Behavioral/Systems/Cognitive

Presynaptic and Postsynaptic Mechanisms of Synaptic Plasticity and Metaplasticity during Intermediate-Term Memory Formation in *Aplysia*

Igor Antonov,[1] Eric R. Kandel,[1,2,3] and Robert D. Hawkins[1,2]

[1]Department of Neuroscience, Columbia University, [2]New York State Psychiatric Institute, and [3]Howard Hughes Medical Institute, New York, New York 10032

Synaptic plasticity and learning involve different mechanisms depending on the following: (1) the stage of plasticity and (2) the history of plasticity, or metaplasticity. However, little is known about how these two factors are related. We have addressed that question by examining mechanisms of synaptic plasticity during short-term and intermediate-term behavioral sensitization and dishabituation in a semi-intact preparation of the *Aplysia* siphon-withdrawal reflex. Dishabituation differs from sensitization in that it is preceded by habituation, and is thus a paradigm for metaplasticity. We find that whereas facilitation during short-term sensitization by one tail shock involves presynaptic covalent modifications by protein kinase A (PKA) and CamKII, facilitation during intermediate-term sensitization by four shocks involves both presynaptic (PKA, CaMKII) and postsynaptic (Ca^{2+}, CaMKII) covalent modifications, as well as both presynaptic and postsynaptic protein synthesis. The facilitation also involves presynaptic spike broadening 2.5 min after either one or four shocks, but not at later times. Dishabituation by four shocks differs from sensitization in several ways. First, it does not involve PKA or CaMKII, but rather involves presynaptic PKC. In addition, unlike sensitization with the same shock, dishabituation by four shocks does not involve protein synthesis or presynaptic spike broadening, and it also does not involve postsynaptic Ca^{2+}. These results demonstrate that not only the mechanisms but also the site of plasticity depend on both the stage of plasticity and metaplasticity during memory formation.

Introduction

Learning-related synaptic plasticity in many systems including *Aplysia* and hippocampus involves a family of different cellular and molecular mechanisms, depending on the following: (1) the duration or frequency of the training stimulation, which can give rise to different stages of plasticity; and (2) the history of plasticity, which is known as "metaplasticity" (Abraham and Bear, 1996; Byrne and Kandel, 1996; Fischer et al., 1997; Lee et al., 2000; Bailey et al., 2008). However, little is known about the relationship between these two factors: that is, how metaplasticity depends on the stage of plasticity or vice versa. We have addressed this question by examining mechanisms of synaptic plasticity during short-term and intermediate-term sensitization and dishabituation in a semi-intact preparation of the *Aplysia* siphon-withdrawal reflex (Antonov et al., 1999, 2001). Dishabituation differs from sensitization in that it is preceded by habituation, and is thus a paradigm for metaplasticity.

Previous *in vitro* studies have found that mechanisms of short-term facilitation at *Aplysia* sensory–motor neuron synapses depend on the state (rested or depressed) of the synapse, illustrating metaplasticity (Byrne and Kandel, 1996). In addition, both the mechanisms and site of facilitation depend on the stage of facilitation. Brief exposure to the modulatory transmitter serotonin (5HT) produces short-term (minutes) facilitation that involves presynaptic covalent modifications and enhancement of transmitter release. In contrast, repeated exposure to 5HT produces long-term (days) facilitation that involves the protein and RNA synthesis-dependent growth of new synapses, which by its nature requires coordinated presynaptic and postsynaptic alterations (Glanzman et al., 1990; Bailey et al., 1992; Trudeau and Castellucci, 1995; Martin et al., 1997).

In addition, intermediate exposure to 5HT produces an intermediate-term (hours) stage of facilitation, which involves elements of the mechanisms of both short-term and long-term facilitation and may form a bridge between them (Ghirardi et al., 1995; Sutton and Carew, 2000; Kim et al., 2003; Li et al., 2009). For example, a single 10 min exposure to 5HT produces intermediate-term facilitation that, unlike short-term facilitation, depends on both presynaptic and postsynaptic covalent modifications and protein synthesis (Byrne and Kandel, 1996; Nakanishi et al., 1997; Chitwood et al., 2001; Jin et al., 2004, 2007, 2008; Li et al., 2005; Villareal et al., 2007, 2009; Fulton et al., 2008). Some of the same mechanisms also contribute to intermediate-term forms of behavioral plasticity (Sutton et al., 2001, 2004).

We have now examined presynaptic and postsynaptic molecular mechanisms of facilitation during intermediate-term behav-

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Correspondence should be addressed to Robert D. Hawkins, Columbia University, 1051 Riverside Drive, New York, NY 10032. E-mail: rdh1@columbia.edu.

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ioral sensitization, and compared them with those during short-term sensitization. In addition, we have asked whether metaplasticity occurs during behavioral learning and extends to the intermediate-term stage: that is, whether intermediate-term dishabituation involves the same mechanisms as intermediate-term sensitization, or whether recruitment of those mechanisms is affected by prior habituation. Collectively, our results show that not only the mechanisms but also the site of plasticity during learning depend on both (1) the stage of plasticity and (2) the history of plasticity. These studies thus reveal novel aspects of metaplasticity during an intermediate-term stage of memory formation.

Materials and Methods

The behavioral and electrophysiological methods were similar to those we have described previously (Antonov et al., 1999, 2003), except that we increased the intertrial interval from 5 to 15 min to minimize baseline habituation, delivered habituation stimuli with an interstimulus interval (ISI) of 10 s to specify the time of habituation more precisely, and used two different levels of tail shock. Briefly, the siphon, tail, and CNS of *Aplysia californica* (100–150 g) were dissected and pinned to the floor of a recording chamber filled with circulating, aerated artificial seawater at room temperature (Fig. 1*A*). The siphon was partially split, and one-half was left unpinned. A controlled force stimulator was used to deliver taps of ~20 g/mm^2 and 500 ms duration to the pinned half, and withdrawal of the other half was recorded with a low-mass isotonic movement transducer 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, which also controlled the stimulation. A fixed capillary electrode was used to deliver constant AC electric shocks of 25 ma and 1 s duration to the tail.

The preparation was rested for at least 1 h before the beginning of training (Fig. 1*B*). In sensitization experiments, the reflex was tested once every 15 min, and either a single shock or a train of four shocks (with a 2 s interval between shocks) was delivered to the tail 2.5 min before the fourth test (post-test). In test-alone control experiments, the shock was omitted. In habituation experiments, a series of 10 siphon stimulations with an ISI of 10 s was given starting 6 min before the fourth test. In dishabituation experiments, tail shock was then delivered 2.5 min before the fourth test. Experiments were continued only if the siphon withdrawal was between 0.5 and 5 mm on the first test, and >3 mm in response to the shock.

