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18 June 1976

Presynaptic Facilitation as a Mechanism for Behavioral Sensitization in *Aplysia*

Abstract. *Sensitization is an elementary form of nonassociative learning, related to behavioral arousal, in which a strong stimulus facilitates a reflex response. Studies of the neural circuit of the gill-withdrawal reflex in the isolated abdominal ganglion of* Aplysia *indicate that short-term sensitization is due to presynaptic facilitation. The facilitation results in a sudden increase in the amount of neurotransmitter released by the sensory neurons at their synapses with motor neurons.*

The ability to study behavior on a cellular level in a number of higher invertebrates (*1*) makes is possible to examine the cellular and synaptic alterations produced by simple forms of behavioral modifications and to begin to explore their molecular mechanisms. This report and its companion (*1a*) represent an attempt to apply this approach in *Aplysia*.

Sensitization is an elementary form of learning in which a strong or noxious stimulus enhances an animal's preexisting reflex responses for periods ranging from minutes to several weeks, depending upon the pattern and duration of training (*2*). Sensitization resembles classical conditioning in that activity in one pathway facilitates reflex activity in another. Unlike classical conditioning, however, reflex facilitation does not require specific temporal association of the two stimuli. As a result of this similarity to classical conditioning, sensitization is thought by some to be closely related to associative learning (*3*). Sensitization can also produce dishabituation, the enhancement of a reflex response that has previously been habituated (*4, 5*). Whereas habituation is restricted to the stimulated pathway, sensitization alters the responsiveness of a variety of related reflex pathways. Because both sensitization and behavioral arousal lead to increased responsiveness that is generalized and sustained, sensitization is thought to be a component of behavioral arousal (*5, 6*).

We have studied the cellular mechanisms underlying sensitization of the gill-withdrawal reflex in the marine mollusk *Aplysia*. Our results indicate that sensitization involves a novel synaptic mechanism, presynaptic facilitation.

Weak or moderate stimulation of the siphon leads to brisk withdrawal of the gill. A single training session of 10 to 15 repeated stimuli results in short-term habituation (15 minutes to several hours) of the reflex response. The reflex response can be abruptly enhanced for many minutes if a single strong sensitizing stimulus is applied to the head (*2*).

The neuronal mechanisms of short-term habituation and sensitization can be studied in the isolated abdominal ganglion (Fig. 1A). The ganglion contains an identified cluster of 24 sensory neurons that innervate the siphon skin and 6 identified motor neurons that mediate the gill-withdrawal reflex (*7*). The sensory neurons make direct monosynaptic excitatory connections with the motor neurons (*8, 9*). With repeated sensory stimulation at rates that produce habituation in the intact animal (once every 10 seconds to once every 3 minutes), the monosynaptic excitatory postsynaptic potential (EPSP) produced in the motor neuron by action potentials in the sensory neuron becomes depressed (Fig. 1B) due to a decrease in transmitter release by the sensory neuron (*9*). Electrical stimulation (6 hertz for 10 seconds) of the neural pathway (connective) from the head ganglion that mediates the effect of a sensitizing stimulus in an intact animal rapidly facilitates the EPSP for about 50 minutes (Fig. 1, B and C) [for a similar phenomenon in another synaptic system in *Aplysia*, see (*10*)]. The facilitating stimulus does not fire the sensory neuron (Fig. 1B); this distinguishes this form of heterosynaptic facilitation from posttetanic facilitation resulting from repetitive activity in the sensory neurons (*5, 10*).

