Mechanoafferent Neurons Innervating Tail of *Aplysia*. II. Modulation by Sensitizing Stimulation

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SUMMARY AND CONCLUSIONS

1. The tail-withdrawal reflex of *Aplysia* can be sensitized by weak stimulation of a site outside the site used to test the reflex or by repeatedly stimulating the test site itself. The sensitization of tail-withdrawal responses is associated with enhanced activation of the tail motor neurons and heterosynaptic facilitation of the monosynaptic connections between the tail sensory neurons and tail motor neurons. This synaptic facilitation can occur under conditions in which neither posttetanic potentiation nor generalized changes in postsynaptic input resistance contribute to the facilitation. In addition to producing monosynaptic excitatory postsynaptic potentials (EPSPs), action potentials in tail sensory neurons often recruit longer latency polysynaptic input to the tail motor neurons during sensitization.

2. Strong, noxious tail shock similar in intensity to that used previously for sensitization and aversive classical conditioning of other responses in *Aplysia* produces more heterosynaptic facilitation than does weak sensitizing stimulation. Heterosynaptic facilitation builds up progressively with multiple trials and lasts for hours.

3. Very strong shocks to the tail can change the response characteristics of tail sensory neurons so that a prolonged, regenerative burst of spikes is elicited by a brief intracellular depolarizing pulse. This burst-

ing response produced by sensitizing stimulation has not been described previously in *Aplysia* sensory neurons and can greatly amplify the synaptic input to tail motor neurons from the sensory neurons. In addition, strong shocks to the tail increase the duration and magnitude of individual sensory neuron action potentials.

4. Sensitizing tail stimulation usually produces long-lasting depolarization of the tail motor neurons and often long-lasting hyperpolarization of the tail sensory neurons. The tail motor and sensory neurons show both increases and decreases of input resistance following sensitizing stimulation. However, the small, occasional increases in input resistance of the motor neuron are insufficient to explain the heterosynaptic facilitation produced by sensitizing stimulation.

5. Serotonin (5-HT) application can mimic many of the effects of sensitizing tail shock, including facilitation of both tail withdrawal and the monosynaptic connections between tail sensory and motor neurons, hyperpolarizing and depolarizing responses in the tail sensory neurons, and an increase in the duration and magnitude of the sensory neuron action potential. In the nearly isolated sensory neuron soma, 5-HT usually produces a slow, decreased conductance depolarizing response, suggesting that the 5-HT-induced hyperpolarizing response seen in the semi-intact preparation is produced indirectly through interneurons.
INTRODUCTION

Behavioral sensitization is a simple form of learning in which a novel or motivationally significant stimulus enhances reflex responsiveness. Although a common form of behavioral modification (30), neural mechanisms contributing to sensitization have been investigated in relatively few preparations (18, 20) and only in the gill- and siphon-withdrawal reflex of *Aplysia* have cellular mechanisms been correlated with behavioral sensitization (10, 14, 16). In the preceding paper (39) we described a newly identified population of mechanoafferent neurons in the ventrocaudal (VC) cluster of each pleural ganglion that innervates the tail of *Aplysia*. In the present paper we examine various changes in these sensory neurons that accompany sensitization of the tail-withdrawal reflex. The possible contribution of plastic changes in the tail sensory neurons to sensitization in *Aplysia* is of interest for two reasons. First, the tail sensory neurons offer the first opportunity to test and extend models of the cellular mechanisms of sensitization that have been proposed on the basis of studies of the *Aplysia* siphon sensory neurons (5, 14–16, 23–25). Second, noxious stimulation of the tail has been used as the reinforcing stimulus in analyses of mechanisms of classical conditioning of gill and siphon withdrawal (11, 13, 19) as well as for analyzing the mechanisms of cellular analogs of conditioning (9, 38) and a complex form of sensitization (40). Consequently, possible changes in the functional effectiveness of the tail sensory neurons during sensitization stimulation may be important for understanding changes that occur during various forms of learning and plasticity in *Aplysia*.

METHODS

The general methods used in this study were described in the preceding paper (39). In experiments in which the membrane potential was controlled or the input resistance of the sensory or motor neurons was measured, each cell was impaled with two independent microelectrodes, one for recording and one for injecting current. In most of these experiments a reduced tail-withdrawal preparation was used, consisting of the left pedal and pleural ganglia connected to the tail solely by the left posterior pedal nerve (LP9; see Ref. 39).

In several experiments (Figs. 1 and 9) tail withdrawals were elicited by weak electrical test stimuli applied through silver electrodes implanted in the left side of the tail. Each stimulus consisted of a 70-ms 60-Hz AC train of about 2 mA. Test stimuli were repeated at 5-min intervals. The sensitizing von Frey hair was applied 3 min after the fourth test (Fig. 1). In a separate experiment 5-HT was used to mimic the sensitizing stimuli used in other experiments and was applied 4 min after the third test (Fig. 9).

In the experiments illustrated in Figs. 2 and 3, sensitizing stimulation (which also served as test stimulation) consisted of an 0.8-s train of 5-ms square-wave pulses (6 Hz) delivered to the tail via implanted silver electrodes every 60 s. The shock intensity was set by applying progressively more intense stimuli to the tail until the threshold for the resulting EPSPs in the motor neuron was reached. This voltage was then doubled, and after 2 min the sensitization training procedure was begun using this new intensity. In other experiments the same methods were used to determine more-intense shock intensities (approximately 5 times the threshold for eliciting EPSPs in the motor neuron from tail stimulation in Figs. 4 and 5 and approximately 10 times this threshold in Fig. 6); 1.5-s trains of 60-Hz AC electric shock were used to test threshold. During sensitization training the sensitizing stimuli were delivered at 5-min intervals.