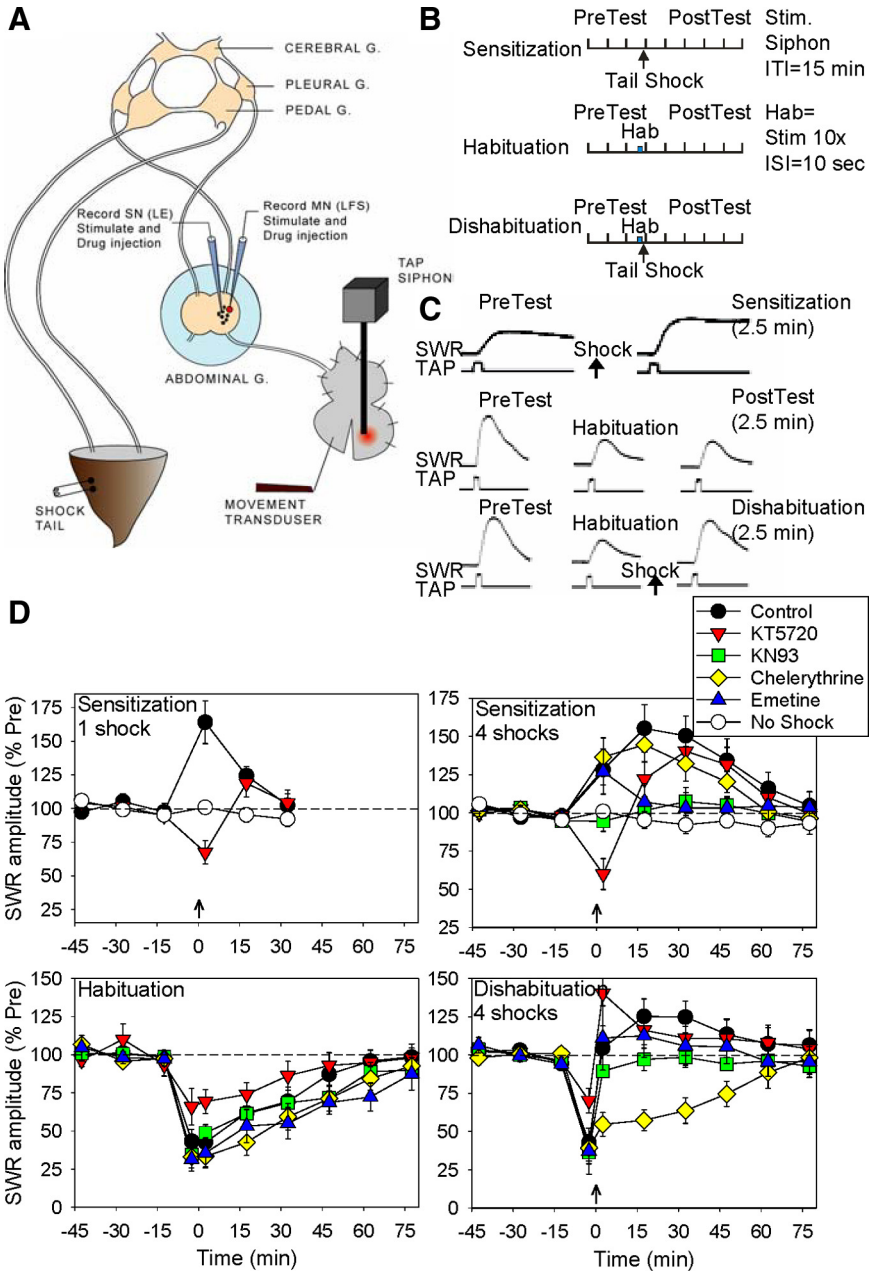

Figure 1. Behavioral pharmacology of sensitization and dishabituation in the siphon-withdrawal preparation. ***A***, The preparation. ***B***, Behavioral protocols. See the text for details. ***C***, Examples of siphon withdrawal (SWR) before (PreTest) and 2.5 min after (PostTest) tail shock (Sensitization), 10 closely spaced siphon stimuli (Habituation), or habituation followed by tail shock (Dishabituation). ***D***, Average results from experiments like the ones shown in ***C*** with the abdominal ganglion bathed in normal saline (Control), the PKA inhibitor KT5720, the CaMKII inhibitor KN93, the PKC inhibitor chelerythrine, or the protein synthesis inhibitor emetine, and no shock control. There were significant overall effects of group during sensitization following a single tail shock ($F_{(2,15)} = 8.04, p < 0.01, n = 6, 5,$ and 7), sensitization following four tail shocks ($F_{(5,33)} = 4.54, p < 0.01, n = 6, 7, 6, 6, 7,$ and 7), dishabituation following four tail shocks ($F_{(4,26)} = 4.19, p < 0.01, n = 6, 5, 6, 7,$ and 7), and a marginal effect during habituation following 10 closely spaced siphon stimuli ($F_{(4,25)} = 2.56, p < 0.10, n = 7, 5, 5, 7,$ and 6). The point at −2 min indicates the response to the 10th habituation stimulus. The amplitude of siphon withdrawal has been normalized to the average value on the three pretests in each experiment. The overall average pretest value was 2.4 mm, which was not significantly different in experiments with the different inhibitors by a one-way ANOVA. The average response to the tail shock was 5.5 mm. The error bars indicate SEMs.

In pharmacological experiments, the abdominal ganglion was surrounded by a circular well with the nerves led through a Vaseline seal, so that the ganglion could be bathed in a different solution than the rest of the preparation. Drugs were applied for 30 min before and during the experiments. In electrophysiological experiments, the abdominal ganglion was partially desheathed and an LE siphon sensory neuron and LFS siphon motor neuron were impaled with double-barreled microelectrodes. The recording barrel (7–15 MΩ) contained 2.5 M KCl. On each test trial, we measured siphon withdrawal, evoked firing of the LE and LFS neurons, the membrane resistance of each neuron, the duration of

the action potential in the LE neuron, and the amplitude and shape of the monosynaptic EPSP produced in the LFS neuron by direct stimulation of the LE neuron (Figs. 2*A*, 4*A*). In some experiments, we pressure injected drugs into the LE or LFS neuron from the second barrel of the electrode 30 min before the start of the experiment. The injection barrel contained 0.8 M KCl, 0.1% fast green to monitor the injections, and in some experiments a peptide kinase inhibitor, BAPTA, or gelonin. Emetine, protein kinase A (PKA) 6-22 (Sigma), KT5720, chelerythrine, KN93, CaMKII 281-309, PKC 19-31 (Calbiochem), BAPTA (Invitrogen), and gelonin (Aczon) were prepared as stock solutions in distilled water and diluted in ASW or electrode solution immediately before use.

The data from each type of experiment were analyzed with two-way or three-way ANOVAs with one repeated measure (test), followed by planned comparisons of the difference between the training groups and the reduction of that difference by the drug (the drug × training interaction) at each test. One-tailed statistics were used when the direction of the effect was predicted from previous results (shock vs no shock comparisons).

Results

Behavioral pharmacology of sensitization and dishabituation

We first examined the effects of different pharmacological inhibitors on behavioral sensitization and dishabituation in the siphon-withdrawal preparation (Fig. 1*C*,*D*). To obtain a stable baseline response and thus get better estimates of the time courses of sensitization and dishabituation, we modified our previous behavioral protocols (Antonov et al., 1999) by testing the reflex once every 15 min. We also used two different levels of tail shock: a single shock (25 ma, 1 s) or a train of four closely spaced shocks (ISI, 2 s). A single shock produced sensitization that lasted ~30 min ($p < 0.05$ at 2.5 and 17.5 min after the shock compared with no shock control) with a peak at 2.5 min. Bathing the abdominal ganglion in an inhibitor of PKA, KT5720 (2 μM) blocked the sensitization ($p < 0.01$ at 2.5 min compared with saline control) and revealed transient behavioral inhibition ($p < 0.05$ at 2.5 min only after the shock compared with no shock control). These results suggest that one shock produces PKA-dependent short-term sensitization.

A train of four shocks produced sensitization that lasted ~1 h ($p < 0.05$ from 2.5 to 62.5 min) with a peak at 17.5 min. Bathing the abdominal ganglion in an inhibitor of protein synthesis, emetine (200 μM) had no effect on sensitization 2.5 min after the shock, but blocked sensitization at all later times ($p < 0.05$ from 17.5 to 47.5 min). As with one shock, KT5720 blocked the early part of sensitization after four shocks ($p < 0.05$ at 2.5 and 17.5 min) and revealed transient behavioral inhibition ($p < 0.01$ at 2.5 min only), but had less effect on the late part of sensitization. Bathing the ganglion in an inhibitor of PKC, chelerythrine (50 μM) did not have a significant effect, although it tended to reduce sensitization at times >2.5 min after the shock. However, bathing the ganglion in an inhibitor of CaMKII, KN93 (10 μM) blocked both the early and late parts of sensitization after four shocks ($p < 0.01$ from 2.5 to 47.5 min). These results suggest that four shocks produce PKA-dependent short-term sensitization 2.5 min after the shock, followed by PKA-dependent and protein synthesis-dependent intermediate-term sensitization. In addition, they suggest that CaMKII contributes to both stages of sensitization.

