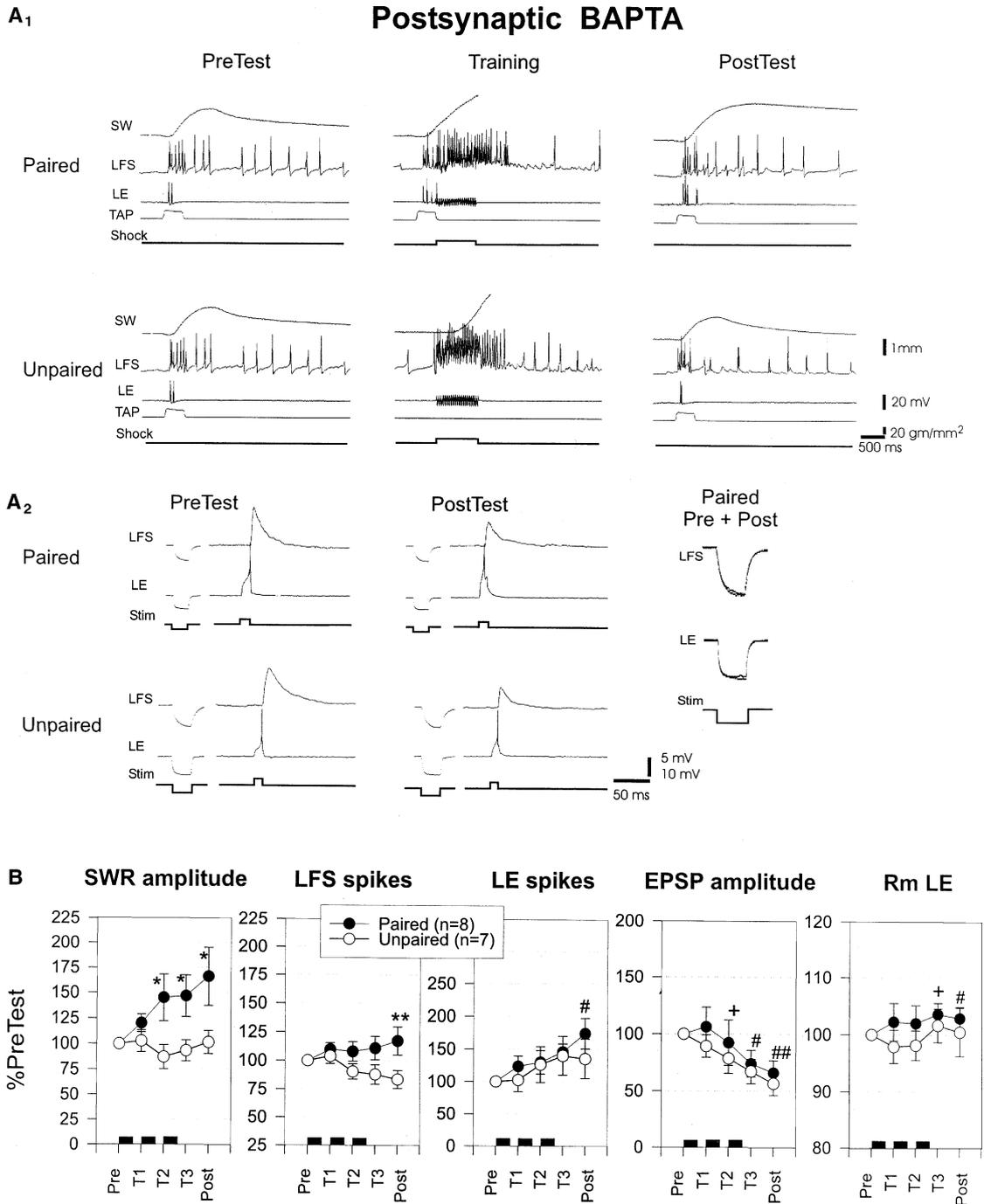
Postsynaptic BAPTA

A₁

PreTest Training PostTest

Paired

SW
LFS
LE
TAP
Shock

Unpaired

SW
LFS
LE
TAP
Shock

| 1 mm
| 20 mV
| 20 gm/mm²
500 ms

A₂

PreTest PostTest Paired Pre + Post

Paired

LFS
LE
Stim

LFS
LE
Stim

Unpaired

LFS
LE
Stim

5 mV
10 mV
50 ms

B

SWR amplitude   LFS spikes   LE spikes   EPSP amplitude   Rm LE

Paired (n=8)
Unpaired (n=7)

