It will therefore be of theoretical interest to determine whether these advanced features of learning can be demonstrated in a simple behavior mediated by a neural circuit containing a small number of nerve cells.

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8. *Aplysia californica* (80 to 300 g) were used. The parapodia of all animals were surgically removed to permit complete visualization and direct access to the mantle shelf and siphon. All animals were then housed in individual pans in a 200-gallon aquarium for approximately 1 week before an experiment was begun. For details of housing and preparation of animals, see (*5*).
9. The tactile stimulus to the siphon was applied with a nylon bristle (*5*); the electrical stimulus to the mantle was a very weak electric shock (60 Hz a-c) with an intensity of approximately 15 mA for 0.5 second delivered in seawater with bipolar capillary electrodes separated by approximately 5 mm. The electrode was in series with a 10-kilohm resistor to provide relatively constant current. This level of shock was chosen because it produced siphon withdrawals comparable to those elicited by the tactile stimulus to the siphon. The US was delivered with spanning electrodes across the tail (*5*) and consisted of a 1.0-second pulse of 60-Hz a-c current at an intensity of 75 mA in seawater.
10. Siphon withdrawal was measured with a stopwatch from stimulus onset until the siphon stopped contracting and began to relax. All responses were measured (blind) by an observer who did not know the experimental history of the animals (*5*).
11. Both CS's and the US were delivered by hand, with the timing of the two stimuli guided by electronically controlled signals. Thus the interstimulus interval is approximate, although it was usually accurate to within 0.25 second. Moreover, the intertrial interval in group studies was also somewhat variable (between 5 and 6 minutes on average). In other experiments in which both the interstimulus and the intertrial intervals were precisely controlled (*18*), comparable results were obtained (Fig. $2B_2$).
12. For all experiments, the results of two independent replications were pooled. Data were analyzed for each animal by first computing a difference score for each pathway (each test score minus the pretest score for that pathway) and then determining the difference between CS+ and CS− scores. Thus each animal provided a single score that reflected the difference in that animal's response to the paired and unpaired conditioned stimuli. Since multiple statistical comparisons were not made, within-group and between-group statistical comparisons were made by means of *t*-tests for correlated means or independent means respectively. All probability values are two-tailed.
13. These results confirm earlier findings (*5*) of significant sensitization 30 minutes after 15 trials of US-alone training [see figure 6B in (*5*)]. In addition to sensitization (a generalized nonassociative increase in responsiveness), increased responsiveness in the CS− pathway may also be due to stimulus generalization. On the other hand the CS− pathway may underestimate sensitization because of habituation. Additional behavioral experiments are in progress to distinguish among these possibilities.
14. Sensitization can readily be observed after one trial in other experimental contexts such as when stronger unconditioned stimuli are used or when sensitization is superimposed on a previously habituated response (dishabituation) [T. J. Carew, V. F. Castellucci, E. R. Kandel, *Int. J. Neurosci.* **2**, 79 (1971); *Science* **205**, 417 (1979)].
15. An example of this can be seen by comparing the 15-trial data in Fig. 1D (where testing was done 30 minutes after training) with those in Fig. 2A (the same animals as in Fig. 1D 24 hours later); both sensitization and differential conditioning significantly increased. Similar though less dramatic results were also seen after five trials; no sensitization was evident 15 minutes after training, while modest sensitization was evident 24 hours later (Fig. 2A), and the differential effect also showed a slight increase.
16. The US-alone control serves as "pure" sensitization control (*13*) since this pathway has not been exposed to a CS during training.
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18. Electrodes were implanted in the siphon skin as described (*5*). Overlap in the receptive fields of the two sites (*17*) was minimized by implanting one electrode on the dorsal surface of the siphon (near the caudal margin of the shell) and the other in the base of the siphon (near the anus). For each site, current was passed between the implanted electrode and a ground electrode in the tank. Electrical stimulation consisted of a 0.5-second pulse of 60-Hz a-c current stepped down from a variable transformer and dropped across a 10-kilohm resistor in series with the electrode to produce relatively constant current. Currents used ranged between 5 and 10 mA. The site at the base of the siphon usually required slightly less current to produce a withdrawal comparable to that produced from the other site. Stimulus intensities were first adjusted to produce siphon withdrawal pretest scores from each site comparable to those in previous experiments (Figs. 1D and 2A) and then one site was randomly chosen to be CS+ or CS−. The intensity of the US [60-Hz a-c current for 1 second, delivered by a second pair of electrodes implanted in the tail (*5*)] was adjusted before training so that it produced a pronounced withdrawal of the whole animal. Typical current range for the US was 20 to 30 mA. Five trials were delivered (intertrial interval, 5 minutes), and testing was carried out 15 to 30 minutes after training. Timing of stimuli was controlled by a programmable timing device (*5*).
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20. A CS-US interval of 0.5 second produced optimal learning; an interval of 1.0 second produced significant but reduced learning; and intervals of 2.0, 5.0, and 10.0 seconds as well as backward intervals (US-CS pairings) of 0.0 and 0.5 second (US offset to CS onset) produced no learning (T. J. Carew, R. D. Hawkins, E. R. Kandel, in preparation).
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22. Supported by NIH career awards 5KO2 M0081 (to T.J.C.) and 5KO5 MH18558 (to E.R.K.), by NIH grants NS 12744 and GM 23540, and a grant from the McKnight Foundation. We thank L. Bursztajn for technical assistance, J. H. Schwartz for comments on an earlier draft of this manuscript, H. Ayers for manuscript preparation, and L. Katz and K. Hilten for the illustrations.