To explore mechanisms contributing to intermediate-term dishabituation and compare them to sensitization, we first used 10 closely spaced siphon stimuli (ISI, 10 s) to produce habituation that lasted ~1 h ($p < 0.01$ from 4.5 to 34.5 min after the stimuli compared with pretest). Bathing the abdominal ganglion in KT5720 reduced short-term habituation during the repeated siphon stimulation ($p < 0.01$ compared with saline control on the 10th stimulus) but had no significant effect on the rate of recovery from habituation. Emetine, chelerythrine, and KN93 had no significant effects on habituation or recovery from habituation. A train of four shocks produced dishabituation that also lasted ~1 h ($p < 0.05$ from 2.5 to 47.5 min after the shock compared with no shock control) with a peak at 17.5 min. Thus, dishabituation had approximately the same time course as intermediate-term sensitization with the same shock. However, unlike sensitization, dishabituation was blocked by the PKC inhibitor chelerythrine ($p < 0.05$ for the drug × shock interaction from 2.5 to 32.5 min), whereas emetine, KT5720, and KN93 had no significant effects.

Table 1. Average shock and pretest responses for the different drug treatments in Figure 1

	Control	KT5720	KN93	Chelerythrine	Emetine
Shock					
SWR (mm)					
Mean	5.7	6.3	4.4*	5.4	5.2
SE	0.3	0.4	0.3	0.4	0.4
N	19	17	14	13	12
Pretest					
SWR (mm)					
Mean	2.1	2.6	2.7	2.5	2.2
SE	0.2	0.3	0.2	0.2	0.2
N	33	22	20	20	17

SWR, Siphon withdrawal. *Significantly different from control.

As controls, none of the drugs had significant effects on the amplitude of siphon withdrawal during the pretest or in response to the shock, except that KN93 produced a 23% reduction in the shock response ($p < 0.05$ compared with control by a Dunnet's test following a one-way ANOVA) (Table 1). However, the results for sensitization and dishabituation were not altered when differences due to the shock response were factored out in an ANCOVA. These results are similar to those for facilitation at nondepressed and depressed synapses *in vitro* (Byrne and Kandel, 1996), and provide direct evidence that PKA and PKC contribute preferentially to behavioral sensitization and dishabituation, respectively. In addition, they suggest that dishabituation does not depend on CaMKII or protein synthesis, even though intermediate-term sensitization with the same shock does.

Cellular mechanisms and molecular pathways involved in intermediate-term sensitization

To examine cellular mechanisms contributing to intermediate-term sensitization and compare them to those of short-term sensitization, we recorded the evoked firing and input resistance of an LE siphon sensory neuron and an LFS siphon motor neuron as well as the monosynaptic EPSP between them during behavioral learning (Fig. 2*A*). The behavioral results replicated those in the pharmacological studies: a single shock produced sensitization that lasted ~30 min ($p < 0.05$ 2.5 and 17.5 min after the shock compared with no shock control) with a peak at 2.5 min. A train of four shocks produced sensitization that lasted ~1 h ($p < 0.05$ from 2.5 to 47.5 min) with a peak at 17.5 min (Fig. 2*B*). Either one or four shocks also produced increases in the evoked firing of the LFS motor neuron and the amplitude of the LE–LFS EPSP that approximately paralleled the increase in siphon withdrawal ($p < 0.05$ from 2.5 to 62.5 min after four shocks for each measure), and the three measures correlated significantly with each other ($p < 0.05$ for each pairwise comparison). Furthermore, when those correlations were factored out in ANCOVAs, there was no longer

a significant effect of shock on siphon withdrawal, suggesting that most of the increase in withdrawal was due to increases in evoked firing of the LFS motor neurons and facilitation of the sensory–motor neuron EPSPs.

We then investigated presynaptic and postsynaptic molecular mechanisms involved in the facilitation by injecting different inhibitors into the sensory or motor neuron before sensitization (Fig. 3). With one or four tail shocks, injecting either a peptide inhibitor of PKA (PKA 6-22) or a peptide inhibitor of CaMKII (CaMKII 281-309) into the sensory neuron reduced facilitation of the EPSP over its entire time course ($p < 0.05$ for the inhibitor × shock interaction from 2.5 to 32.5 min after four shocks for both inhibitors). With four tail shocks, injecting an inhibitor of protein synthesis (gelonin) into the sensory neuron significantly reduced facilitation at times >2.5 min after the shock ($p < 0.05$ from 17.5 to 32.5 min). Injecting a peptide inhibitor of PKC (PKC 19-31) into the sensory neuron did not have a significant effect on facilitation by four tail shocks, although it tended to reduce the facilitation after 2.5 min.

With a single tail shock, injecting either BAPTA or CaMKII 281-309 into the motor neuron had no effect. However, with four tail shocks, injecting either BAPTA or CaMKII 281-309 into the motor neuron reduced the facilitation after 2.5 min ($p < 0.05$ from 17.5 to 32.5 min after the shock in both cases). Injecting the protein synthesis inhibitor gelonin into the motor neuron also reduced facilitation after 2.5 min ($p < 0.05$ from 17.5 to 32.5 min after four shocks). Injecting a peptide inhibitor of PKA into the motor neuron did not have a significant effect, although it also tended to reduce facilitation after 2.5 min.

As controls, none of the presynaptic or postsynaptic injections had significant effects on the test-alone (no-shock) control groups, the LFS membrane resistance, the siphon withdrawal in response to the shock, or the pretest responses for siphon withdrawal, LFS spikes, or EPSP amplitude (Table 2). Injecting a single LE sensory neuron or a single LFS motor neuron also did not have a significant effect on the increase in siphon withdrawal during sensitization [presumably, because the reflex is mediated by approximately five to eight LE neurons and three LFS neurons in this preparation (Byrne et al., 1974; Hickie et al., 1997; Antonov et al., 1999)], indicating that the preparations were otherwise healthy.