In this study only tail mechanoefferent neurons in the left pleural ganglion were examined, and these were identified by the criteria established in the preceding paper (39). In every case the putative tail mechanoefferent met the following criteria: 1) Location of the orange, dark-rimmed soma (40–80 μm diameter) within the VC cluster, on or near its medial border. 2) A resting potential between −35 and −55 mV. 3) A complete lack of spontaneous excitatory input or activity. 4) An intracellularly stimulated action potential between 50 and 80 mV in amplitude, followed immediately by a rapid afterhyperpolarization ranging between 2 and 10 mV, and finally a slower afterdepolarization, which could be 0–4 mV in amplitude (all amplitudes relative to initial resting potential). 5) The eliciting by intracellular stimulation of short-latency (5–8 ms) EPSPs in identified tail motor neurons P5, P6, or P7 in the left pedal ganglion. In many cases, additional evidence for the identity of these cells with the tail mechanoefferents described in the preceding paper (39) was obtained by showing hyperpolarizing responses to cutaneous stimulation, a restricted excitatory receptive field on the tail, or an all-or-none impulse in response to posterior pedal nerve stimulation.

The presumptive tail motor neurons (P5, P6, and P7) were identified by the following criteria
(Ref. 39 and unpublished observations). 1) Location of the relatively pale soma (100–150 μm diameter) on the track of axons converging into the pedal-pleural connective (see Fig. 3 in Ref. 39). 2) A resting potential between −45 to −65 mV. 3) Spontaneous excitatory input. 4) Powerful excitatory input in response to weak mechanical or electrical stimulation of the tail. 5) The eliciting by intracellular stimulation of strong longitudinal contractions of the ipsilateral side of the tail. P5 causes contraction of the tail tip, P6 contracts the entire tail, and P7 causes contraction of the base of the tail.

In experiments examining the effects of serotonin (5-HT) on tail sensory neuron properties three preparations were used. 1) A semi-intact preparation (Figs. 9, 10A) similar to the one described in the preceding paper (39) with an inner chamber to separate the central nervous system (CNS) from the peripheral tissues (connected to the CNS by the left posterior pedal nerve traversing a small slot, which was sealed with Vaseline). Each compartment had its own perfusion system. This preparation allowed pharmacological isolation of the CNS from the periphery while allowing continued neural interaction between the two parts of the preparation. 2) An isolated left pleural-pedal ganglia preparation (Fig. 10B) in which all nerves and connectives (except the left pedal-pleural connective) were excised. 3) A nearly isolated sensory cell soma preparation (Fig. 10C) in which part of the pleural ventrocaudal (PlVC) cluster (39) was undercut with a fine blade and removed from the pleural ganglia. This procedure usually freed a group of 10–20 sensory cell somata that was then pinned to a piece of fine filter paper. No other cell bodies and apparently very little neuropil were brought along. While not a perfectly isolated soma preparation, this preparation probably excludes most of the synaptic inputs to the sensory cell soma.

Serotonin (5-hydroxytryptamine creatinine sulfate complex or 5-HT) and 4-aminopyridine (4-AP) were obtained from Sigma Chemical Co., St. Louis, MO. Tetraethylammonium chloride (TEA) was obtained from Eastman Kodak Co., Rochester, NY. All solutions were made up in artificial seawater (Instant Ocean, Aquarium Systems, Eastlake, OH).

Unless otherwise noted, numerical data are expressed as the mean ± SE. Statistical comparisons were made using a two-tailed t test for correlated means.

RESULTS

Sensitization and heterosynaptic facilitation from weak tail stimulation

As described in the previous paper (39), the tail-withdrawal reflex involves the activation of mechanosensory neurons in the pleural VC cluster. These tail sensory neurons make direct and indirect synaptic connections to identified tail motor neurons that cause the tail to contract. We have found that synaptic connections from the tail sensory neurons are facilitated heterosynaptically during sensitization of the tail-withdrawal reflex produced by weak mechanical or electrical stimulation of the tail. Heterosynaptic facilitation is seen during two types of sensitization: 1) sensitization produced by stimulation outside the site used to test the reflex and 2) sensitization produced by repeated stimulation of the test site itself.