%PreTest

Figure 6. Electrophysiological Studies of Classical Conditioning in Experiments in which the Ca²⁺ Chelator BAPTA Had Been Injected into the LFS Neuron

Postsynaptic BAPTA blocked the pairing-specific increases in the monosynaptic EPSP, the membrane resistance of the LE neuron, and evoked firing of the LE neuron (see Figure 4).

components (and even different types of siphon with-drawal) can undergo opposite types of short-term plas-ticity following tail shock (Illich et al., 1994; Hawkins et al., 1998a). For this reason, one must compare plasticity of a specific behavioral component of learning with plas-ticity of the particular neurons that mediate that compo-nent. This is best achieved in simple systems for which

it is possible to describe a neural "circuit diagram," as we have been able to do for the siphon-withdrawal reflex (Antonov et al., 1999). Such circuit information also makes it possible to draw inferences about the causal relation between the neural and behavioral plasticity. Thus, if certain neural elements are known to mediate the behavioral response, then plasticity of those ele-
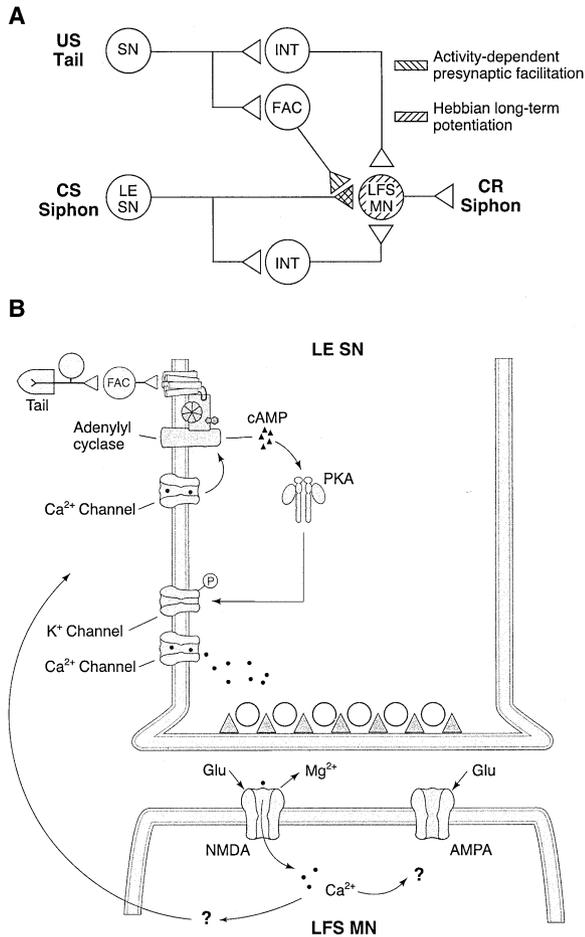
Figure 7. Cellular and Molecular Mechanisms that Are Thought to Contribute to Classical Conditioning of the Siphon-Withdrawal Reflex

(A) Partial circuit diagram for conditioning of the reflex. The conditioned stimulus (CS) activates LE siphon sensory neurons (SN), which make monosynaptic excitatory connections onto both LFS siphon motor neurons (MN) and interneurons (INT) that contribute to a polysynaptic component of the reflex. The unconditioned stimulus (US) activates tail sensory neurons, which excite both facilitatory interneurons (FAC) that produce presynaptic facilitation of the LE neurons and conventional interneurons that excite the LFS neurons. The shading indicates neuronal elements that must be activated conjointly for the induction of either (1) activity-dependent presynaptic facilitation or (2) Hebbian LTP at the LE−LFS synapses.
(B) Molecular mechanisms that are thought to contribute to associative plasticity at the LE-LFS synapses during conditioning. Facilitatory interneurons release several transmitters, including serotonin, that bind to receptors coupled to adenylyl cyclase on the sensory neuron, stimulating production of cAMP, activation of cAMP-dependent protein kinase (PKA), phosphorylation and closure of K^+ channels, broadening of subsequent action potentials, increased Ca^{2+} influx, and increased transmitter release. Spike activity in the sensory neuron just before the serotonin causes an influx of Ca^{2+} that "primes" the cyclase, leading to enhanced activation of the cAMP cascade. The spike activity also causes release of glutamate, which binds with AMPA and NMDA-type receptors on the motor neuron. Depolarization of the motor neuron relieves the Mg^{2+} block of the NMDA receptor channels, allowing the glutamate to stimulate Ca^{2+} influx. The Ca^{2+} may have postsynaptic actions, but it also appears to stimulate production of a retrograde messenger that interacts with the cAMP cascade in the sensory neuron.

ments should contribute to plasticity of the behavior. Furthermore, because the circuit for the siphon-withdrawal reflex is so simple, we have been able to test that inference more rigorously than has previously been possible and have found that blocking specific molecular mechanisms of plasticity in a few key neurons can block behavioral learning.