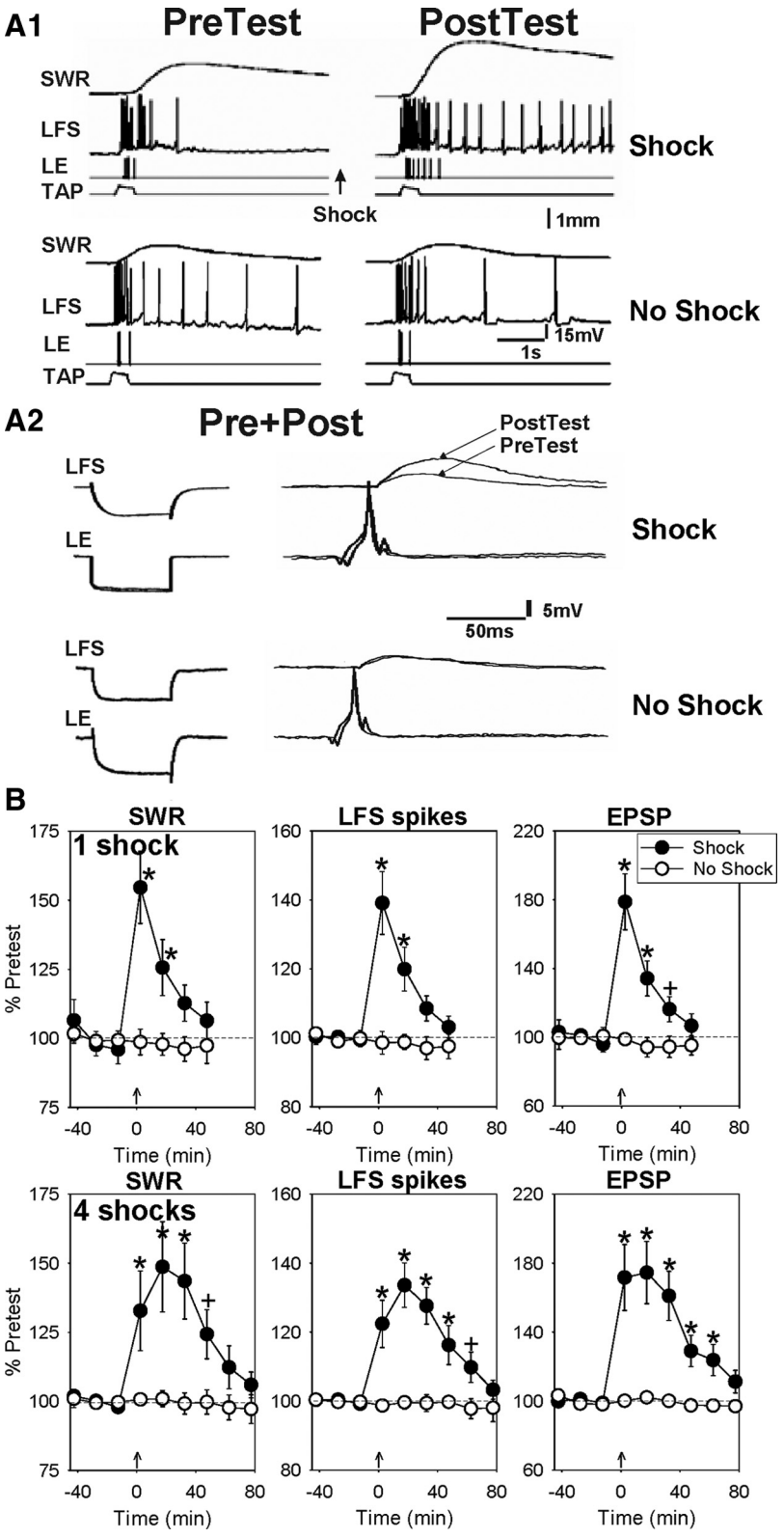

Figure 2. Cellular mechanisms involved in sensitization. ***A***, Examples of siphon withdrawal (SWR), evoked firing of an LFS siphon motor neuron, and the monosynaptic EPSP from an LE sensory neuron to the LFS neuron before (PreTest) and 2.5 min after (PostTest) four tail shocks (top) or no shock control (bottom). ***B***, Average results from experiments like the ones shown in ***A*** with either a single shock ($n = 6$ for shock; $n = 6$ for no shock) or four shocks ($n = 7$ for shock; $n = 6$ for no shock). There was a significant overall effect of shock in each case. The overall average pretest values were 1.8 mm for siphon withdrawal, 15 spikes for evoked LFS firing, and 5.9 mV for the amplitude of the EPSP, which were not significantly different in experiments with shock and no shock. In this and Figs. 4, 5, and 6, $^{*}p < 0.05$. $^{+}p < 0.05$ one tail for the difference between shock and no shock.

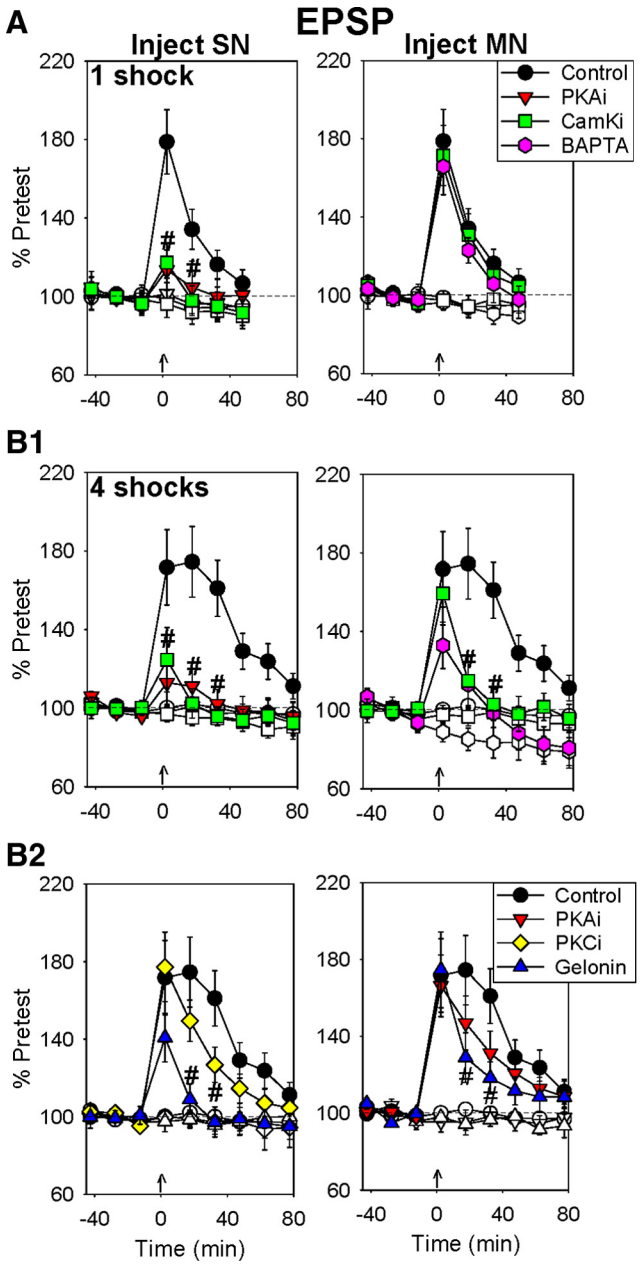

Figure 3. Presynaptic and postsynaptic molecular mechanisms of the facilitation during sensitization. ***A***, Average facilitation of the EPSP by one shock (colored symbols) and no shock controls (white symbols) following no injection (Control) or intracellular injection of a peptide inhibitor of PKA (PKAi), CaMKII (CamKi), or the Ca^{2+} chelator BAPTA into the sensory neuron (SN) or motor neuron (MN). There was a significant overall effect of group ($F_{(4,52)} = 2.75$, $p < 0.05$, $n = 12, 12, 13, 10$, and 15), and a marginal effect for the group × shock interaction ($F_{(4,52)} = 2.45$, $p < 0.10$). ***B***, Average facilitation by four tail shocks following no injection (Control) or intracellular injection of a peptide inhibitor of PKA (PKAi), CaMKII (CamKi), PKC (PKCi), the protein synthesis inhibitor gelonin, or the Ca^{2+} chelator BAPTA into the SN or MN. There were significant overall effects of group ($F_{(8,89)} = 3.91$, $p < 0.01$, $n = 13, 10, 13, 13, 11, 12, 13, 11$, and 11) and the group × shock interaction ($F_{(8,89)} = 2.15$, $p < 0.05$). The overall average pretest value was 6.1 mV. The average response to the tail shock was 5.1 mm. These values were not significantly different in experiments with different injections. In this and Figs. 5 and 6, $^{\#}p < 0.05$ for the interaction between the inhibitor and shock at each test.

These results suggest that whereas short-term facilitation of the EPSP by one tail shock involves presynaptic PKA and CaMKII, intermediate-term facilitation by four tail shocks involves both presynaptic (PKA, CaMKII, and protein synthesis) and postsynaptic (Ca^{2+}, CaMKII, and protein synthesis) mechanisms, which have somewhat different but overlapping time courses. Presynaptic PKA and CaMKII contribute to the entire time course of facilitation, whereas postsynaptic Ca^{2+} and CaMKII, as well as both presynaptic and postsynaptic protein synthesis, contribute at times >2.5 min after the shock. After 2.5 min, the facilitation by four tail shocks is almost completely blocked by either presynaptic or postsynaptic inhibitors, suggesting that the presynaptic and postsynaptic mechanisms are not simply additive.

Possible mechanisms of expression of the facilitation during intermediate-term sensitization

These results suggest that both presynaptic and postsynaptic kinases and protein synthesis can contribute to the facilitation during sensitization, but they do not specify where or how that facilitation is ultimately expressed. As one way to address that question, we measured changes in several properties of the LE siphon sensory neuron during these experiments, including evoked firing, membrane resistance, and action potential duration (Fig. 4*A*). Results with one or four tail shocks were similar: either shock produced transient increases in all of these measures that did not last as long as facilitation of the EPSP ($p < 0.05$ 2.5 min only after four shocks for each measure) (Fig. 4*B*). The increases in LE firing and action potential duration correlated with the increase in membrane resistance ($p < 0.05$ in each case), suggesting that they involve similar mechanisms.