SENSITIZATION FROM STIMULATING OUTSIDE TEST SITE. To examine the sensitizing effect of stimulation applied at a distance from the test site, we repeatedly delivered a subthreshold test stimulus to the tail and asked whether a sensitizing stimulus applied to another site on the tail would bring the test response above threshold. The test stimulus was a very weak electric shock delivered to the lower left margin of the tail at 5-min intervals. The intensity of this shock was chosen so that it elicited synaptic input to a tail motor neuron being recorded from but did not reach threshold for evoking a measurable withdrawal of the tail (Fig. 1). To allow measurement of EPSP amplitude without superimposed action potentials, the motor neuron was hyperpolarized 30 mV from resting potential by passing current through a second microelectrode. By selecting a very weak subthreshold (for tail withdrawal) test stimulus we could examine the effects of a superimposed sensitizing stimulus applied to another site on the tail without the complication of additional sensitization produced by the test stimulus itself. After four tests without any withdrawal responses or change in the amount of synaptic input to the tail motor neuron in response to the test shock (Fig. 1A, trial 4), a weak mechanical stimulus (5-g von Frey hair) was briefly applied to the dorsal surface of the tail (about 5 cm away from the test site). This weak sensitizing stimulus elicited a withdrawal of the tail that lasted about 30s. When the next electrical test stimulus was delivered (2 min after the sensitizing mechanical stimulus), a large withdrawal of the tail resulted (lasting 25 s) that was associated with a large increase in synaptic input to the tail motor neuron (Fig. 1A, trial 5).
FIG. 1. Sensitization and synaptic facilitation produced by a weak mechanical stimulus. A: test responses of the tail and a tail motor neuron 3 min before (trial 4) and 2 min after (trial 5) brief application of a 5-g von Frey hair as a sensitizing stimulus to the tail. The test stimulus was a 70-ms shock (approximately 1 mA) delivered at 5-min intervals through an insulated electrode implanted in the tail. The sensitizing stimulus was applied after four tests in which neither a withdrawal response nor a change in the motor neuron response was observed. In order to reduce the likelihood of action-potential initiation and observe the underlying synaptic input, the motor neuron was kept hyperpolarized 30 mV from resting potential throughout the experiment. B: EPSPs produced by a single tail sensory neuron spike 30 s before and 90 s after von Frey hair application. Test EPSPs were elicited every 60 s by intracellular activation of single sensory neuron action potentials. The short-latency component is the monosynaptic EPSP. After sensitizing stimulation, the sensory neuron spike evoked longer latency polysynaptic EPSPs in addition to the monosynaptic EPSP. Spikes in the motor neuron are clipped by the pen recorder.

In the same experiment we examined changes in the monosynaptic connection between a tail sensory neuron and the tail motor neuron. The excitatory receptive field of the sensory neuron was outside of the region stimulated by the electrode and thus the sensory neuron was not activated by the tail shock used as the test stimulus. In addition, it was not activated by the sensitizing mechanical stimulus. This connection was tested every 60 s by stimulating the sensory cell soma intracellularly with a brief depolarizing pulse that elicited a single action potential. From an initial amplitude of 24 mV, the EPSP gradually declined with repeated testing to about 20 mV (Fig. 1B, trial 4) immediately before the sensitizing stimulus. Thirty seconds after the sensitizing stimulus the amplitude of the test EPSP could not be measured because the EPSP elicited a spike in the motor neuron, but the next test EPSP (Fig. 1B, trial 5, 90 s after sensitizing stimulation) was 28 mV, an increase of 40% over its last base-line value. This facilitation is of heterosynaptic rather than homosynaptic origin, since the sensory neuron was not itself activated by the sensitizing stimulus (ruling out posttetanic potentiation). In addition, the facilitation is not due to general voltage-dependent changes in postsynaptic properties (such as anomalous rectification, Ref. 22), since the motor neuron was held at a constant hyperpolarized level throughout the experiment.

Another common effect of sensitizing stimulation is the recruitment of polysynaptic excitatory input to the tail motor by a single sensory neuron action potential (Fig. 1B). In this experiment the sensory neuron never elicited late EPSPs before the sensitizing stimulus but reliably recruited a long-lasting (200–400 ms duration) barrage of EPSPs when tested at 60-s intervals for over 5 min after application of the sensitizing von Frey hair.

SENSITIZATION FROM STIMULATING TEST SITE ITSELF. During the experiments described in the preceding paper (39) we made the unex-
petted observation that repeated elicitation of the tail-withdrawal reflex with a weak constant-intensity stimulus often led to progressive sensitization of the elicited withdrawal responses. To examine mechanisms contributing to this sensitization we monitored changes in 1) tail withdrawal, 2) tail motor neuron activity, and 3) monosynaptic connections from tail sensory neurons to the tail motor neurons during repeated application (at 60-s intervals) of relatively weak electric shock to a point on the tail through an implanted electrode (Fig. 2). To avoid possible complications introduced by homosynaptic plasticity (depression or potentiation) in the sensory neuron's synapses, we stimulated a region of the tail outside the excitatory receptive field of the impaled sensory neuron. An intensity was chosen that was approximately twice the threshold for eliciting observable EPSPs in the tail motor neuron prior to training (see METHODS). This intensity initially produced only a few spikes in the motor neuron and a very weak contraction of the tail (Fig. 2A, trial 1). Coinciding with the onset of each tail shock we injected a brief depolarizing current pulse

![Figure 2](image)

**FIG. 2.** Sensitization and synaptic facilitation in response to repeated application of a weak electrical stimulus. **A:** responses of the tail, a tail motor neuron, and a tail sensory neuron to repeated tail shock at 60-s intervals. Each trial consisted of the simultaneous stimulation of 1) a tail sensory neuron with a brief intracellular depolarizing pulse (small bar) and 2) the tail by electrical stimulation through implanted electrodes (arrows). Because of the conduction delay from the tail to the motor neuron, the monosynaptic EPSP from the sensory neuron action potential precedes the complex EPSP from the tail shock. The tail shock was applied outside the tail sensory neuron's excitatory receptive field. Tail-withdrawal magnitude, motor neuronal spike activity, and the monosynaptic EPSP amplitude increased between trials 1 and 10. The motor neuron also showed a persistent depolarization on trial 10. Spikes in the motor neuron are clipped by the pen recorder. **B:** monosynaptic EPSPs during and after training. Responses on trials 1 and 10 are the same as shown in A. Following trial 10 the motor neuron was hyperpolarized by current injection to its original resting potential to test whether the facilitation was independent of the motor neuron depolarization (post 1 min and post 2 min).
into the sensory neuron to elicit a single action potential so that the monosynaptic connection between the sensory and motor neuron could be examined as a function of this form of sensitization training. Because of the conduction delay from the tail to the motor neuron in the pedal ganglion (195 ms) it was possible to observe the resulting monosynaptic EPSP (see Ref. 39) before synaptic input from tail shock reached the motor neuron (Fig. 2A).