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A Cellular Mechanism of Classical Conditioning in *Aplysia*: Activity-Dependent Amplification of Presynaptic Facilitation

Abstract. *A training procedure analogous to differential classical conditioning produces differential facilitation of excitatory postsynaptic potentials (EPSP's) in the neuronal circuit for the siphon withdrawal reflex in* Aplysia. *Thus, tail shock (the unconditioned stimulus) produces greater facilitation of the monosynaptic EPSP from a siphon sensory neuron to a siphon motor neuron if the shock is preceded by spike activity in the sensory neuron than if the shock and spike activity occur in a specifically unpaired pattern or if the shock occurs alone. Further experiments indicate that this activity-dependent amplification of facilitation is presynaptic in origin and involves a differential increase in spike duration and thus in* Ca^{2+} *influx in paired versus unpaired sensory neurons. The results of these cellular experiments are quantitatively similar to the results of behavioral experiments with the same protocol and parameters, suggesting that activity-dependent amplification of presynaptic facilitation may make a significant contribution to classical conditioning of the withdrawal reflex.*

One of the major goals of neuroscience has been to specify the cellular mechanisms of associative learning. Although cellular correlates and analogs of associative learning have been observed in several vertebrate species (*1, 2*), it has been difficult to study these events with intracellular techniques, and it has been impossible to establish a causal relationship between the cellular events and behavior. In the last decade, neural correlates of associative learning have been

investigated in a number of invertebrate species in which intracellular analysis is more feasible, but the behaviors studied have generally been complex (*3, 4*). It has therefore not been possible, even in these cases, to specify a minimal neuronal circuit for the learned behavior, making a complete analysis of the cellular mechanisms of the learning difficult. Recently, classical conditioning of a very simple behavior, the gill and siphon withdrawal reflex of *Aplysia*, was demonstrated, with weak tactile stimulation of the siphon as the conditioned stimulus (CS) and electric shock to the tail as the unconditioned stimulus (US) (*5*). Carew *et al.* (*6*) extended this finding by demonstrating differential conditioning of the siphon withdrawal reflex. Thus, if stimulation of one site on the siphon or mantle shelf was temporally paired with tail shock (the US) while stimulation of another site was specifically unpaired with the tail shock, the withdrawal response to the paired CS showed significantly greater enhancement than did the response to the unpaired CS. The neuronal circuit for the withdrawal reflex has been described; identified siphon mechanoreceptor neurons make monosynaptic excitatory synaptic connections onto both gill and siphon motor neurons as well as onto interneurons that contribute to the reflex (*7–9*). Sensory neurons that mediate input from the tail have also been identified (*10*).

In addition to classical conditioning, the gill and siphon withdrawal reflex also exhibits sensitization, a nonassociative form of learning, when tail shock is presented either alone or unpaired with siphon stimulation (*5, 11*). The biophysical and molecular mechanisms of sensitization have been explored in some detail. Tail shock excites a group of facilitator neurons that are thought to be serotonergic, the L29 cells, that produce presynaptic facilitation of the siphon sensory neurons (*12, 13*). This facilitation is due to adenosine 3′,5′-monophosphate (cyclic AMP)–dependent protein phosphorylation (*14*), which produces a decrease in K^+ conductance, leading to an increase in the duration of action potentials in the sensory neurons. The increase in spike duration in turn leads to an increase in Ca^{2+} influx and increased transmitter release (*15, 16*). Because sensitization and classical conditioning both involve an increase in the response to one stimulus as a result of presentation of a second stimulus, it is attractive to think that classical conditioning might also involve presynaptic facilitation as a mechanism for strengthening the CS pathway. Conditioning and sensitization differ, however, in that conditioning requires that the two stimuli be presented in a temporally paired fashion, whereas sensitization does not. One mechanism that might confer temporal specificity on presynaptic facilitation would be enhancement or amplification of the facilitation by preceding spike activity in the facilitated neurons, as proposed by Kandel and Tauc (*17*). To examine whether this mechanism contributes to differential conditioning of the withdrawal reflex, we investigated whether an experimental protocol based on the one used in the conditioning experiments would produce differential facilitation of excitatory postsynaptic potentials (EPSP's) in the circuit for the withdrawal reflex. The experimental protocol and parameters were the same as in the behavioral experiments (*6*), except that we substituted intracellularly produced spike activity in two individual siphon sensory neurons for cutaneous stimulation of the siphon, and we measured the size of the EPSP's in a siphon motor neuron instead of siphon withdrawal. As in the behavioral experiments, we used a differential training procedure. Thus, spike activity in one sensory neuron was paired with the US, while spike activity in a second sensory neuron was specifically unpaired with the US. This procedure allowed us to subtract any nonspecific effects from the effect of temporal pairing in each experiment.