The changes in LE neuron membrane properties were all reduced by injecting peptide inhibitors of PKA or CaMKII into the sensory neuron ($p < 0.05$ after one and four shocks for LE firing and action potential duration in both cases), but not by presynaptic injection of a peptide inhibitor of PKC or the protein synthesis inhibitor gelonin (Fig. 5*A*). The changes in LE membrane properties were also not significantly reduced by postsynaptic injection of BAPTA, peptide inhibitors of PKA or CaMKII, or gelonin. As controls, none of the injections had significant effects on the test-alone control groups or the pretest responses for LE spikes, membrane resistance, or spike width, except that presynaptic injection of a peptide inhibitor of CaMKII produced a 26% reduction in the pretest spike width ($p < 0.05$ compared with control by a Dunnet's test) (Table 2). However, the results for LE spike broadening were not altered when differences due to the pretest were factored out in an ANCOVA. These results suggest that the early phase of facilitation 2.5 min after either one or four tail shocks involves PKA-dependent and CaMKII-dependent broadening of presynaptic action potentials, whereas facilitation at later times involves some other process that requires either a presynaptic (one shock) mechanism or both presynaptic and postsynaptic (four shocks) mechanisms.

A possible postsynaptic mechanism, membrane insertion of AMPA-type glutamate receptors, has been proposed to contribute to facilitation in an *in vitro* analog of intermediate-term sensitization (Li et al., 2005). To investigate whether that process also contributes to facilitation during behavioral sensitization, we examined changes in the shape of the EPSPs. The sensory–motor neuron EPSPs are glutamatergic (Dale and Kandel, 1993; Trudeau and Castellucci, 1993; Conrad et al., 1999), and have an early component that is selectively blocked by the AMPA antagonist CNQX and a later component that is selectively blocked by the NMDA antagonist APV in this preparation (Antonov et al., 2003). Thus, if sensitization involves insertion of AMPA-type glutamate receptors, one would predict that the early part of the EPSP would be enhanced more than the late part.

Table 2. Average shock and pretest responses for the different drug injections in Figures 3 and 5

		SN				MN			
	Control	PKAi	CamKi	PKCi	Gelonin	BAPTA	CamKi	PKAi	Gelonin
Shock									
SWR (mm)									
Mean	5.6	5.6	5.1	4.6	4.9	5.0	5.3	4.7	4.6
SE	0.4	0.3	0.4	0.4	0.5	0.3	0.4	0.4	0.4
N	13	12	15	7	6	12	12	6	6
Pretests									
SWR (mm)									
Mean	1.8	1.8	1.8	1.9	1.9	2.0	1.9	1.8	1.9
SE	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.2	0.2
N	25	22	26	13	11	22	23	11	11
LFS spikes									
Mean	14.6	14.3	14.5	14.7	14.8	14.3	14.3	14.8	15.1
SE	0.4	0.3	0.3	0.4	0.3	0.3	0.4	0.5	0.3
N	25	22	26	13	11	27	23	11	11
LFS Rm (au)									
Mean	3.6	3.1	3.6	3.3	3.3	3.4	3.3	3.5	3.2
SE	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1
N	23	22	26	13	11	27	23	11	11
EPSP (mV)									
Mean	5.9	6.2	6.5	6.0	5.4	5.9	6.5	6.6	5.9
SE	0.3	0.4	0.3	0.4	0.4	0.2	0.3	0.4	0.3
N	25	22	26	13	11	27	23	11	11
LE spikes									
Mean	3.8	4.4	3.7	3.9	3.8	4.4	3.9	3.5	3.8
SE	0.2	0.3	0.2	0.3	0.4	0.2	0.3	0.3	0.3
N	25	22	26	13	11	27	23	11	11
LE Rm (au)									
Mean	3.7	3.4	3.7	3.4	3.6	3.4	3.5	3.6	3.5
SE	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.2
N	23	22	26	13	11	27	23	11	11
LE width (ms)									
Mean	2.3	2.3	1.7*	2.1	2.2	2.0	1.9	1.6	1.8
SE	0.1	0.2	0.1	0.2	0.2	0.2	0.1	0.1	0.2
N	22	18	21	13	11	19	19	11	11

SN, Sensory neuron; MN, motor neuron; SWR, siphon withdrawal; PKAi, peptide inhibitor of PKA; CamKi, peptide inhibitor of CaMKII. *Significantly different from control.

To test that prediction, we measured the ratio of the late (75 ms after peak) and early (peak) parts of the EPSPs. As expected, the ratio is decreased by the NMDA antagonist APV and increased by the AMPA antagonist CNQX (Antonov et al., 2007). Somewhat surprisingly, however, there was a transient increase in the ratio (i.e., there was greater enhancement of the late part of the EPSP) 2.5 min after sensitization by either one or four shocks ($p < 0.05$ compared with no shock) (Fig. 5*B*). That effect was blocked by presynaptic injection of a peptide inhibitor of PKA ($p < 0.05$ for the inhibitor × shock interaction), with no significant effect of postsynaptic injection of BAPTA or CaMKII 281-309. In all of these respects, the changes in the late/peak ratio paralleled changes in the duration of presynaptic action potentials. Furthermore, the increase in the ratio can be mimicked by the K^+ channel blocker 4-amino-pyridine, which causes broadening of presynaptic action potentials (Antonov et al., 2007). These results suggest that the increase in the late/peak ratio 2.5 min after the shock is due to action potential broadening at that time. At later times, however, there was no significant effect of shock on the late/peak ratio. That result suggests that facilitation of the EPSP at times >2.5 min after the shock may not be due to either postsynaptic AMPA receptor insertion or presynaptic action potential broadening but, rather, involves some other mechanism such as modulation of presynaptic transmitter release (Zhao and Klein, 2002; Jin et al., 2006; Leal and Klein, 2009).

Cellular mechanisms and molecular pathways involved in intermediate-term dishabituation

Studies of *in vitro* analogs of short-term sensitization and dishabituation have shown that they involve different molecular mechanisms (Byrne and Kandel, 1996). To investigate that question *in vivo* and extend it to the intermediate-term range, we recorded an LE siphon sensory neuron, an LFS siphon motor neuron, and the monosynaptic EPSP between them during dishabituation by four tail shocks (Fig. 6*A*). Again, the behavioral results replicated those in the pharmacological studies: 10 closely spaced siphon stimuli (ISI, 10 s) produced habituation that lasted ~1 h ($p < 0.05$ from 4.5 to 79.5 min after the stimuli compared with pretest), and a train of four shocks produced dishabituation that also lasted ~1 h ($p < 0.05$ from 2.5 to 62.5 min after the shock compared with no shock control) with a peak at 17.5 min (Fig. 6*B1*). Habituation produced decreases in the evoked firing of the LFS motor neuron and the amplitude of the LE–LFS EPSP that approximately paralleled the decrease in siphon withdrawal ($p < 0.05$ from 4.5 to 64.5 min after the 10 stimuli for each measure). Similarly, tail shock produced increases in the evoked firing of the LFS motor neuron and the amplitude of the LE–LFS EPSP that approximately paralleled the increase in siphon withdrawal during dishabituation ($p < 0.01$ from 2.5 to 47.5 min after the shock for each measure). These results suggest that the changes in withdrawal during habituation and dishabituation, like sensitization, were due in part to changes in evoked firing of