With repeated stimulation of the tail the amplitude of the resulting tail withdrawal progressively increased. This sensitization was accompanied by an increase in the number of spikes generated in the motor neuron and an increase in the amplitude of the monosynaptic EPSP from the sensory neuron. In addition, the motor neuron showed a residual depolarization (9 mV on trial 10) persisting from previous trials (Fig. 2A). If this depolarization were accompanied by an increase in input resistance of the postsynaptic cell (anomalous rectification), the depolarization itself might explain the increase in EPSP amplitude (22). However, as shown in Fig. 2B, anomalous rectification in the tail motor neuron cannot explain the synaptic facilitation because the facilitation was still present when the membrane potential was artificially hyperpolarized to the resting level (post 1 and 2 min). In addition, a comparison of the amplitude of responses to constant-current hyperpolarizing pulses before and after the sensitizing stimulation revealed no generalized increase in postsynaptic input resistance.

Figure 3 shows pooled results of six experiments in six animals in which we examined the effects of repeated weak tail shock (10 trials) on 1) tail-withdrawal magnitude, 2) tail motor neuron activity, and 3) sensory-to-motor neuron EPSP amplitude. The progressive increase in the behavioral response was accompanied by increases in the amplitude of the monosynaptic EPSP from the tested sensory neuron and in the firing of the tail motor neuron. After eight trials the withdrawal and motor neuronal responses declined somewhat from their peak levels, but nevertheless the responses on the 10th trial were significantly different from their initial values (withdrawal: $t_5 = 4.78$, $P < 0.005$; motor neuronal response: $t_5 = 4.26$, $P < 0.01$). The monosynaptic EPSP amplitude also increased significantly to nearly 3 times its initial value ($279 \pm 27\%$; $t_5 = 4.47$, $P < 0.01$).

The facilitation of the EPSP is not due to posttetanic potentiation because none of these mechanoreceptive neurons was activated by the tail shock during training (shock was applied outside these cells' excitatory receptive fields). Furthermore, as described in the preceding paper (39), repeated activation of individual cells by intracellular pulses at the 60-s test intervals results in depression rather than facilitation of the EPSP. Thus the enhancement of the monosynaptic EPSP by tail stimulation appears to be due to heterosynaptic facilitation. However, the possibility remains that the testing procedure may have influenced the degree of heterosynaptic facilitation observed because of a possible interaction between the test spike in the sensory neuron and the closely paired sensitizing stimulus applied to the tail (see Refs. 19 and 38). This potential complication was minimized in the studies described below by using longer intertrial intervals that allowed long intervals between each test spike and the sensitizing stimulation.
Heterosynaptic facilitation from noxious tail shock

Although sensitization has been observed in response to relatively weak stimuli (18), most studies of sensitization have employed strong or noxious stimuli (10, 11, 17, 18, 28, 30, 36, 40). In addition, the noxious unconditioned stimuli that have been used to produce aversive classical conditioning in a variety of animals often produce sensitization as well (11, 17, 18, 30, 36). Therefore, we were curious to see if the synaptic changes described above for sensitization produced by weak stimuli in Aplysia also occur during sensitization produced by noxious stimulation.

For the sensitizing stimulus we selected strong tail shock similar to that previously used for aversive classical conditioning in Aplysia (11). This shock was considerably stronger than that used in the experiment of Figs. 2 and 3 (5 times threshold for the motor neuron EPSP rather than 2 times threshold) but less than that used in experiments described below (Fig. 6). To eliminate the possible contribution of anomalous rectification, all EPSPs were measured at a constant level of membrane potential in the motor neuron. To avoid possible complications of synaptic potentiation or depression produced by spike activity in the sensory neuron in response to the tail shock, we restrict our analysis here to the effects on tail mechanodetector neurons that were not activated by the tail shock.

A single strong shock to the tail produced clear facilitation of the monosynaptic EPSP measured 4 min after the shock compared to the response in the base-line test given 1 min before the shock (Fig. 4A). The amplitude of the EPSP then declined gradually over the next 25 min, with no significant differences between the base-line EPSPs and test EPSPs observed 19 min after the shock ($t = 1.99$). In a separate group of animals ($n = 8$) we examined the effects of applying the same shock 5 times at 5-min intervals (Fig. 4B). Again, the test pulse used to fire an action potential in each sensory neuron was given 1 min prior to each shock (4 min after the last shock). Repeated shocks produced a progressive increase in the amplitude of the EPSP. Comparison of Fig. 4A and B suggests that part of this buildup might be due to summation of the facilitatory effects of each shock with the residual facilitation persisting from prior shocks.

Multiple trials increased not only the magnitude of the facilitation but also its duration. Figure 5 shows the acquisition and retention of heterosynaptic facilitation for those cells from the group shown in Fig. 4B whose monosynaptic connections could be reliably

![Figure 4](image-url)
FIG. 5. Retention of heterosynaptic facilitation after multiple training trails with noxious tail shock. Procedures were identical to those described in Fig. 4B except that testing was continued for at least 74 min after the fifth tail shock. n = 6. Test EPSPs were normalized to the mean of the two base-line EPSP elicited 6 and 1 min prior to the first shock (first arrow). Times indicated are relative to the test 1 min before the last shock (fifth arrow).

measured for at least 75 min following the last tail shock (n = 6). These cells still showed significant facilitation, about 200% of their base-line level, 74 min after the end of training (t = 2.15, P < 0.05). One animal showed facilitated EPSPs (200% of its base-line level) 3 h after the fifth shock. By contrast, the animals receiving a single shock showed no significant facilitation 19 min after the shock (Fig. 4A).