Second, neural plasticity is often studied with protocols that may not correspond to what happens physiologically during learning. For example, previous studies on *Aplysia* have provided evidence that both activity-dependent presynaptic facilitation (Hawkins et al., 1983; Clark et al., 1994) and long-term potentiation (Murphy and Glanzman, 1996, 1997, 1999) contribute to a cellular analog of conditioning, but those studies have not shown that either mechanism contributes to behavioral learning. Although the temporal parameters of the cellular analogs are generally very similar to those of behavioral training, they differ in at least one respect: the "CS" in the analogs consists of 5–12 spikes produced by intracellular stimulation of a sensory neuron (Hawkins et al., 1983; Walters and Byrne, 1983; Carew et al., 1984; Clark et al., 1994; Murphy and Glanzman, 1996, 1997, 1999), whereas the siphon tap CS causes the sensory neuron to fire only about three spikes (Figure 4). Thus, it was not previously known whether sensory neuron firing that occurs during behavioral training would support either activity-dependent facilitation or long-term potentiation. We have now provided evidence that both mechanisms occur with physiological patterns of activity during behavioral learning.

Activity-Dependent Presynaptic Facilitation and Hebbian LTP Interact during Learning

The second major conclusion from our results is that activity-dependent presynaptic facilitation and long-term potentiation are not independent but interact during conditioning. Activity-dependent presynaptic facilitation is accompanied by associative changes in presynaptic membrane properties (Hawkins et al., 1983; Clark et al., 1994) that are mediated by cAMP and PKA (Byrne and Kandel, 1996). Similar associative changes in membrane properties (Eliot et al., 1994) and also cAMP levels (Kandel et al., 1983; Ocorr et al., 1985; Abrams et al., 1998) can be observed in isolated sensory neuron clusters or homogenates, indicating that the adenylyl cyclase molecule acts as an associative mechanism that is intrinsic to the sensory neurons themselves (Figure 7B). Consistent with these previous studies in vitro, we have observed an associative change in sensory neuron membrane resistance during conditioning and have found that it is blocked by presynaptic injection of PKAi. In addition, however, we have found that the change in sensory neuron membrane resistance is also blocked by postsynaptic injection of BAPTA. This result suggests that a postsynaptic rise in Ca^{2+} somehow gates or amplifies the presynaptic associative mechanism, presumably through retrograde signaling. The rise in Ca^{2+}, in turn, is thought to result from activation of a postsynaptic associative molecular mechanism, the NMDA receptor channel. These results therefore suggest that there are both pre- and postsynaptic associative mechanisms that act in series during conditioning.

What might be the advantage of such a hybrid mechanism? One possibility is coordinate regulation of pre- and postsynaptic plasticity. There is increasing evidence that learning-related plasticity in a variety of systems has both pre- and postsynaptic components. Thus, activity-dependent facilitation in isolated *Aplysia* cell culture (Bao et al., 1998) and long-term potentiation at synapses between hippocampal pyramidal neurons in culture (Pavlidis et al., 2000; Arancio et al., 2001) can both be blocked by injecting either BAPTA into the postsynaptic neuron or kinase inhibitors into the presynaptic neuron. Similarly, LTP at hippocampal mossy fiber synapses (Huang et al., 1994; Weisskopf et al., 1994; Yeckel et al., 1999) and in amygdala (Huang and Kandel, 1998) appears to involve both postsynaptic Ca^{2+} and presynaptic PKA. Because the synapse forms a functional unit, the pre- and postsynaptic components may be coordinated by transynaptic signaling, as occurs during synaptic development (Sanes and Lichtman, 1999). Our results suggest retrograde signaling from the postsynaptic to the presynaptic neuron, and a variety of evidence indicates that hippocampal potentiation may also involve retrograde signaling (Hawkins et al., 1998c; Contractor et al., 2002). In particular, similar to our results in *Aplysia*, hippocampal potentiation in culture is accompanied by a change in the membrane properties of the presynaptic neuron that can be blocked by either pre- or postsynaptic injections (Ganguly et al., 2000).