The preparation we used was the isolated central nervous system attached to the tail by the posterior pedal nerves (*18*). We measured the amplitudes of the

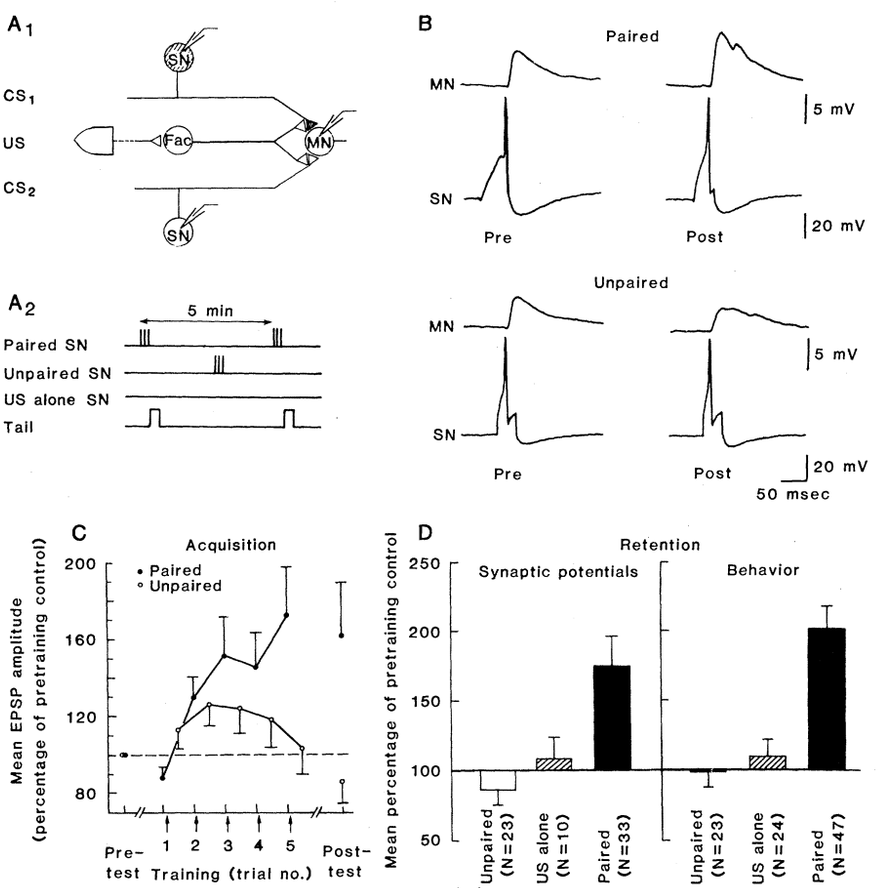

Fig. 1. Differential facilitation of monosynaptic EPSP's in the neuronal circuit for the withdrawal reflex. (A_1) Experimental arrangement and (A_2) training protocol. The US also excites the motor neurons through pathways that are not shown. Shading indicates that activity in the neuron is paired with the US. See text for details. (B) Examples of the EPSP's produced in a common postsynaptic siphon motor neuron (*MN*) by action potentials in a paired and an unpaired sensory neuron (*SN*) before (*Pre*) and 1 hour after training (*Post*). Facilitation of the EPSP from the paired sensory neuron is greater than that of the EPSP from the unpaired sensory neuron in the same experiment. (C) Average acquisition of differential facilitation in 23 experiments similar to the one shown in (B). Bars indicate S.E.M. Arrows show times at which the US (tail shock) was delivered. (D) Comparison of cellular data showing differential facilitation of EPSP's and behavioral data showing differential conditioning of the withdrawal reflex. PSP data are pooled from two types of experiments: paired versus unpaired (23 experiments) and paired versus US alone (ten experiments). Behavioral data are from experiments on conditioning of the withdrawal reflex with the same experimental protocol and parameters (*6*).

monosynaptic EPSP's produced in a common postsynaptic neuron by intracellular stimulation of each of two siphon sensory neurons both before and after training (*19*) (Fig. 1, A_1 and B). The two sensory neurons were chosen arbitrarily from an homogeneous cluster of siphon mechanoreceptor cells (*7*). In most experiments, the postsynaptic neuron was one of a group of recently identified siphon motor neurons (*20*), but in a few experiments it was L7, a gill and siphon motor neuron. During training each sensory neuron was stimulated intracellularly, causing it to fire a train of five action potentials once every 5 minutes. Stimulation of one of the sensory neurons immediately preceded shock to the tail or posterior pedal nerve, while stimulation of the other (unpaired) neuron followed tail shock by 2.5 minutes (*21*) (Fig. $1A_2$). On average, the EPSP's from both sensory neurons were facilitated during training, but facilitation of the EPSP from the paired neuron was greater than that of the EPSP from the unpaired neuron (Fig. 1C). In fact, while the EPSP from the unpaired neuron was usually facilitated early in training, it often began to decrease in amplitude toward the end of training, probably as a result of the dominance of homosynaptic depression caused by repeated firing of the sensory neuron (*7*). When tested either 5 or 15 minutes after a series of five training trials, the EPSP from the paired neuron was significantly facilitated compared to its pretraining amplitude, whereas the EPSP from the unpaired neuron was not significantly facilitated. In 23 experiments, the mean ± standard error of the mean (S.E.M.) was 161 ± 27 percent of pretraining control for the paired neurons ($t_{22} = 2.25$, $P < .05$) and 86 ± 11 percent for the unpaired neurons (*22*). Moreover, the percent change in the amplitude of the EPSP from the paired neuron was significantly greater than that for the EPSP from the unpaired neuron ($t_{22} = 2.77$, $P < .02$). This differential facilitation of EPSP's was maintained for at least 45 minutes after training in the six experiments in which all three neurons were held that long (Fig. 1B).