A novel effect of sensitization—regenerative bursting responses in sensory neurons

Occasionally we observed a sustained, regenerative burst of spikes in response to brief depolarization of the sensory neuron following sensitizing stimulation. This effect of sensitization has not been described previously in mechanoafferent neurons in *Aplysia* (although an isolated observation of a bursting response appears in Ref. 8). The regenerative responses in the tail mechanoafferents usually followed very strong sensitizing stimulation (approximately 10 times EPSP threshold in the tail motor neurons). An example is shown in Fig. 6 where, following strong tail shock, a brief (50 ms) intracellular pulse triggered a train of nine action potentials that greatly outlasted the eliciting pulse. The burst is regenerative in the sense that a single spike appears to lead to the generation of additional spikes. During the burst the action potentials showed a progressive increase in amplitude and duration, and the amplitude of the hyperpolarizing afterpotentials increased (Fig. 6B). The bursting response was accompanied by facilitation of the monosynaptic connection to the tail motor neuron, an increase in excitability of the sensory neuron (as judged by the reduced latency to initiate a spike with a constant-current de-

FIG. 6. Regenerative spike discharge in a tail sensory neuron following very strong tail stimulation. *A*: response to brief intracellular depolarization prior to sensitizing tail shock. *B*: response to the same stimulus 2 min after tail shock. The 50-ms depolarizing pulse elicited a burst of 9 spikes that continued for about 500 ms. Note the recruitment of polysynaptic input to the motor neuron. *C*: response 15 min after the tail shock. The bursting response to the brief intracellular depolarizing pulse ceased but synaptic facilitation, an increase in spike amplitude and duration (see text), and an augmentation of the depolarizing afterpotential in the sensory neuron were still present.
polarizing pulse), and an increase in the amplitude of the depolarizing afterpotential. In addition, examination of the responses on a storage oscilloscope revealed that the first spike in the burst showed a 30% increase in spike amplitude and a 17% increase in spike duration (measured at half-amplitude) compared to the previous test spike. Both the spike amplitude and duration then increased progressively during the burst (by 15 and 450%, respectively). The capacity to trigger a sustained discharge lasted approximately 150 s after each sensitizing shock in this cell and could be repeatedly reinstated by reapplication of the sensitizing shock. When the bursting response could no longer be triggered after each sensitizing shock, the sensory neurons showed persistent synaptic facilitation, enhanced excitability (decreased latency to fire to a constant-current depolarizing pulse), and increased spike magnitude and duration (Fig. 6C) compared to the test response preceding the sensitizing stimulus (Fig. 6A). The degree to which these effects are due to homosynaptic factors (spike activity), heterosynaptic factors (neuromodulation), or an interaction of homo- and heterosynaptic factors is not yet known.

Regenerative bursting responses to brief soma stimulation were observed in 57 of 982 sensory neurons examined (6%) and occurred in 35 of 207 preparations (17%). They were only observed following sensitizing stimulation. Up to five tail sensory neurons showed bursting responses in a single preparation, and in some preparations three concurrently recorded sensory neurons acquired and later lost the bursting responses simultaneously. The bursts comprised from 2 to 16 spikes and ranged from 0.1 to 1.5 s in duration. In every case the cell had a relatively large depolarizing afterpotential (1–4 mV) before the development of the sustained discharge (Fig. 6A). The bursting response itself produced a large depolarizing afterpotential (10–20 mV) (Fig. 6B), and after the regenerative bursts could no longer be triggered (Fig. 6C) the depolarizing afterpotential was larger (4–10 mV) than it had been prior to the sensitizing stimulation. Thus the generation of the burst may involve an enhancement of the depolarizing afterpotential to a point where firing threshold is reached, similar to effects described in some gastropod bursting neurons (6, 35). The development of the bursting response is not due to a progressive depolarization of the cell, since these cells, like those described below (Figs. 7 and 8) often showed a persistent hyperpolarizing response to sensitizing stimulation.

It is possible that the sensory neurons that show the regenerative bursts comprise a distinct subpopulation of the tail sensory neurons. However, all the bursting cells also showed nonregenerative responses prior to and after the sensitizing stimulation. In addition, on the basis of soma location and EPSP properties, it seems that many of the tail sensory neurons in the left pleural ganglion showed regenerative responses in some preparations but not in others. These observations are consistent with the possibility that regenerative bursting is an extreme manifestation of sensitization that most or all of the sensory neurons can express under appropriate conditions.

While the mechanism of the sustained discharge is not yet known, its functional implications are clear. As shown in Fig. 6B, the burst, by producing additional EPSPs, results in a dramatic further amplification of the excitation of the motor neuron by the sensory neuron (in addition to heterosynaptic facilitation), which, in this case, brought the motor neuron to spike threshold and also recruited additional interneuronal input.

Membrane changes accompanying heterosynaptic facilitation

Sensitizing stimulation of the tail with either electrical or mechanical stimuli usually caused characteristic modulation of the electrophysiological properties of both the motor neurons and sensory neurons involved in tail withdrawal. The effects of pinching the tail with fine forceps are illustrated in Fig. 7. This mechanical stimulus produced a long-lasting depolarization of the tail motor neuron that was paralleled by a persistent hyperpolarization of the sensory neuron (Fig. 7A). These hyperpolarizations of the sensory neurons and depolarizations of the motor neurons have been observed to last as long as 20 min after a single sensitizing stimulus.