A second possible advantage of a hybrid mechanism is the generation of synaptic learning rules with more desirable functional characteristics. Thus, although activity-dependent presynaptic facilitation by widely projecting facilitatory neurons provides a natural mechanism for associating a stimulus with a global reinforcing event, that mechanism may not provide a high degree of synapse specificity. By contrast, Hebbian potentiation is a natural mechanism for more local associations and can be highly synapse-specific. Combining the two mechanisms may give rise to a more restrictive learning rule with some of the best properties of both. The combination may also have similar advantages for the long-term retention of plasticity (Bailey et al., 2000). Because activity-dependent presynaptic facilitation is itself a hybrid combination of two simpler mechanisms (posttetanic potentiation and presynaptic facilitation), these examples suggest that such combinations of synaptic mechanisms may occur more generally and thus provide a broader range of functional capabilities during learning.

Experimental Procedures

The experimental preparation (Figure 1A) has been described previously (Antonov et al., 1999). Briefly, the siphon, tail, and central nervous system of *Aplysia californica* (100–150 gm; Marinus, Long Beach, CA) were dissected out in 50% $MgCl_2$ and 50% artificial seawater and then pinned to the floor of a recording chamber filled with circulating, aerated artificial seawater at room temperature. The siphon was partially split, and one-half was left unpinned. The CS (a tap of approximately 20 gm/mm^2, 500 ms duration, produced by a controlled force stimulator; Cohen et al., 1997) was delivered to the pinned half of the siphon, and withdrawal of the other half was recorded with a low mass isotonic movement transducer (Harvard Apparatus, South Natick, MA) attached to the siphon with a silk suture. The peak amplitude of withdrawal was measured using a laboratory interface to a microcomputer and commercially available software (Hilal Associates, Englewood, NJ), which also controlled the stimulation. The US (an AC electric shock of 25 ma, 1 s duration) was delivered to the tail via a fixed capillary electrode. Preparations were considered unhealthy or damaged and not used if the first shock produced a siphon withdrawal of less than 3 mm (the maximal withdrawal was usually about 7 mm).

The preparation was rested for at least 1 hr before the beginning of training (Figure 1B). There were three blocks of four training trials each, with a 5 min interval between trials in a block and a 20 min rest between blocks. The response to the CS was measured in a pretest 5 min before the first block (Pre), in test trials 15 min after each block (T1–T3), and in a final post-test 45 min after the last block (Post). Experiments were continued only if the siphon withdrawal on the pretest was between 0.5 and 5 mm, and were excluded if there was any visible evidence of damage to the siphon at the end of the experiment. During paired training, the CS began 500 ms before the US on each trial. During unpaired training, the interstimulus interval was 2.5 min. Animals were randomly assigned to the training conditions. Because the stimulation was controlled by a computer that also recorded and measured the responses, a blind procedure was not considered necessary.

In pharmacological experiments on conditioning (Figure 1), the abdominal ganglion was surrounded by a circular well with the nerves led through a Vaseline seal so that the ganglion could be bathed (without perfusion) in a different solution than the rest of the preparation. The well contained KT5720 (Biomol, Plymouth Meeting, PA), APV (Sigma, St. Louis, MO), or vehicle (normal seawater) from the beginning of the 1 hr rest period to the end of the experiment.

In electrophysiological experiments (Figures 4–6), the abdominal ganglion was partially desheathed, and LE and LFS neurons were impaled with double-barreled microelectrodes. The recording barrel (7–15 Mohm) contained 2.5 M KCl. Evoked firing of the LE and LFS neurons was measured during the first 1 s after the start of the response to siphon stimulation, which included the peak of the siphon withdrawal on most trials. The membrane resistance of the LE and LFS neurons was monitored by measuring the hyperpolarization produced by an intracellular current pulse delivered through the second barrel 20 s before the siphon tap on each test. The synaptic connection between the two neurons was examined by using a depolarizing intracellular current pulse to fire a single action potential in the LE neuron approximately 10 s before the siphon tap and measuring both the amplitude and area of the unitary monosynaptic PSP in the LFS neuron. The amplitudes and durations of the intracellular current pulses were adjusted for each neuron at the beginning of the experiment and then kept constant. The LFS neuron was hyperpolarized approximately 30mV below resting potential while the PSP was tested to prevent the generation of action potentials. There were no consistent differences in LFS resting potentials (measured at the beginning and end of the recording) in the different experimental groups. Experiments were included only if healthy recordings were maintained from both neurons through the final post-test.