To determine whether the facilitation occurs presynaptically or postsynaptically, we have used a quantal analysis. This analysis is technically difficult to achieve in most central neurons because they receive many synaptic in-

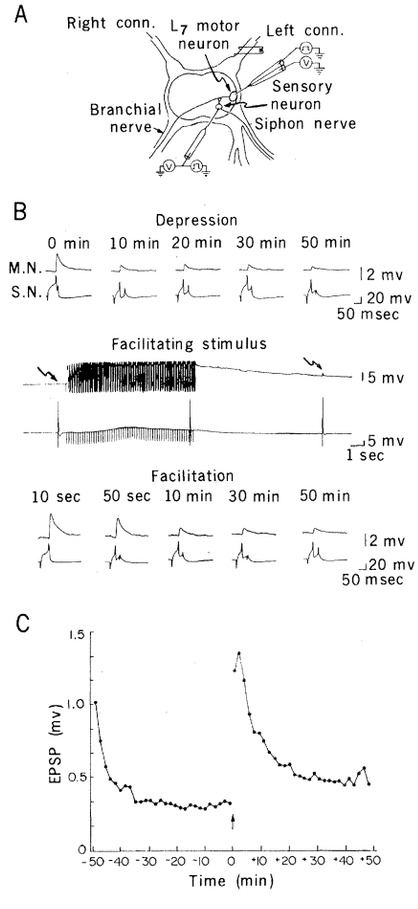


Fig. 1. Synaptic facilitation at the synapse between mechanoreceptor neurons and motor neurons. (A) Ventral aspect of the abdominal ganglion of *Aplysia* illustrating simultaneous recording from gill motor neuron L_7 and a mechanoreceptor sensory neuron. (B) Depression and subsequent facilitation of a monosynaptic EPSP after a strong stimulus. Arrows indicate the last EPSP before the facilitating stimulus and the first EPSP after the stimulus. Abbreviations: *S.N.*, sensory neuron; *M.N.*, motor neuron. (C) Time course of facilitation. Data were obtained from an experiment similar to the one illustrated in (B). Each point represents the average amplitude of ten successive evoked EPSP's. Facilitation occurred at time 0, when the left connective was stimulated as in (B). The EPSP's are facilitated beyond the initial control amplitude. We do not know to what extent the decline after the facilitating stimulation is due to continued testing or to gradual spontaneous recovery from facilitation.

puts, making it difficult to analyze reliably the spontaneous release from a single presynaptic source. In addition, the evoked quantal size is usually small in relation to the background synaptic bombardment produced by neural activity. We increased the signal-to-noise ratio by examining fluctuations in the synaptic potentials produced by a single sensory neuron in one motor neuron using solutions with elevated concentrations of divalent cations (165 m*M* Mg^{2+}, 10 to 33 m*M* Ca^{2+}) to depress the release of transmitter. In these solutions the total content of divalent cations is tripled compared to normal (55 m*M* Mg^{2+}, 10 m*M* Ca^{2+}). This high concentration of divalent cations increases the threshold of neurons and reduces the activity of spontaneously active cells (*9, 11*). The facilitation observed is similar in amplitude to that observed when the ganglion is bathed in artificial seawater.

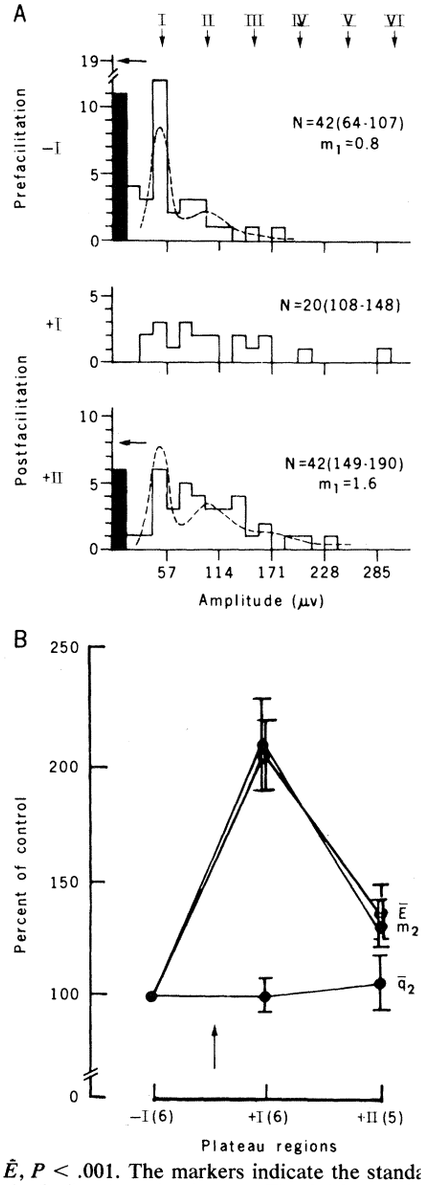
According to the quantal hypothesis (*12*), the average amplitude of the EPSP ($\bar{E}$) is equal to $m \times \bar{q}$ where m is the quantal content, and $\bar{q}$ is the average size of the transmitter quantum. We estimated m and $\bar{q}$ (i) from amplitude histograms of the evoked EPSP's (m_1 and q_1) and (ii) from an analysis of failures in synaptic transmission (m_2 and q_2) (*13*).