Figure 7B shows that both types of slow membrane responses coincide with heterosynaptic facilitation of the monosynaptic
connection from the tail sensory neuron to the tail motor neuron. This figure also shows that the slow hyperpolarizing response in the sensory neuron can block action-potential initiation. Before and after the tail pinch, single test action potentials were elicited by intracellular current pulses in the sensory neuron at 60-s intervals (five tests are shown). Following the tail pinch, the hyperpolarization of the sensory neuron prevented the sensory neuron from reaching firing threshold during the next intracellular pulse (star in Fig. 7A), so that the current injected into the sensory neuron had to be increased (test 3) to fire the cell. On the subsequent tests (tests 3 and 4) the synaptic connection was clearly facilitated.

The tail pinch, like tail shock (Fig. 2), produced a long-lasting depolarization of the motor neuron, which might itself be responsible for the synaptic enhancement if the motor neuron exhibited anomalous rectification (22). To rule out a possible contribution of anomalous rectification in producing the synaptic facilitation, the motor neuron was then hyperpolarized by current injection back to the resting level (test 5). Rather than diminishing the EPSP, hyperpolarization of the motor neuron resulted in a further increase in EPSP amplitude. In addition, since the background synaptic activity had decreased at this point, it was possible to see the recruitment of polysynaptic EPSPs (which immediately follow the monosynaptic EPSP) by the single sensory neuron action potential (see also Fig. 1B).

While the tail motor neurons invariably show a persistent depolarization following sensitizing stimulation (Figs. 2A, 7A, 8A), the tail sensory neurons show various responses to sensitizing stimulation, including early hyperpolarization, late hyperpolarization, and late depolarization. We estimated the relative frequencies of these responses to moderate-intensity tail shock outside the excitatory re-
FIG. 8. Changes in input resistance and membrane potential produced by sensitizing stimulation. A: simultaneous recordings from a tail motor neuron and tail sensory neuron. In both cells the input resistance measured by constant-current hyperpolarizing pulses injected through a second microelectrode showed a very slight increase (about 5%) several minutes after strong shock of the tail (outside of the excitatory receptive field of the sensory neuron). This motor neuron showed the largest apparent increase in input resistance observed (four of six cells examined showed a decrease in input resistance), an increase that is unlikely to account for the large changes in EPSP amplitude produced by similar tail shock (e.g., Fig. 4). In this example, the depolarization of the motor neuron lasted about 8 min and the hyperpolarization of the sensory neuron nearly 15 min (recovery not shown). B: early hyperpolarizing response to sensitizing stimulation (shock artifacts visible) in the sensory neuron (expansion of record in A). This record also shows the brief depolarizing phase that often separates the early and late hyperpolarizing responses in the sensory neuron.

We examined changes in input resistance in response to moderate tail shock in six tail motor neurons and seven tail sensory neurons. Following tail shock, the motor neurons fired a train of action potentials and usually showed a decreased input resistance (dropping as much as 50%) associated with a long-lasting depolarization, produced at least in part by a barrage of fast synaptic input. Occasionally (two of six cells) the long-lasting depolarization was associated with a very small (about 5%) increase in input resistance (Fig. 8A). Such a small and unreliable increase in input resistance of the motor neuron is unlikely to account for the synaptic facilitation produced by moderate-intensity tail shock, which reliably ranges from 100 to 300% after a single shock (Fig. 4A).

The modulation of input resistance in the sensory neurons is also variable. Four of seven cells examined showed a decrease in input resistance 60 s after sensitizing stimu-
lation, two showed a small increase (Fig. 8), and one showed no change. The increases in input resistance might be explained by the contribution of an underlying decreased conductance postsynaptic potential (PSP) like that observed in the ink motor neurons (7, 12) and in the siphon sensory neurons (24). However, this and other possibilities cannot be directly assessed using these data because the apparent input resistance varies dramatically with membrane potential. Thus, the modulation by sensitizing stimulation of both the sensory neuron and motor neuron conductances must be examined further with the membrane potential controlled by voltage clamp.

**Serotonin application mimics effects of sensitization**

Since the effects of sensitization in the gill- and siphon-withdrawal circuit can be mimicked by application of serotonin (5-HT) (5, 24), we have conducted preliminary experiments to determine whether the effects of sensitization in the tail-withdrawal circuit can also be mimicked by 5-HT application. For the test stimulus, a weak intensity of tail shock was selected that produced stable tail withdrawal and tail motor neuron responses prior to 5-HT application (Fig. 9). Because of the tendency for test stimuli themselves to produce sensitization (see above), stable base-line responses could be obtained in only a few animals. After three tests (given at 5-min intervals) in which the withdrawal response remained at a constant amplitude, $5 \times 10^{-3} \text{ M}$ 5-HT was perfused into the inner chamber containing the left pedal and pleural ganglia. When tested 60 s after the beginning of 5-HT perfusion, the tail-withdrawal response had increased by 180% and the motor neuron response had increased from 7 to 10 spikes (Fig. 9A).