The EPSP's from both the paired and the unpaired neurons probably undergo a combination of facilitation (caused by the US) and synaptic depression (caused by repeated firing of the sensory neurons). Thus the differential effect shown in Fig. 1, B and C, could be due either to increased facilitation or to decreased depression of the EPSP's from the paired neurons, compared to those from the unpaired neurons. To distinguish between these possibilities, we carried out another series of experiments in which stimulation of one sensory neuron was paired with the US as before, but the other sensory neuron was not stimulated during training (it received US alone or sensitization training). Fifteen minutes after the end of training, facilitation of the EPSP from the paired sensory neuron was significantly greater than that of the EPSP from the sensory neuron receiving US-alone training. In ten experiments, the mean ± S.E.M. was 204 ± 36 percent of pretraining control for the paired neurons and 108 ± 16 percent for the US-alone neurons ($t_9 = 3.45$, $P < .01$). Since the EPSP's from neurons receiving US alone do not undergo synaptic depression during training, this differential effect can not be due to differential depression of the EPSP's. Rather, these results demonstrate that facilitation of the EPSP from a sensory neuron to a postsynaptic neuron is enhanced if the sensory neuron is active just before the facilitating stimulus (the US) is presented. The amplitude of this effect suggests that it can completely account for the differential facilitation of EPSP's from paired and unpaired sensory neurons shown in Fig. 1, B and C, although we cannot rule out the possibility that differential protection from synaptic depression might also contribute to those results.

These experiments demonstrate activity-dependent amplification of facilitation of the EPSP's from sensory neurons to motor neurons in the circuit for the siphon withdrawal reflex. The protocol and parameters used in these experiments are the same as those that have been used in behavioral experiments demonstrating differential conditioning of the withdrawal reflex (*6*). A comparison of the results of our cellular experiments with results from the behavioral experiments shows that they are similar quantitatively as well as qualitatively (Fig. 1D). This fit between the cellular and behavioral results and the fact that the sensory and motor neurons examined in this study mediate the withdrawal reflex suggest that activity-dependent amplification of facilitation may contribute to behavioral conditioning of the reflex (*23*).

Activity-dependent amplification of facilitation could result from either a presynaptic or a postsynaptic mechanism. Facilitation at these synapses underlying behavioral sensitization is presynaptic in origin (*12*) and is due to broadening of the action potentials, which leads to an increase in Ca^{2+} influx in the sensory neurons (*15*). We therefore investigated the possibility that this presynaptic mechanism might also be involved in the activity-dependent amplification of facilitation described above. We examined the durations of the action potentials in the sensory neurons in the presence of tetraethylammonium (TEA), which decreases K^+ current and thus broadens the action potential, making any changes in spike duration more apparent. Moreover, in the presence of TEA the late inward current during an action potential is carried predominantly by Ca^{2+} ions, so that a change in spike duration is indicative of a change in Ca^{2+} influx (*15*).

Our experimental protocol was similar to that described for the experiments demonstrating differential facilitation of EPSP's, except that we now (i) measured the durations of the action potentials in the sensory neurons instead of the amplitudes of the EPSP's in the motor neuron, (ii) carried out both training and testing with the abdominal ganglion (which contains the siphon sensory neurons) bathed in seawater containing 50 m*M* TEA, and (iii) increased the number of training trials from 5 to 15. We found that additional training trials were necessary to maximize the differential effect under these conditions, perhaps because the presence of TEA in the abdominal ganglion altered the efficacy of the CS or the US, or both, during training. As in the postsynaptic potential experiments, two arbitrarily selected siphon sensory neurons were stimulated intracellularly, causing them to fire action potentials once every 5 minutes. Stimulation of one sensory neuron immediately preceded tail shock, whereas stimulation of the other neuron was specifically unpaired with tail shock (*24*). Five to 15 minutes after a series of 15 training trials, the action potential in the paired neuron was significantly broadened compared to its pretraining duration, while the action potential in the unpaired neuron was not. In 21 experiments, the mean ± S.E.M. was 123 ± 9 percent of pretraining control for the paired neurons ($t_{20} = 2.45$, $P < .05$) and 98 ± 5 percent for the unpaired neurons. Moreover, the percent change in the duration of the action potential in the paired neuron was significantly greater than that for the unpaired neuron ($t_{20} = 3.15$, $P < .01$). This differential broadening of action potentials was maintained for at least 3 hours after training in the 12 experiments in which both the paired and unpaired neurons were held that long ($t_{11} = 2.31$, $P < .05$) (Fig. 2, A_1 and B). The action potentials in both neurons continued to broaden during the 3 hours after training; this