In injection experiments (Figures 3–6), the second barrel of the electrode contained 0.86 M KCl, sulforhodamine B (1.5%) to monitor the injection, and either nothing else (vehicle control), the PKA inhibitor fragment 6–22 amide (0.5 mM), or BAPTA (200 mM). The electrode was connected to a Picospritzer (Parker Instrumentation), and pulses of pressure (1 s duration, 5–15 psi) were delivered at 2 s intervals for 2–5 min during the rest period, approximately 30–40 min before the pretest. The success of the injection was checked with a fluorescence microscope at the end of the experiments. Sulforhodamine was visible in the synaptic region as well as the cell bodies (not shown in the low power images in Figure 3), although the drug concentrations were presumably lower there. The electrophysiological experiments with failed PKAi injections (no fluorescence) were not significantly different in any comparison with the vehicle control experiments in three-way ANOVAs (injection, pairing, and test) for each response measure and have been combined with them. PKAi was from Sigma (St. Louis, MO) and BAPTA was from Molecular Probes (Eugene, OR).

The data from each type of experiment were analyzed with three-way ANOVAs with one repeated measure (test), followed by planned comparisons of the difference between the paired and unpaired groups and the reduction of that difference by each drug (the drug $\times$

pairing interaction) overall, and then at each test to define the time courses of those effects (Winer et al., 1991). The experiments shown in Figures 4–6 were interleaved and analyzed statistically as a single design.

Pharmacological experiments on glutamate receptors contributing to the sensory neuron-motor neuron PSP (Figure 2) were performed on partially desheathed, isolated abdominal ganglia continuously perfused with normal seawater or seawater containing the different drugs. The LFS motor neuron was hyperpolarized approximately 30mV below resting potential during each test of the PSP. APV, CNQX, and DNQX were from Sigma (St. Louis, MO). The data were analyzed with a two-way ANOVA with one repeated measure (peak/late) followed by planned comparisons of the change in each measure from the control (no drug) level.

References

Abrams, T.W., Yovell, Y., Onyike, C.U., Cohen, J.E., and Jarrard, H.E. (1998). Analysis of sequence-dependent interactions between calcium and transmitter stimuli in activating adenylyl cyclase in *Aplysia*: possible contributions to CS-US sequence requirement during conditioning. Learn. Mem. *4*, 496–509.

Antonov, I., Kandel, E.R., and Hawkins, R.D. (1999). The contribution of facilitation of monosynaptic PSPs to dishabituation and sensitization of the *Aplysia* siphon withdrawal reflex. J. Neurosci. *19*, 10438–10450.

Antonov, I., Antonova, I., Kandel, E.R., and Hawkins, R.D. (2001). The contribution of activity-dependent synaptic plasticity to classical conditioning in *Aplysi*a. J. Neurosci. *21*, 6413–6422.

Arancio, O., Antonova, I., Gambaryan, S., Lohmann, S.M., Wood, I.S., Lawrence, D.S., and Hawkins, R.D. (2001). Presynaptic role of cGMP-dependent protein kinase during long-lasting potentiation. J. Neurosci. *21*, 143–149.

Bailey, C.H., Castellucci, V.F., Koester, J., and Kandel, E.R. (1979). Cellular studies of peripheral neurons in siphon skin of *Aplysia californica*. J. Neurophysiol. *42*, 530–557.

Bailey, C.H., Giustetto, M., Huang, Y.-Y., Hawkins, R.D., and Kandel, E.R. (2000). Is heterosynaptic modulation essential for stabilizing Hebbian plasticity and memory? Nat. Rev. Neurosci. *1*, 11–20.

Bao, J.-X., Kandel, E.R., and Hawkins, R.D. (1997). Involvement of pre- and postsynaptic mechanisms in posttetanic potentiation at *Aplysia* synapses. Science *275*, 969–973.

Bao, J.-X., Kandel, E.R., and Hawkins, R.D. (1998). Involvement of presynaptic and postsynaptic mechanisms in a cellular analog of classical conditioning at *Aplysia* sensory-motor neuron synapses in isolated cell culture. J. Neurosci. *18*, 458–466.

Bliss, T.V.P., and Collingridge, G.L. (1993). A synaptic model of memory: long-term potentiation in hippocampus. Nature *361*, 31–39.

Byrne, J. (1987). Cellular analysis of associative learning. Physiol. Rev. *6*, 329–439.

Byrne, J.H., and Kandel, E.R. (1996). Presynaptic facilitation revisited: state and time dependence. J. Neurosci. *16*, 425–435.