At low levels of transmitter release, when synaptic transmission occasionally fails, the data obtained can be roughly approximated by a Poisson distribution (*9, 14*). The application of this distribution assumes that the average response level is constant. At these synapses, however, the amplitude of the synaptic potential decreases gradually with repeated stimulation. Therefore, we collected 100 to 300 responses both before and after presenting a facilitating stimulus. We grouped the first 20 responses together and divided the other responses into equal consecutive regions of relative stability, each containing 20 to 100 responses. In each region the amplitude of the responses did not change by more than 15 percent.

To compare the estimates m_1 and q_1, obtained from the amplitude histograms with those obtained from the failure analysis, we focused on the cases in which failures were found (Fig. 2A). We estimated m_1 and q_1 (*15*) in six experiments before and after the facilitating stimulus. In these experiments, $\bar{E}$ increased as a result of the facilitating stimulus from a median control value of 100 percent (in the region before the facilitating stimulus) to 141 percent ($P < .025$, one-tailed Wilcoxon matched-pairs signed-ranks test). This increase was paralleled by an increase in m_1 from 100 to 200 percent ($P < .05$, two-tailed Wilcoxon test). By contrast, q_1 remained at a median value of 100 percent.

We obtained similar results when we estimated m_2 and q_2 using the failure analysis. According to the Poisson distribution, m_2 can be obtained simply from the ratio of failures (n_0) to the total number of trials (N) (*12*), where $m_2 = \ln N/n_0$. Data from six experiments on synaptic facilitation were normalized to the region before the facilitation (Fig. 2B). The estimated values of $\bar{q}_2$ obtained in this way did not vary significantly between successive regions. By contrast, m_2 paralleled $\bar{E}$. Both increased from a control value of 100 percent to a maximum value of 200 percent before returning to control value. Since we only began to evaluate responses once failures occurred, the data do not describe the maximal facilitation observed, in which m is presumably much larger (*16*).

These results suggest that heterosynaptic facilitation underlying sensitization is due to a novel synaptic mechanism, presynaptic facilitation. This facili-