parallels results from behavioral experiments on conditioning of the withdrawal reflex (*5, 6, 25*). When we blocked Ca^{2+} current by bathing the ganglion in 15 m*M* $CoCl_2$ after training, the durations of the spikes in both neurons were greatly reduced, confirming that the inward current during the late phase of the action potential is carried by Ca^{2+} ions (Fig. $2A_2$). These results are consistent with the hypothesis that activity-dependent amplification of facilitation is presynaptic in origin and involves a differential increase in spike duration and Ca^{2+} influx in paired vesus unpaired sensory neurons.

An increase in spike duration (and hence in Ca^{2+} influx) may have as its primary cause either an increase in Ca^{2+} conductance or a decrease in K^+ conductance. Klein and Kandel (*16*) found that presynaptic facilitation underlying sensitization of the withdrawal reflex is due to a decrease in K^+ conductance, which is reflected in a decrease in the outward current elicited by depolarizing pulses in voltage-clamp experiments. Using an experimental protocol similar to that described above for the postsynaptic potential experiments (with five training trials in normal seawater), we found a significantly greater decrease in the outward current in paired than in unpaired sensory neurons under voltage clamp (*26*). This result supports the conclusion from the spike broadening experiments that activity-dependent amplification of facilitation has a presynaptic mechanism. Since the pulse parameters used in this experiment were chosen to maximize the contribution of the K^+ conductance that is modulated during normal presynaptic facilitation, this finding also suggests that the mechanism of differential spike broadening is a differential decrease in K^+ conductance in paired versus unpaired sensory neurons, although it does not rule out the possibility of a differential increase in Ca^{2+} conductance.

The results of these experiments suggest that activity-dependent amplification of presynaptic facilitation (Fig. $2C_1$) is a mechanism of classical conditioning of the withdrawal reflex. However, at least two other types of cellular mechanisms could also explain conditioning of this reflex. First, the CS and US pathways might converge on facilitator neurons in such a way that paired presentation of the CS and US produces substantially greater firing of the facilitators and therefore greater presynaptic facilitation than unpaired presentation of the two stimuli (Fig. $2C_2$). That it is possible to produce differential facilitation of two arbitrarily chosen siphon sensory neurons makes this mechanism unlikely, since it would require a separate facilitator neuron for each sensory neuron. Furthermore, in experiments in which we recorded from identified facilitator neurons in a semi-intact preparation, we found that paired presentation of the CS and US produced no more total firing of the facilitators than did unpaired presentation of the two stimuli (*27*). Thus our evidence does not support a summation