Byrne, J.H., Castellucci, V., and Kandel, E.R. (1974). Receptive fields and response properties of mechanoreceptor neurons innervating skin and mantle shelf of *Aplysia*. J. Neurophysiol. *37*, 1041–1064.

Carew, T.J., and Sahley, C.L. (1986). Invertebrate learning and memory: from behavior to molecules. Annu. Rev. Neurosci. *9*, 435–487.

Carew, T.J., Walters, E.T., and Kandel, E.R. (1981). Classical conditioning in a simple withdrawal reflex in *Aplysia californica*. J. Neurosci. *1*, 1426–1437.

Carew, T.J., Hawkins, R.D., and Kandel, E.R. (1983). Differential classical conditioning of a defensive withdrawal reflex in *Aplysia californica*. Science *219*, 397–400.

Carew, T.J., Hawkins, R.D., Abrams, T.W., and Kandel, E.R. (1984). A test of Hebb's postulate at identified synapses which mediate classical conditioning in *Aplysia*. J. Neurosci. *4*, 1217–1224.

Clark, G.A., Hawkins, R.D., and Kandel, E.R. (1994). Activity-dependent enhancement of presynaptic facilitation provides a cellular mechanism for the temporal specificity of classical conditioning in *Aplysia*. Learn. Mem. *1*, 243–257.

Cohen, T.E., Kaplan, S.W., Kandel, E.R., and Hawkins, R.D. (1997). A simplified preparation for relating cellular events to behavior: mechanisms contributing to habituation, dishabituation, and sensitization of the *Aplysia* gill-withdrawal reflex. J. Neurosci. *17*, 2886–2899.

Colwill, R.M., Absher, R.A., and Roberts, M.L. (1988a). Context US learning in *Aplysia californica*. J. Neurosci. *8*, 4434–4439.

Colwill, R.M., Absher, R.A., and Roberts, M.L. (1988b). Conditional discrimination learning in *Aplysia californica*. J. Neurosci. *8*, 4440–4444.

Conrad, P., Wu, F., and Schacher, S. (1999). Changes in functional glutamate receptors on a postsynaptic neuron accompany formation and maturation of an identified synapse. J. Neurobiol. *39*, 237–248.

Contractor, A., Rogers, C., Maron, C., Henkemeyer, M., Swanson, G.T., and Heinemann, S.F. (2002). Trans-synaptic Eph receptor-ephrin signaling in hippocampal mossy fiber LTP. Science *296*, 1864–1869.

Dale, N., and Kandel, E.R. (1993). L-glutamate may be the fast excitatory transmitter of *Aplysia* sensory neurons. Proc. Natl. Acad. Sci. USA *90*, 7163–7167.

Eliot, L.S., Hawkins, R.D., Kandel, E.R., and Schacher, S. (1994). Pairing-specific, activity-dependent presynaptic facilitation at *Aplysia* sensory-motor neuron synapses in isolated cell cultures. J. Neurosci. *14*, 368–383.

Frost, W.N., and Kandel, E.R. (1995). Structure of the network mediating siphon-elicited siphon withdrawal in *Aplysia*. J. Neurophysiol. *73*, 2413–2427.

Frost, L., Kaplan, S.W., Cohen, T.E., Henzi, V., Kandel, E.R., and Hawkins, R.D. (1997). A simplified preparation for relating cellular events to behavior: contribution of LE and unidentified siphon sensory neurons to mediation and habituation of the *Aplysia* gill- and siphon withdrawal reflex. J. Neurosci. *17*, 2900–2913.

Ganguly, K., Kiss, L., and Poo, M.-M. (2000). Enhancement of presynaptic neuronal excitability by correlated presynaptic and postsynaptic spiking. Nat. Neurosci. *3*, 1018–1026.

Glanzman, D.L. (1994). Postsynaptic regulation of the development and long-term plasticity of *Aplysia* sensorimotor synapses in cell culture. J. Neurobiol. *25*, 666–693.

Hawkins, R.D. (1997). LTP and learning: let's stay together. Behav. Brain Sci. *20*, 620–621.

Hawkins, R.D., and Schacher, S. (1989). Identified facilitator neurons L29 and L28 are excited by cutaneous stimuli used in dishabituation, sensitization, and classical conditioning of *Aplysia*. J. Neurosci. *9*, 4236–4245.

Hawkins, R.D., Abrams, T.W., Carew, T.J., and Kandel, E.R. (1983). A cellular mechanism of classical conditioning in *Aplysia*: activity-dependent amplification of presynaptic facilitation. Science *219*, 400–415.