To monitor EPSP changes, a tail sensory neuron was stimulated 30 s before each test. The monosynaptic EPSP to the tail motor neuron increased by 125% in the presence of the 5-HT (Fig. 9B). In addition, examination of the sensory neuron spike on a storage oscilloscope revealed that the action-potential duration (at half its maximum amplitude) increased from 2.5 to 2.8 ms and its amplitude increased from 53 to 65 mV. While the 5-HT produced no changes in the resting levels of tension of the tail or of the membrane potential of the motor neuron, it did produce a

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**Fig. 9. Sensitization and synaptic facilitation produced by 5-HT.** A: simultaneous recordings of responses of the tail, a tail motor neuron, and a tail sensory neuron to a weak tail shock (outside the excitatory receptive field of the sensory neuron) 4 min before and 1 min after superfusion of the ganglia with $5 \times 10^{-3} \text{ M}$ 5-HT. The 5-HT did not reach the peripheral tissues, which were separated from the inner chamber. In addition to facilitating the tail withdrawal and motor neuronal responses, the 5-HT produced a tonic hyperpolarization of the sensory neuron. B: facilitation of the monosynaptic EPSP from the sensory neuron to the motor neuron. The EPSP was tested 30 s before each test shown in A.
stable 5-mV hyperpolarization of the tail sensory neuron. About 10 min after 5-HT washout, the tail-withdrawal response, motor neuron response, and monosynaptic EPSP returned to the levels seen prior to 5-HT application.

5-HT has been observed to enhance tail withdrawal in three of four experiments in which stable baseline responses were obtained and to facilitate the monosynaptic EPSP from five of five tail sensory neurons examined in these preparations. In addition, 5-HT produced variable effects on the sensory neuron membrane potential, causing a slow hyperpolarization in three of the sensory neurons and a slow depolarization in two sensory neurons. An example of a slow depolarizing response in the sensory neuron associated with facilitation of the monosynaptic EPSP is shown in Fig. 10A. In this case the sensory neuron was stimulated every 60 s. After five test stimuli the 5-HT was superfused over the ganglia (30 s before the next test). The sensory neuron depolarized and the next test stimulus elicited 3 spikes. To observe the unitary EPSP, the stimulus current was then decreased by 25%. In the next test (during washout of the 5-HT) the EPSP was facilitated by about 70% compared to the last pretest. The sensory neuron membrane potential and the EPSP returned to their pretest levels within about 5 min after 5-HT washout.

The 5-HT-induced increases in magnitude and duration of the action potential were seen during both hyperpolarizing (Fig. 9) and depolarizing (Fig. 10A) responses of the tail sensory neurons. In Fig. 10A the sensory neuron depolarized by 5 mV while the action-potential duration increased from 1.5 to 2.0 ms and its amplitude from 64 to 74 mV. As previously shown in the siphon sensory neurons (24), the effects on the action potential are greatly enhanced when repolarizing K+ currents are partially blocked. Figure 10B shows the effects of 10^{-4} M 5-HT on the sensory neuron action potential in a solution of artificial seawater containing 20 mM TEA and 5 mM 4-AP to partially block K+ currents (34). Both the amplitude and duration of the action potential were increased dramatically by 5-HT.

The variability of the effects of 5-HT on the sensory neuron resting potential was considerably reduced when the sensory cell soma was isolated from the nervous system by axotomy and physical separation (see METHODS). The isolated sensory cell soma almost always showed a slow, decreased-conductance depolarizing response to 5-HT perfusion (Fig. 10C), suggesting that the hyperpolarizing responses to 5-HT seen in less isolated preparations (Figs. 9 and 10B) may not be direct effects of 5-HT but, instead, indirect effects mediated through other cells. Alternatively, distal parts of the sensory neuron (removed in the nearly isolated soma preparation) may have 5-HT receptors that can mediate the hy...
perpolarizing responses in the less-isolated preparations.

DISCUSSION

Neuronal correlates of sensitization

The tail-withdrawal reflex in *Aplysia* can be sensitized by stimulating a site outside the site used to test the reflex or by repeatedly stimulating the test site itself. In each case the increase in the amplitude of tail withdrawal is correlated with an increase in activity of identified tail motor neurons in response to tail stimulation. This increased motor neuronal response seems likely to be due, at least in part, to the concomitant facilitation of the monosynaptic connections between the tail sensory neurons and the tail motor neurons. Comparison of the amount of heterosynaptic facilitation produced by weak and by noxious stimulation suggests that the degree of facilitation is graded with the intensity of the sensitizing stimulus.

Factors that could also contribute to the facilitation of the motor neuron response and sensitization of the tail-withdrawal reflex are long-lasting depolarization of the motor neuron, the recruitment of excitatory interneuronal activity, and enhanced sensory or motor function in the periphery (the tail). While these effects could operate independently, it is interesting to note that an increase in the functional effectiveness of the sensory neurons mediating the afferent input for the reflex might play an important role in producing the other effects. In particular, enhanced synaptic transmission from the sensory neurons to their follower cells would be expected to increase the recruitment of excitatory interneurons, which in turn might prolong the depolarization of the motor neuron. Little is known about the peripheral organization of the tail-withdrawal reflex, but if the VC sensory neurons make parallel connections to peripheral motor neurons as well as to the central motor neurons (as has been described in the siphon-withdrawal reflex, Ref. 1) general facilitation of the sensory neuron connections might also be expressed as enhanced peripheral responsiveness. Thus enhanced transmission from the sensory neurons may produce several complementary effects contributing to behavioral sensitization.

All these effects, including enhancement of the monosynaptic input to the tail motor neurons from the tail sensory neurons, would be further enhanced if (under conditions of extreme sensitization) the central processes of the tail sensory neurons began firing regenerative bursts in response to individual action potentials arriving from the periphery. Regenerative bursts in the sensory neurons might also be expected to lead to yet another facilitatory mechanism—posttetanic potentiation. Posttetanic potentiation in the tail sensory neurons has recently been observed following intracellularly evoked bursts of action potentials that are similar in frequency and duration to the regenerative bursts observed after strong sensitizing stimulation (Ref. 37; unpublished observations).