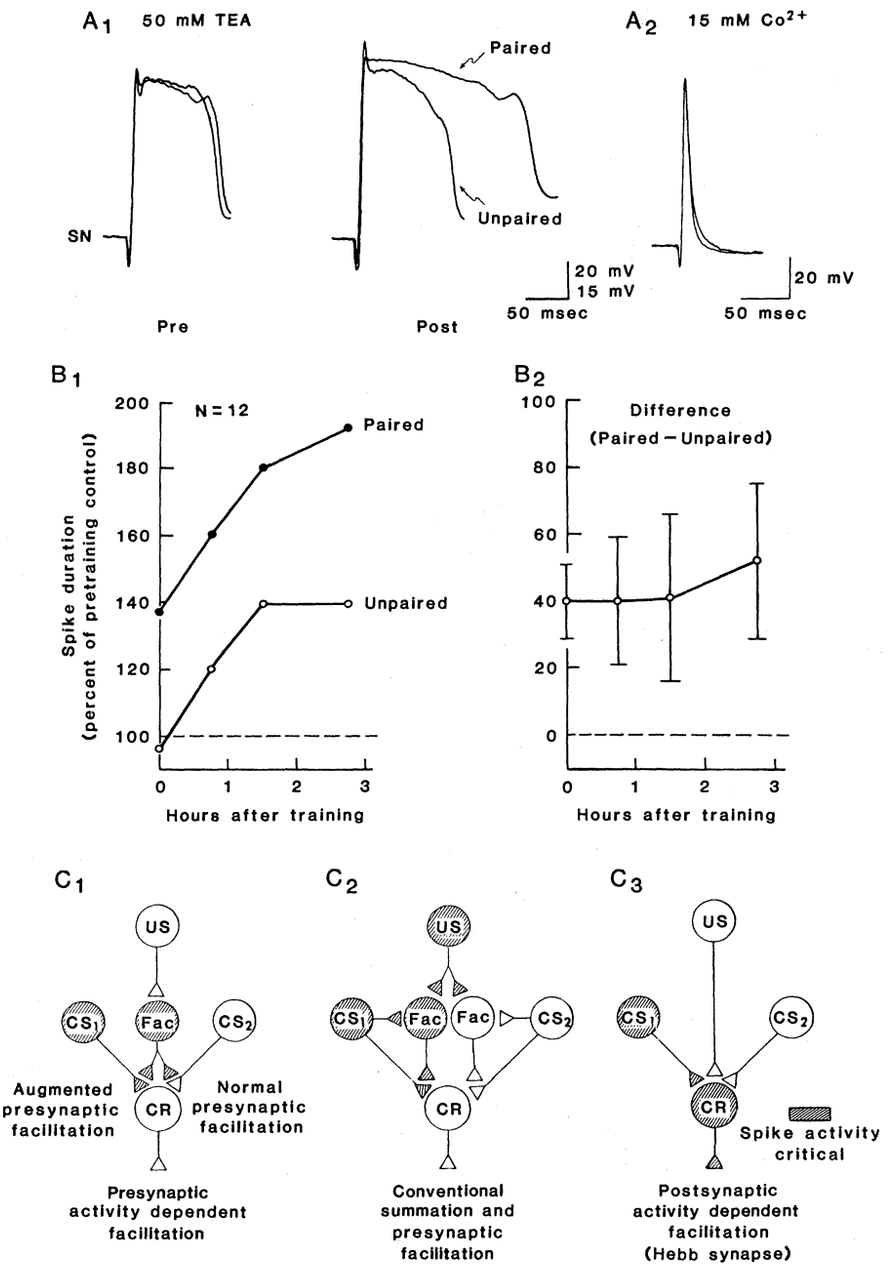

Fig. 2. Differential broadening of the action potentials in two sensory neurons in the presence of 50 m*M* tetraethylammonium (TEA). (A_1) Examples of the action potentials in a paired and unpaired sensory neuron before (*Pre*) and 3 hours after training (*Post*). The action potentials in the two neurons have been superimposed. Broadening of the action potential in the paired neuron is greater than that of the action potential in the unpaired neuron in the same experiment. (A_2) The action potentials in paired and unpaired neurons with 15 m*M* $CoCl_2$ added to the bath after the 3-hour posttest, from another experiment with action potentials similar to those shown in (A_1). (B_1 and B_2) Average differential spike broadening and time course of retention in 12 experiments in which both neurons were held for at least 3 hours after training. (B_1) Average spike broadening in the paired and unpaired neurons. (B_2) Average difference between the spike broadening in the paired neuron and that in the unpaired neuron in the same experiment (from the experiments shown in B_1). Bars indicate S.E.M. (C_1, C_2, and C_3) Diagram of activity-dependent presynaptic facilitation and two other possible cellular mechanisms of classical conditioning. These mechanisms require the occurrence of temporally paired spike activity in the shaded neurons. In (C_2), paired activity in neurons CS_1 and US causes firing of the left (but not the right) facilitator neuron. See text for discussion.

mechanism, although we cannot rule out the possibility that summation in interneurons might contribute to the behavioral conditioning. A second mechanism that might explain the temporal specificity of conditioning was first proposed by Hebb (*28*). In the Hebb model (Fig. $2C_3$), the strength of a particular synaptic connection is increased if use of that synapse contributes to the occurrence of an action potential in the postsynaptic (CR) neuron. Thus, if stimulation of a CS neuron is immediately followed by a US that causes neuron CR to fire, the CS-CR synapse will be strengthened. This mechanism is not necessary for conditioning to occur in our system, since in some of our experiments the postsynaptic neuron was held at a hyperpolarized level and did not fire any action potentials in response to the US. Furthermore, this mechanism is not sufficient, since intracellular stimulation of the postsynaptic neuron does not serve as an effective US (*29*).

Our experiments indicate that the mechanism of classical conditioning of the withdrawal reflex may simply be an elaboration of the mechanism of sensitization of the reflex, namely, presynaptic facilitation caused by an increase in action potential duration and Ca^{2+} influx in the sensory neurons. These experiments also suggest that the pairing specificity characteristic of classical conditioning results from amplification of the facilitation by temporally paired spike activity in the sensory neurons. We do not know which aspect of the action potential in a sensory neuron interacts with the process of presynaptic facilitation to amplify it. Four possibilities are depolarization, Na^+ influx, Ca^{2+} influx, and K^+ efflux. We also do not know which step in the biochemical cascade leading to presynaptic facilitation is sensitive to one or more of these aspects of the action potential. As a working hypothesis we propose that the influx of Ca^{2+} with each action potential may provide the signal for activity and that it may interact with the serotonin-sensitive adenylate cyclase in the terminals of the sensory neuron so that the cyclase subsequently produces more cyclic AMP in response to serotonin (*30*). Alternatively, the catalytic unit of the cyclase, the regulatory unit (which couples the catalytic unit to the serotonin receptor) and the receptor itself, all of which are membrane-associated proteins, may be modulated by the transmembrane voltage changes that occur during an action potential.