Hawkins, R.D., Carew, T.J., and Kandel, E.R. (1986). Effects of interstimulus interval and contingency on classical conditioning of the *Aplysia* siphon withdrawal reflex. J. Neurosci. *6*, 1695–1701.

Hawkins, R.D., Lalevic, N., Clark, G.A., and Kandel, E.R. (1989). Classical conditioning of the *Aplysia* siphon-withdrawal reflex exhibits response specificity. Proc. Natl. Acad. Sci. USA *86*, 7620–7624.

Hawkins, R.D., Kandel, E.R., and Siegelbaum, S.A. (1993). Learning

to modulate transmitter release: themes and variations in synaptic plasticity. Annu. Rev. Neurosci. *16*, 625–665.

Hawkins, R.D., Cohen, T.E., Greene, W., and Kandel, E.R. (1998a). Relationships between dishabituation, sensitization, and inhibition of the gill- and siphon-withdrawal reflex in Aplysia: effects of response measure, test time, and training stimulus. Behav. Neurosci. *112*, 24–38.

Hawkins, R.D., Greene, W., and Kandel, E.R. (1998b). Classical conditioning, differential conditioning, and second-order conditioning of the *Aplysia* gill-withdrawal reflex in a simplified mantle organ preparation. Behav. Neurosci. *112*, 636–645.

Hawkins, R.D., Son, H., and Arancio, O. (1998c). Nitric oxide as a retrograde messenger during long-term potentiation in hippocampus. Prog. Brain Res. *118*, 155–172.

Hickie, C., Cohen, L.B., and Balaban, P.M. (1997). The synapse between LE sensory neurons and gill motoneurons makes only a small contribution to the *Aplysia* gill-withdrawal reflex. Eur. J. Neurosci. *9*, 627–636.

Huang, Y.-Y., and Kandel, E.R. (1998). Postsynaptic induction and PKA-dependent expression of LTP in the lateral amygdala. Neuron *21*, 169–178.

Huang, Y.-Y., Li, X.-C., and Kandel, E.R. (1994). CAMP contributes to mossy fiber LTP by initiating both a covalently mediated early phase and macromolecular synthesis-dependent late-phase. Cell *79*, 69–79.

Illich, P.A., Joynes, R.L., and Walters, E.T. (1994). Response-specific inhibition during general faclitation of defensive responses in *Aplysia*. Behav. Neurosci. *108*, 614–623.

Kandel, E.R., Abrams, T., Bernier, L., Carew, T.J., Hawkins, R.D., and Schwartz, J.H. (1983). Classical conditioning and sensitization share aspects of the same molecular cascade in *Aplysia*. Cold Spring Harb. Symp. Quant. Biol. *48*, 821–830.

Kelso, S.R., Ganong, A.H., and Brown, T.H. (1986). Hebbian synapses in hippocampus. Proc. Natl. Acad. Sci. USA *83*, 5326–5330.

Lin, X.Y., and Glanzman, D.L. (1994a). Hebbian induction of long-term potentiation of *Aplysia* sensorimotor synapses: partial requirement for activation of a NMDA-related receptor. Proc. R. Soc. Lond. B Biol. Sci. *255*, 215–221.

Lin, X.Y., and Glanzman, D.L. (1994b). Long-term potentiation of *Aplysia* sensorimotor synapses in cell culture: regulation by postsynaptic voltage. Proc. R. Soc. Lond. B Biol. Sci. *255*, 113–118.

Lin, X.Y., and Glanzman, D.L. (1997). Effect of interstimulus interval on pairing-induced LTP of *Aplysia* sensorimotor synapses in cell culture. J. Neurophysiol. *77*, 667–674.

Mackey, S.L., Glanzman, D.L., Small, S.A., Dyke, A.M., Kandel, E.R., and Hawkins, R.D. (1987). Tail shock produces inhibition as well as sensitization of the siphon-withdrawal reflex of *Aplysia*: possible behavioral role for presynaptic inhibition mediated by the peptide Phe-Met-Arg-Phe-NH$_2$. Proc. Natl. Acad. Sci. USA *84*, 8730–8734.

Mackey, S.L., Kandel, E.R., and Hawkins, R.D. (1989). Identified serotonergic neurons LCB1 and RCB1 in the cerebral ganglia of *Aplysia* produce presynaptic facilitation of siphon sensory neurons. J. Neurosci. *9*, 4227–4235.

Mayford, M.A., Bach, M.E., Huang, Y.-Y., Wang, L., Hawkins, R.D., and Kandel, E.R. (1996). Control of memory formation through regulated expression of a CaMKII transgene. Science *274*, 1678–1683.