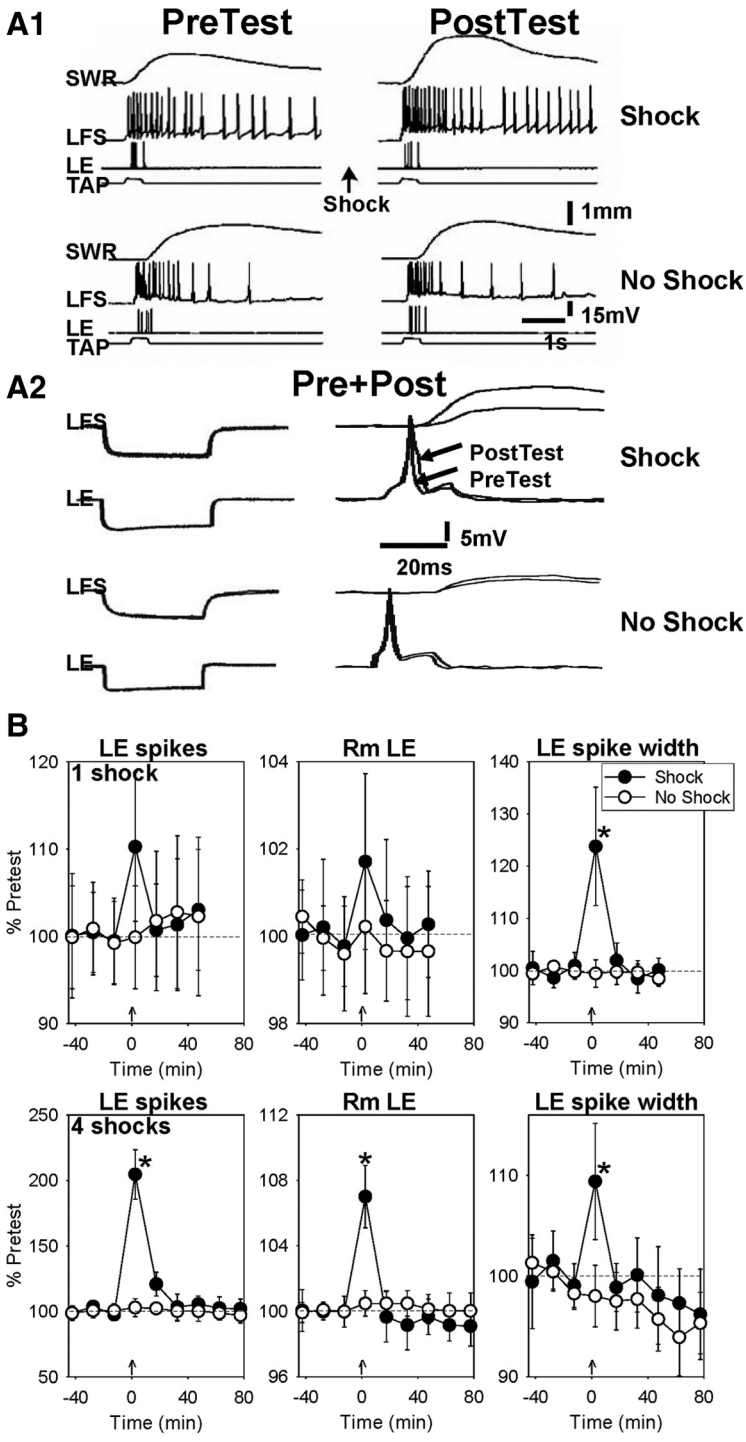

Figure 4. Changes in sensory neuron membrane properties during sensitization. ***A***, Examples of evoked firing, membrane resistance, and action potential duration of an LE siphon sensory neuron before (PreTest) and 2.5 min after (PostTest) one tail shock (top) or no shock control (bottom). ***B***, Average results from experiments like the ones shown in ***A*** with either a single shock ($n = 6$ for shock; $n = 6$ for no shock) or four shocks ($n = 7$ for shock; $n = 6$ for no shock). The overall average pretest values were 3.8 spikes for evoked LE firing and 2.3 ms for action potential duration, which were not significantly different in experiments with shock and no shock.

the LFS motor neurons and the amplitude of the sensory–motor neuron EPSPs.

We next investigated whether presynaptic or postsynaptic molecular mechanisms were involved in depression and facilitation of the EPSP during these forms of learning. Because dishabituation was blocked by bath application of the PKC inhibitor chelerythrine but not by inhibitors of PKA or CaMKII (Fig. 1*D*), we first examined the role of PKC. Injecting a peptide inhibitor of PKC (PKC 19-31) into the sensory neuron had no effect on depression of the EPSP during habituation but reduced facilitation of the EPSP during dishabituation over its entire time course ($p < 0.05$ for the inhibitor × shock interaction from 2.5 to 17.5 min after the shock) (Fig. 6*C*). In contrast, injecting BAPTA into the motor neuron had no effect on either depression of the EPSP during habituation or facilitation of the EPSP during dishabituation. As controls, the injections did not have significant effects on the LFS membrane resistance, the siphon withdrawal in response to the shock, or the pretest response on any measure. Injecting a single LE sensory neuron or LFS motor neuron also did not have a significant effect on the increase in siphon withdrawal during dishabituation, indicating that the preparations were otherwise healthy. These results suggest that presynaptic PKC contributes importantly to facilitation of the EPSP during behavioral dishabituation, even though it contributes very little during sensitization (Fig. 3*B*). On the other hand, postsynaptic Ca^{2+} contributes very little to facilitation of the EPSP during dishabituation, even though it contributes importantly during sensitization with the same tail shock (four shocks). Thus, whereas facilitation during intermediate-term sensitization is both presynaptic and postsynaptic, facilitation during dishabituation is presynaptic.

During these experiments, we also measured several properties of the LE siphon sensory neuron including evoked firing, membrane resistance, and action potential duration. None of those measures changed significantly during habituation or dishabituation (Fig. 6*B2*). Similarly, there were no significant changes in the late/peak ratio of the EPSP (Fig. 6*D*). Because all of these measures increased 2.5 min after the same shock during sensitization (Figs. 4*B*, 5*B*), these results suggest that prior habituation somehow blocks the increases. They also suggest that plasticity of the EPSP during habituation and dishabituation, like sensitization at times >2.5 min after the shock, may not be due to changes in either presynaptic spike width or postsynaptic AMPA receptor insertion, but rather involves some other mechanisms.

Discussion

Presynaptic and postsynaptic mechanisms contributing to intermediate-term sensitization

In agreement with previous studies (Antonov et al., 1999), our electrophysiological results provide strong support for the idea that plasticity of monosynaptic sensory–motor neuron EPSPs