Facilitation of transmission from tail sensory neurons

We have found that the facilitation of the monosynaptic connections between the tail sensory neurons and motor neurons is due to a heterosynaptic mechanism. Heterosynaptic facilitation has now been described in a variety of synapses in *Aplysia* (14, 16, 21, 32) and may also be present in mammalian neurons (3, 31). Behavioral dishabituation and sensitization of the gill-withdrawal reflex in *Aplysia* have been shown to involve heterosynaptic facilitation of the mecanoafferent neurons from the siphon (10, 16). Thus, the demonstration of heterosynaptic facilitation of the VC mecanoafferent neurons from the tail during sensitization of the tail-withdrawal reflex suggests that heterosynaptic facilitation may be a general mechanism contributing to behavioral sensitization and arousal in *Aplysia* and perhaps in other animals.

As with the siphon sensory neurons in *Aplysia* (10, 16), heterosynaptic facilitation of the tail sensory neuron connections does not appear to require a general modulation of postsynaptic properties, since facilitation is observed when there is no change in membrane potential or increase of input resistance in the motor neuron. In addition, activation of the motor neuron does not appear to be sufficient for producing the synaptic facilitation, since direct intracellular activation of the motor neuron alone in the isolated pedal-pleural ganglia produces no synaptic facili-
tation, even though nerve stimulation facilitates sensory neuron connections in the same preparation (unpublished observations). These observations are consistent with the involvement of presynaptic rather than postsynaptic mechanisms during heterosynaptic facilitation.

Additional support for a possible presynaptic mechanism of facilitation comes from the effects of 5-HT application. Our preliminary studies indicate that 5-HT usually produces synaptic facilitation in the tail sensory neurons even in the apparent absence of motor neuronal alterations. In the nearly isolated soma preparation (in which secondary synaptic interactions are minimized), 5-HT usually produced a slow decreased conductance depolarization of the sensory neuron. This effect suggests that 5-HT, a putative sensitizing neuromodulator (2, 5), directly affects at least part of the presynaptic neuron. In addition, the effect of this neuromodulator on the tail sensory neuron soma parallels the decreased conductance depolarizing effects of both 5-HT and sensitizing stimulation on the siphon sensory neuron soma, effects that have been shown to play a role in presynaptic facilitation in the siphon- and gill-withdrawal reflex (24). While conclusive evidence for a presynaptic locus of facilitation in the tail sensory neurons must await a quantal analysis, the similarities during sensitizing procedures of the tail sensory neurons to the siphon sensory neurons (where quantal analysis has been performed, Ref. 14) suggests that the two populations of sensory neurons have similar responses and roles during sensitization. Recently these similarities have extended to the apparent involvement during sensitization of a common intracellular messenger, cyclic AMP (4, 9, 29), and a specific serotonin-sensitive K⁺ channel (29). Indeed, it is attractive to think that the novel regenerative bursting responses produced during extreme sensitization represent an extreme effect on the depression of the serotonin-sensitive K⁺ current that has been examined in the siphon sensory neurons (23-25, 33).

Although there are extensive similarities between the siphon and tail sensory neurons, it is too early to conclude that the mechanisms of sensitization in these cells are identical in all respects. For example, in the tail sensory neurons a possible role for a direct 5-HT-evoked increase in Ca²⁺ current (see Ref. 27) has not been excluded.

Additional implications

Our investigation of the effects of sensitizing stimulation on the tail sensory neurons has revealed several correlates of sensitization that may have interesting functions and thus deserve further study. One finding is the ability of the sensory neurons to show regenerative bursting responses during extreme sensitization. Another finding is the occurrence of early and late hyperpolarizing responses in the sensory neuron, which can elevate the threshold for eliciting a spike by soma stimulation. The observation of hyperpolarizing responses to cutaneous stimulation was also made in the siphon sensory neurons (10), but hyperpolarizing effects have not been examined further in that system. It will be interesting to determine whether the hyperpolarizing responses correspond to a form of afferent inhibition and what the role of such inhibition might be during sensitization. The recent discovery (unpublished observations) of interneurons that can produce early and late hyperpolarization of the tail sensory neurons and that are activated by tail stimulation should aid in such an analysis.

Another interesting observation is that sensitization and heterosynaptic facilitation build up rapidly during the repeated application of a constant-intensity sensitizing stimulus (see Figs. 3, 4, and 5). This buildup may play a role in the development of long-term forms of learning, such as the sensitization of siphon withdrawal that has been shown to last for weeks (28). It also suggests that when similar tail stimulation is used as an unconditioned stimulus for the aversive classical conditioning of behaviors such as gill and siphon withdrawal (11, 13), the efficacy of the reinforcing pathway may increase with continued training. Since the intensity of the reinforcer is usually correlated with the effectiveness of conditioning (26), such buildup could contribute to the rate or strength of conditioning. A buildup of facilitation in Aplysia might be due to various factors, including summation with the after-effects of prior sensitizing stimulation (Fig. 4). Buildup of sensitization and synaptic facilitation might also be promoted by positive
feedback between the sensory neurons and the facilitatory system that modulates the sensory neurons. If the facilitatory system is activated by synaptic input from the sensory neurons, facilitation of input from the sensory neurons should then lead to greater activation of the facilitatory system, which should further enhance subsequent sensory input. While the primary mechanisms underlying the sensitization of simple defensive reflexes in *Aplysia* seem likely to reside in presynaptic facilitation of the sensory neurons, the rate, degree, and extent of sensitization may depend on various interactions between the sensory neurons and different classes of interneurons activated by sensitizing stimulation.

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