Murphy, G.G., and Glanzman, D.L. (1996). Enhancement of sensorimotor connections by conditioning-related stimulation in *Aplysia* depends on postsynaptic Ca^{2+}. Proc. Natl. Acad. Sci. USA *93*, 9931–9936.

Murphy, G.G., and Glanzman, D.L. (1997). Mediation of classical conditioning in *Aplysia californica* by long-term potentiation of sensorimotor synapses. Science *278*, 467–471.

Murphy, G.G., and Glanzman, D.L. (1999). Cellular analog of differential classical conditioning in *Aplysia*: disruption by the NMDA receptor antagonist DL-2-amino-5-phosphono-valerate. J. Neurosci. *19*, 10595–10602.

Ocorr, K.A., Walters, E.T., and Byrne, J.H. (1985). Associative condi-

tioning analog selectively increases cAMP levels of tail sensory neurons in *Aplysia*. Proc. Natl. Acad. Sci. USA *82*, 2548–2552.

Oda, Y., Kawasaki, K., Morita, M., Korn, H., and Matsui, H. (1998). Inhibitory long-term potentiation underlies auditory conditioning of goldfish escape behavior. Nature *394*, 182–185.

Pavlidis, P., Montgomery, J., and Madison, D.V. (2000). Presynaptic protein kinase activity supports long-term potentiation at synapses between individual hippocampal neurons. J. Neurosci. *20*, 4497–4505.

Perlman, A.J. (1979). Central and peripheral control of siphon-withdrawal reflex in *Aplysia californica*. J. Neurophysiol. *42*, 510–529.

Rogan, M.T., Staubli, U.V., and LeDoux, J.E. (1997). Fear conditioning induces associative long-term potentiation in the amygdala. Nature *390*, 604–607.

Sanes, J.R., and Lichtman, J.W. (1999). Development of the neuromuscular junction. Annu. Rev. Neurosci. *20*, 389–442.

Schacher, S., Wu, F., and Sun, Z.-Y. (1997). Pathway-specific synaptic plasticity: activity-dependent enhancement and suppression of long-term heterosynaptic facilitation at converging inputs on a single target. J. Neurosci. *17*, 597–606.

Trudeau, L.E., and Castellucci, V.F. (1993). Excitatory amino acid neurotransmission of sensory-motor and interneuronal synapses of *Aplysia californica*. J. Neurophysiol. *70*, 1221–1230.

Tsien, J.Z., Huerta, P.T., and Tonegawa, S. (1996). The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. Cell *87*, 1327–1338.

Tsvetkov, E., Carlezon, W.A., Benes, F.M., Kandel, E.R., and Bolshakov, V.Y. (2002). Fear conditioning occludes LTP-induced presynaptic enhancement of synaptic transmission in the cortical pathway to the lateral amygdala. Neuron *34*, 289–300.

Walters, E.T. (1989). Transformation of siphon responses during conditioning of *Aplysia* suggests a model of primitive stimulus-response association. Proc. Natl. Acad. Sci. USA *86*, 7616–7619.

Walters, E.T., and Byrne, J.H. (1983). Associative conditioning of single sensory neurons suggests a cellular mechanism for learning. Science *219*, 405–408.

Weisskopf, M.G., Castillo, P.E., Zalutsky, P.A., and Nicoll, R.A. (1994). Mediation of hippocampal mossy fiber long-term potentiation by cAMP. Science *265*, 1878–1882.

Wigstrom, H., Gustafsson, B., Huang, Y.-Y., and Abraham, W.C. (1986). Hippocampal long-lasting potentiation is induced by pairing single afferent volley with intracellularly injected depolarizing current pulses. Acta Physiol. Scand. *126*, 317–319.

Winer, B.J., Brown, D.R., and Kenneth, M.M. (1991). Statistical Principles in Experimental Design (New York: McGraw-Hill).

Yeckel, M.F., Kapur, A., and Johnston, D. (1999). Multiple forms of LTP in hippocampal CA3 neurons use a common postsynaptic mechanism. Nat. Neurosci. *2*, 625–633.

Zamanillo, D., Sprengel, R., Hvalby, O., Jensen, V., Burnashev, N., Rozov, A., Kaiser, K.M., Koster, H.J., Borchardt, T., Worley, P., et al. (1999). Importance of AMPA receptors for hippocampal synaptic plasticity but not for spatial learning. Science *284*, 1805–1811.