An attractive feature of the hypothesis that activity-dependent presynaptic facilitation is a mechanism of conditioning is that it is a type of mechanism which could be very general, for three reasons. First, it requires little special circuitry since the mechanism of pairing specificity is intrinsic to neurons in the CS pathway. The minimum requirements of this model are (i) facilitatory neurons, such as the L29 cells, which are excited by motivationally significant stimuli and which may project very diffusely (in principle, a single L29 neuron that produced facilitation in all of the sensory neurons would be sufficient to explain our results) and (ii) differential activity in the neurons that receive facilitatory input. We do not feel that there is anything unique about the siphon sensory neurons and believe that this mechanism is likely to operate throughout the nervous system wherever these two requirements are satisfied. Indeed, Walters and Byrne (*31*) report independent and similar results in another group of neurons in *Aplysia*. This mechanism may also operate in the vertebrate nervous system, with the diffusely projecting aminergic or cholinergic systems playing the role that the L29 neurons play in the abdominal ganglion of *Aplysia*.

Second, the mechanism that we propose underlies activity dependence of presynaptic facilitation is amplification of a cyclic AMP–dependent decrease in K^+ conductance by the voltage or ion fluxes that occur during an action potential. If the same mechanism occurred in locations other than the terminals of the sensory neurons, it could produce other aspects of learned behavior—for instance, if it occurred in the integrative region of interneurons or motor neurons it could produce features of operant conditioning (*32*). This speculation is rendered more plausible by the fact that cyclic nucleotides and decreases in K^+ conductance have been implicated in both classical and operant conditioning in other preparations (*4, 33*). Of particular relevance are experiments on *Drosophila* which show that both sensitization and avoidance conditioning are affected by mutations that alter cyclic AMP metabolism (*34*).

Finally, the aspects of a mechanism of associative learning that are most likely to be general phylogenetically are those at the fundamental molecular and biophysical level. Activity-dependent amplification of presynaptic facilitation probably involves the same cascade of biochemical and biophysical processes that mediate conventional presynaptic facilitation at the sensory neuron synapses: cyclic AMP–dependent protein phosphorylation and decreased ionic conductance. These processes are widespread phylogenetically and seem to be highly conserved (*35*). It thus seems possible that activity-dependent amplification of these processes may have similar generality. If so, it could provide a mechanism of conditioning in a wide range of species, including vertebrates.

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18. *Aplysia californica,* weighing 100 to 300 g (supplied by Pacific Biomarine, Venice, Calif.), were anesthetized with isotonic $MgCl_2$ (50 percent of body weight) before surgery. The central nervous system (the pedal, pleural, cerebral, and abdominal ganglia and connectives) was dissected free from all of the body except the tail, to which it was left attached by the posterior pedal nerves. The tail was then separated from the rest of the body (which was discarded) by a transverse cut at the level of the posterior insertion of

the parapodia. The abdominal ganglion, which contains the siphon sensory and motor neurons, was partially desheathed, and the preparation was thoroughly washed with normal artificial seawater (Instant Ocean) before the experiment was begun.