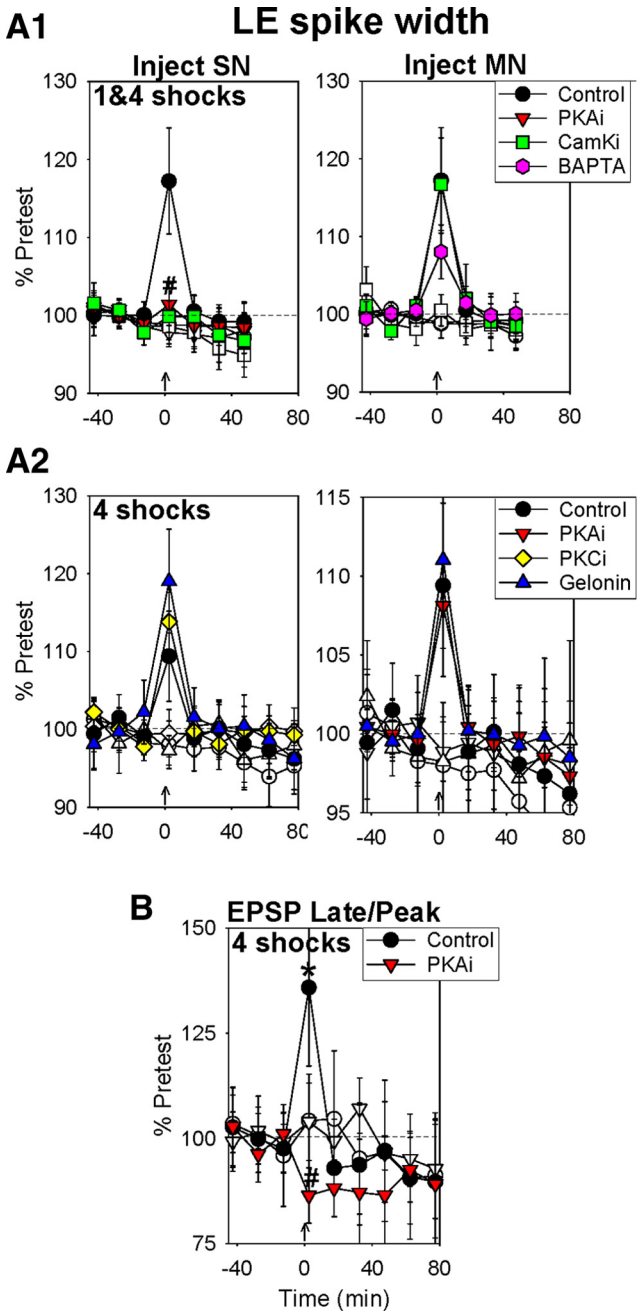

Figure 5. Molecular mechanisms of sensory neuron spike broadening and changes in the shape of the EPSP during sensitization. ***A1***, Average LE spike broadening by one and four tail shocks (colored symbols) and no shock controls (white symbols) following no injection (Control) or intracellular injection of a peptide inhibitor of PKA (PKAi), CaMKII (CamKi), or the Ca^{2+} chelator BAPTA into the sensory neuron (SN) or motor neuron (MN). Results with one and four shocks were similar and have been pooled. There was a significant main effect of group ($F_{(4,79)} = 2.73$, $p < 0.05$, $n = 22$, 18, 21, 19, and 19) and a marginal effect for the group × shock interaction ($F_{(4,79)} = 2.12$, $p < 0.10$) at 2.5 min. ***A2***, Average LE spike broadening by four tail shocks following no injection (Control) or intracellular injection of a peptide inhibitor of PKC (PKCi), PKA (PKAi), or the protein synthesis inhibitor gelonin into the sensory neuron or motor neuron ($n = 13$, 11,11, and 11). The overall average pretest value was 2.0 ms. ***B***, Average ratio of the late (75 ms after peak) and peak amplitude of the EPSP in experiments with four tail shocks (colored symbols) and no shock controls (white symbols). There was a marginal main effect of group ($F_{(1,19)} = 3.16$, $p < 0.10$, $n = 13$ and 10) and group × shock interaction ($F_{(1,19)} = 3.09$, $p < 0.10$) at 2.5 min. The overall average pretest value was 0.31, not significantly different in experiments with PKAi injections.

makes important contributions to behavioral habituation, dishabituation, and sensitization of the siphon withdrawal reflex. In addition, we now find that inhibitors of protein kinases and protein synthesis also have generally similar effects on behavior (Fig. 1) as they do on the monosynaptic EPSP (Figs. 3, 6), further supporting the idea that the two types of plasticity are causally related. However, in a few cases (all involving inhibitors of PKA), changes in behavior and the monosynaptic EPSP were dissociated. Although other explanations are possible, a likely explanation is that plasticity in the monosynaptic and polysynaptic pathways makes different (and perhaps opposing) contributions to the behavioral results in those cases (Mackey et al., 1987; Wright et al., 1991; Cohen et al., 1997).

We also began to define the behavioral conditions under which presynaptic and postsynaptic mechanisms contribute, which has been controversial (Kullmann and Siegelbaum, 1995; Roberts and Glanzman, 2003). We find that facilitation during short-term sensitization by one shock appears to be entirely presynaptic, and facilitation during intermediate-term sensitization by four shocks also involves presynaptic mechanisms. However, during intermediate-term sensitization the facilitation after 2.5 min also involves postsynaptic mechanisms. Furthermore, both the presynaptic and postsynaptic mechanisms are required, suggesting that they are more than additive and therefore interact in some way. These results are similar to those during short-term and intermediate-term facilitation *in vitro*, in which case spontaneous transmitter release from the presynaptic neuron recruits some of the postsynaptic mechanisms of intermediate-term facilitation (Jin et al., 2007, 2008).

One hypothesis that could account for our results with four shocks is that presynaptic mechanisms of short-term sensitization contribute 2.5 min after the shock and similarly recruit the postsynaptic mechanisms of intermediate-term sensitization, which are then responsible for maintaining the facilitation at later times. However, presynaptic injection of a protein synthesis inhibitor preferentially reduced facilitation after 2.5 min, suggesting that presynaptic mechanisms continue to contribute at later times. Furthermore, facilitation following one shock lasts 30 min and is entirely presynaptic. Thus, changing from one to four shocks may instead convert a purely presynaptic mechanism into one that is also dependent on postsynaptic processes, as occurs *in vitro* (Jin et al., 2004).

To begin to examine those alternatives, we investigated two possible mechanisms of expression of the facilitation: presynaptic spike broadening and postsynaptic membrane insertion of AMPA-type glutamate receptors. Although indirect, our results suggest that selective insertion of AMPA receptors does not make an important contribution to the facilitation during behavioral sensitization. Spike broadening is involved in facilitation 2.5 min after either one or four shocks but not at later times, when the facilitation presumably involves some third mechanism. Evidence from experiments on isolated cell culture suggests that modulation of the presynaptic Ca^{2+} conductance or transmitter release process could contribute (Zhao and Klein, 2002; Jin et al., 2006; Leal and Klein, 2009). Because the facilitation during sensitization by four shocks also requires postsynaptic mechanisms, a presynaptic mechanism of expression would imply signaling from the postsynaptic back to the presynaptic neuron, as occurs during long-term facilitation (Cai et al., 2008; Wang et al., 2009).

We found that presynaptic PKA and CaMKII both contribute to the spike broadening 2.5 min after either one or four shocks and to a spike broadening-independent mechanism of facilitation at later times, although inhibitors of CaMKII had additional effects that could complicate interpretation of its role. These results