19. Standard electrophysiological techniques were used (*7–9*). Sensory neurons and motor neurons were impaled with single-barreled glass microelectrodes filled with 2.5*M* potassium chloride and beveled to a resistance of 10 to 20 megohms. A Wheatstone bridge circuit was used for recording while passing current either to depolarize neurons (the sensory cells) or to hyperpolarize them (the motor neuron). In experiments in which the motor neuron was hyperpolarized to prevent it from firing, it was held at the same level while testing the PSP's from the two sensory neurons before and after training.
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21. The siphon sensory neurons are a cluster of about 24 cells that have similar properties and synaptic connections (*7*). These cells do not receive conventional excitatory synaptic input from each other or from the US (tail shock). Two neurons were picked arbitrarily and assigned to be paired or unpaired in such a way as to balance the average amplitudes of the paired and unpaired EPSP's before training (4.2 ± 0.7 mV for the paired neurons and 4.9 ± 0.8 mV for the unpaired neurons). Typically each sensory neuron was made to fire a train of five action potentials by intracellular injection of 40- to 50-msec depolarizing current pulses at 10 Hz. Current intensity was adjusted so that each current pulse produced one action potential. Two types of US were used: either 1.5 seconds of 50 mA, 60-Hz, a-c shock delivered to the tail through bipolar capillary electrodes, or a 1.5-second, 10-Hz train of 3-msec pulses delivered to the posterior pedal nerves through bipolar Ag-AgCl electrodes. The intensity of the tail or posterior pedal nerve stimulation was sufficient to produce brisk firing in the postsynaptic neuron when it was not hyperpolarized. Onset of the US coincided with offset of intracellular stimulation of the paired neuron and followed stimulation of the unpaired neuron by 2.5 minutes.
22. The summary statistics given are means and standard errors of the means. In all cases statistical comparisons are *t*-tests for correlated means. Thus each experimental score was compared to a control value in the same experiment. For within-cell comparisons, the control value was the pretest score, while for between-cell comparisons, the control value was the score for the other cell in the experiment. The EPSP's from the paired and unpaired neurons were tested 2.5 minutes apart, either 5 or 15 minutes after the last US. The order in which the two PSP's were tested was counterbalanced. Only the monosynaptic component of the EPSP (as judged by short and constant latency and smooth rise) was measured, although additional changes were sometimes observed in the polysynaptic response.
23. The degree of correlation between the cellular data and the behavioral data is better than might be expected, since in one case we measured EPSP amplitude and in the other case we measured duration of siphon withdrawal. However, a good correlation between these two measures has also been observed in previous experiments [for example, T. J. Carew and E. R. Kandel, *Science* **182**, 1158 (1973)] and could be explained by several possible mechanisms: (i) Frost *et al.* (*20*) identified interneurons in the siphon withdrawal circuit that fire an extended train of spikes in response to brief excitatory synaptic input from the sensory neurons. An increase in the amplitude of synaptic potentials from the sensory neurons could produce an increase in the duration of the resulting train of spikes in some of these interneurons, thus producing an increase in the duration of siphon withdrawal. (ii) The same cellular change that underlies increased transmitter release from the sensory neurons (for example, a decrease in K^+ conductance) could also lead to repetitive firing of the sensory neurons in response to a brief tactile stimulus. (iii) The activity-dependent change that occurs in the sensory neurons could occur in some interneurons as well, and might prolong interneuronal firing in a similar fashion. These possibilities are neither mutually exclusive nor exhaustive.
24. The abdominal ganglion was placed in a small well and perfused with TEA solution while the rest of the preparation was perfused with normal seawater. The pleural-abdominal connectives were led through a silicon grease seal under the walls of the well. Sensory neurons were assigned to be paired or unpaired in such a way as to balance the durations of their action potentials before training (71 ± 9 msec for the paired neurons and 77 ± 10 msec for the unpaired neurons). Intracellular stimulation consisted of a single 5-msec depolarizing pulse. This typically produced a burst of several spikes in the sensory neuron, of which only the first was measured. The value of the spike duration for the pretest and each of the posttests was the mean of three measurements taken at 5-minute intervals. The US was 1.5 seconds of 50 mA, 60-Hz a-c shock to the tail. Onset of the US followed intracellular stimulation of the paired neuron by 500 msec and followed stimulation of the unpaired neuron by 2.5 minutes
25. Continued broadening of the action potentials may have a number of possible explanations including (i) the onset of a process of consolidation of the memory into a long-term form, (ii) recovery from spike narrowing caused by repeated stimulation of the sensory neurons during training, (iii) prolonged exposure of the neurons to TEA, or (iv) progressive deterioration of the preparation. Preliminary experiments indicate that US-alone training does not produce continued broadening, which suggests that it may be due to recovery from spike narrowing.
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32. A synaptically induced long-lasting decrease in K^+ conductance in an interneuron or motor neuron would lead to (i) a long-lasting increase in the ease with which the neuron could be excited by synaptic input [T. J. Carew and E. R. Kandel, *J. Neurophysiol.* **40**, 721 (1977)], and (ii) a long-lasting increase in the firing rate or bursting frequency of a spontaneously active neuron. A consequence of the mechanism we are proposing is that these increases would be amplified if the neuron happened to be firing action potentials at the time of the modulatory synaptic input. This result would correspond formally to (and could underlie) an increase in the probability, rate, or frequency of a behavior by contingent reinforcement of that behavior.
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Associative Conditioning of Single Sensory Neurons Suggests a Cellular Mechanism for Learning

Abstract. *A cellular analog of associative learning has been demonstrated in individual sensory neurons of the tail withdrawal reflex of* Aplysia. *Sensory cells activated by intracellular current injection shortly before a sensitizing shock to the animal's tail display significantly more facilitation of their monosynaptic connections to a tail motor neuron than cells trained either with intracellular stimulation unpaired to tail shock or with tail shock alone. This associative effect is acquired rapidly and is expressed as a temporally specific amplification of heterosynaptic facilitation. The results suggest that activity-dependent neuromodulation may be a mechanism underlying associative information storage and point to aspects of subcellular processes that might be involved in the formation of neural associations.*

The use of intracellular techniques to investigate neuronal changes produced by classical and operant conditioning paradigms (*1*) encourages the belief that mechanisms of associative information storage can be analyzed on the cellular level. Because associative learning is usually quite sensitive to motivational and attentional factors, some psychologists have assumed that the formation of associations depends, in part, upon the contiguous activation of sensory "analyzers" and modulatory "arousal centers" (*2*). We have tested this general idea on the neuronal level in the mollusk *Aplysia californica* by examining the associative interaction of electrophysiological activity in individual sensory neurons with neuromodulatory concomitants of defensive arousal. Our results suggest a cellular mechanism for associative information storage, activity-dependent neuromodulation, that may be of general significance.

We applied a cellular analog of a differential classical conditioning procedure simultaneously to three mechanoafferent neurons—which innervate the tail (*3*)—in the left pleural ganglion of each animal. Because noxious tail stimulation produces defensive arousal and modulates (heterosynaptically facilitates) (*3, 4*) synaptic connections of these sensory neurons, we used tail shock as the reinforcing or unconditioned stimulus (US) (*5*). Training and testing were conducted in a reduced "split-foot" preparation (*6*). Associative and nonassociative effects of training were measured by testing the amplitude of single monosynaptic excit-