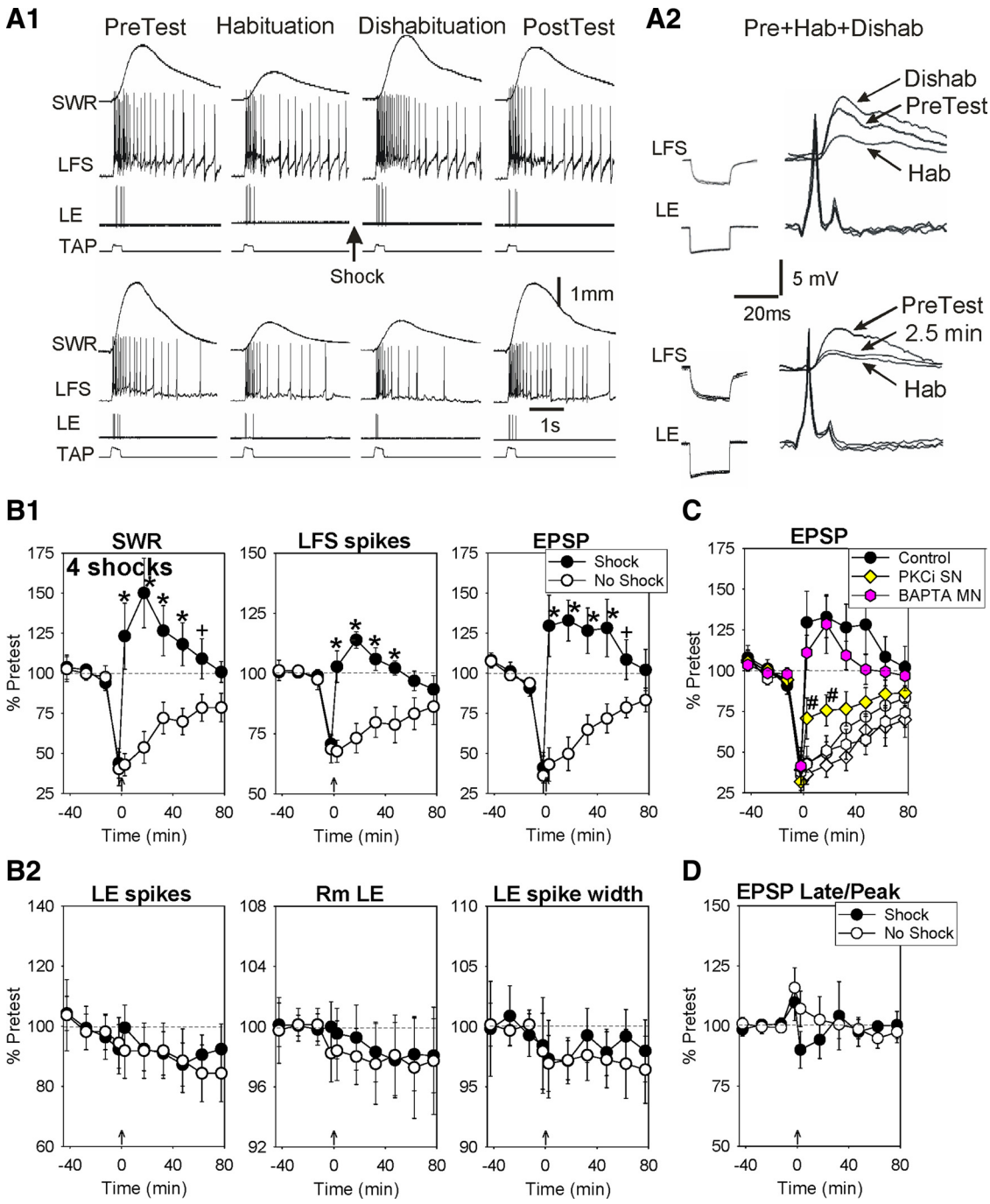

Figure 6. Cellular and molecular mechanisms involved in dishabituation. ***A***, Examples of siphon withdrawal (SWR), evoked firing and membrane resistance of an LE siphon sensory neuron and an LFS siphon motor neuron, and the monosynaptic EPSP from the LE sensory neuron to the LFS motor neuron before (PreTest) and at the end of a series of 10 siphon stimuli [Habituation (Hab)], and 2.5 min [Dishabituation (Dishab)] and 77.5 min (PostTest) after four tail shocks (top) or no shock control (bottom). ***B***, Average results from experiments like the ones shown in ***A*** ($n = 7$ for shock; $n = 6$ for no shock). ***B1***, Siphon withdrawal, evoked LFS firing, and EPSP amplitude. There were significant effects of habituation and shock in each case. The average pretest values were 2.0 mm for siphon withdrawal, 15 spikes for evoked LFS firing, and 6.6 mV for the amplitude of the EPSP, which were not significantly different in experiments with shock and no shock. ***B2***, Evoked LE firing, LE membrane resistance, and LE action potential duration. The average pretest values were 4.2 spikes for evoked LE firing and 2.4 ms for action potential duration, which were not significantly different in experiments with shock and no shock. ***C***, Average depression and facilitation of the EPSP in experiments with tail shock (colored symbols) or no shock (white symbols) following no injection (Control) or intracellular injection of a peptide inhibitor of PKC (PKCi) into the sensory neuron or BAPTA into the motor neuron. There was a significant overall effect of group for the facilitation during dishabituation ($F_{(2,29)} = 4.40, p < 0.05, n = 13, 11,$ and 11). The overall average pretest value was 6.6 mV. The average response to the tail shock was 5.0 mm. These values were not significantly different in experiments with different injections. ***D***, Average ratio of the late (75 ms after peak) and peak amplitude of the EPSP. The average pretest value was 0.56, which was not significantly different in experiments with shock and no shock.

are consistent with previous studies *in vitro* (Byrne and Kandel, 1996; Nakanishi et al., 1997; Angers et al., 2002; Jin et al., 2004) and suggest that the two kinase pathways may somehow be linked (Saitoh and Schwartz, 1983, 1985; Edmonds et al., 1990; Blitzer et al., 1998; Chang et al., 2000). In addition, during intermediate-term sensitization CaMKII has a postsynaptic role that is independent of PKA. In contrast, although PKC contributes to both presynaptic and postsynaptic mechanisms of intermediate-term

facilitation *in vitro* (Byrne and Kandel, 1996; Chitwood et al., 2001; Jin et al., 2004; Villareal et al., 2009), it did not make an important contribution to intermediate-term behavioral sensitization in our experiments.

Both presynaptic and postsynaptic protein synthesis also contribute to intermediate-term sensitization, similar to intermediate-term and long-term facilitation *in vitro* (Trudeau and Castellucci, 1995; Sherff and Carew, 2004; Villareal et al., 2007; Cai et al., 2008; Bailey et al., 2008) (I. Jin, personal communication). Protein synthesis during intermediate-term facilitation may be involved in "tagging" for long-term facilitation (Casadio et al., 1999; Sherff and Carew, 2004; Villareal et al., 2007; Wang et al., 2009), consistent with the idea that intermediate-term plasticity recruits some of the early steps in a program that can lead to stable synaptic growth during long-term plasticity (Bailey et al., 2008).

Mechanisms contributing to intermediate-term dishabituation and metaplasticity

Dishabituation differs from sensitization in that it is preceded by habituation and is thus a possible paradigm for metaplasticity. Historically, the relationship between mechanisms of sensitization and dishabituation has been controversial. Behavioral studies have suggested they may involve either the same or different processes (Carew et al., 1971; Marcus et al., 1988; Hawkins et al., 2006), but most studies of *in vitro* analogs of short-term sensitization and dishabituation have found that they involve different molecular mechanisms (Byrne and Kandel, 1996). However, it has not been known whether the different mechanisms *in vitro* contribute to synaptic plasticity *in vivo*, or whether these differences extend to the intermediate-term range: that is, whether intermediate-term dishabituation involves the same mechanisms as intermediate-term sensitization, or whether recruitment of those mechanisms is affected by prior habituation.

We have directly addressed both of those questions. First, we investigated cellular and molecular mechanisms contributing to facilitation during behavioral dishabituation. Our results suggest that, whereas facilitation of the EPSP during sensitization requires presynaptic PKA and CaMKII but not PKC (Fig. 3), facilitation during dishabituation requires presynaptic PKC (Fig. 6*C*). These results are similar to facilitation at rested and depressed synapses *in vitro* (Byrne and Kandel, 1996), in which cases presynaptic PKC is thought to contribute to vesicle mobilization that counteracts vesicle depletion during the depression.

In addition, we examined dishabituation produced by the same shock and in the same time range as intermediate-term sensitization. Our results suggest that intermediate-term dishabituation and sensitization differ from each other in several additional and unexpected ways at the cellular and molecular levels. First, the protein synthesis inhibitor emetine had no effect on dishabituation, even though emetine blocked most of sensitization with the same tail shock (Fig. 1*D*). Dishabituation is thus more similar to intermediate-term, site-specific sensitization, which is also PKC dependent but not protein synthesis dependent (Sutton et al., 2004).

Second, postsynaptic BAPTA had no effect on facilitation of the EPSP during dishabituation, even though postsynaptic BAPTA blocked facilitation during sensitization by the same tail shock (Figs. 3*B*, 6*C*). Therefore, whether postsynaptic mechanisms are recruited depends not only on the stage of plasticity, but also on the history of plasticity (rested or depressed) of the synapse. Recent results suggest that spontaneous transmitter release from the presynaptic neuron can recruit postsynaptic Ca^{2+}-dependent mechanisms of facilitation *in vitro* (Jin et al., 2007, 2008). Thus, one possibility is that depletion of the readily releasable pool of synaptic vesicles during habituation (Bailey and Chen, 1988) may reduce spontaneous transmitter release during dishabituation, so that the postsynaptic mechanisms are not recruited.

Third, we observed no spike broadening or other changes in LE membrane properties during dishabituation, even though the same shock produced reliable changes during sensitization (Figs. 4*B*, 6*B2*). These results suggest that the PKA pathway is somehow inhibited at depressed synapses, so that shock does not produce spike broadening and other changes in LE membrane properties. Prolonged exposure to 5HT can also inhibit the PKA pathway, in part at the level of adenylyl cyclase (Sugita et al., 1997). Recent results from experiments in cell culture suggest that this effect may be due to an increase in spontaneous release of glutamate, which then acts via type II metabotropic glutamate autoreceptors negatively coupled to adenylyl cyclase (I. Jin, personal communication). Thus, one possibility is that glutamate released during habituation inhibits presynaptic adenylyl cyclase in a similar way.

Additional experiments will be necessary to investigate these novel aspects of plasticity during dishabituation. However, they provide further support for the idea that sensitization and dishabituation can involve different mechanisms at the cellular and molecular levels, and extend that idea to the intermediate-term stage. In addition, they show that not only the mechanisms but also the site of plasticity during learning may depend on both the stage of plasticity and the history of plasticity. Two of the mechanisms that are involved in intermediate-term sensitization but not dishabituation (protein synthesis and recruitment of postsynaptic mechanisms) may be early steps in a program that can lead to stable synaptic growth during long-term sensitization. Therefore, it will now be interesting to perform similar studies of metaplasticity during long-term memory formation.

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