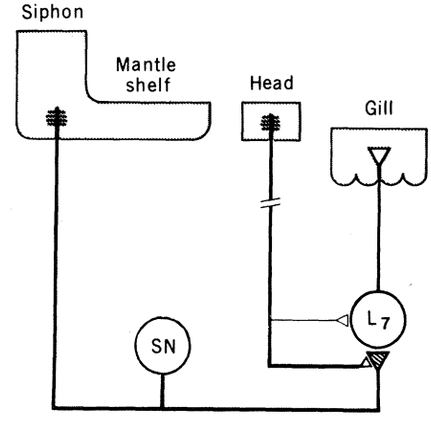


Fig. 2 (left). Quantal analysis of synaptic facilitation. (A) Histograms illustrate the region preceding the facilitating stimulation (−I) and two subsequent regions (+I and +II). Before facilitation, synaptic transmission often fails to occur (26 percent) (dark bar), but these failures probably include part of the adjacent bin. In the first region after the facilitating stimulus there is no failure. There are still proportionately fewer failures (14 percent) in region +II, but the amplitude of the unit peak is not altered, which indicates that m_1 increases while $\bar{q}_1$ does not change (*15*). The interstimulus interval was 10 seconds. The number of stimuli is N, and the two values in parentheses refer to the first and last EPSP's of the plateau region. The dashed lines illustrate theoretical curves based upon the Poisson distribution (with m_1) obtained by assuming a coefficient of variation of 20 percent for q_1 (*9*). The arrows on the ordinate indicate the number of predicted failures. Roman numerals and vertical arrows refer to the successive multiples of q_1. (B) Data based on estimates of m_2 and $\bar{q}_2$ derived according to the failure method. Numbers in parentheses indicate the number of experimental preparations. A *t*-test of correlated pairs of means was performed on the nonnormalized values of $\bar{q}_2$, m_2, and $\bar{E}$. Only $\bar{E}$ and m_2 were significantly different during successive regions; comparison between regions −I and +I; m_2, $P < .005$; $\bar{E}$, $P < .001$. The markers indicate the standard errors of the means, and the arrow indicates the facilitating stimulus. Fig. 3 (above). Postulated changes in the neuronal network of the gill reflex underlying behavioral habituation and sensitization. Only one sensory neuron and motor neuron are indicated. Habituation is postulated to result from a decrease in transmitter output at the synapse between the sensory neuron and the motor neuron (cross-hatched synapse); sensitization produced by presynaptic facilitation is postulated to result from an increase in transmitter output at the same synapse. Sensitization can be produced by a strong stimulus to any part of the animal's body surface, but only the head is indicated in the diagram. The exact neural pathway from the head, indicated by the interrupted line, has not yet been determined.

tation results in a sudden increase in the amount of transmitter released by the synapses of the sensory neurons as a result of activity in another neuronal pathway (Fig. 3). We suggest that these pathways contact the presynaptic terminals of the sensory neurons and regulate their transmitter release. The existence of presynaptic facilitation has been suggested in several synaptic systems on the basis of indirect evidence (*5, 10*); our data provide direct evidence for its occurrence.

We have so far examined only the synaptic connections made between sensory neurons and motor cells. We have not yet examined the connections between sensory neurons and interneurons or between interneurons and motor cells. Thus we cannot exclude other mechanisms as contributing to the behavioral sensitization.

Our results indicate that, although habituation and sensitization are different, they act on a common locus: the presynaptic terminals of the sensory neurons (Fig. 3). Both of these short-term behavioral modifications act by modulating the release of transmitter for periods ranging from several minutes to an hour or more.

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8. The connections between sensory and motor neurons were studied by inserting a single microelectrode into a sensory neuron; the electrode was connected to a Wheatstone bridge circuit for intracellular stimulation and recording. A low-resistance, double-barreled electrode was placed into a motor cell, usually gill motor neuron L_7 (Fig. 1A). The signals from the motor cell were led to a cathode follower and then to a d-c and a high gain a-c amplifier (Tektronix No. 2A61). To minimize baseline noise, the band width of the a-c amplifier was set between 0.6 and 60 hertz. Data were tape-recorded and later displayed (Brush recorder No. 440).
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13. Since the evoked signal is small, failures might have not been recognized accurately by visual inspection. We therefore averaged all the failures encountered in five experiments with a PDP-8 computer. We also averaged the background activity 2 seconds before the occurrence of the triggered presynaptic spikes. In all cases, the averages of the failures and of the background activity were indistinguishable. We observed no time-locked depolarizations, which might have suggested an undisclosed evoked EPSP hidden in the baseline noise (*9*). In addition we examined the possibility that the failures occurred because of a conduction block in the terminals of the sensory cells. If this were so, the failures should have occurred in groups. To determine whether the failures tended to occur in groups, we used the Poisson equation e^{-m}, e^{-3m}, . . . and found that the number of successive failures did not exceed that predicted by the Poisson equation.
14. By assuming that the first peak in a histogram was q, we also examined the fit between the observed data in a histogram of EPSP amplitudes and the values predicted by a Poisson distribution. Knowing $\bar{E}$, we could derive m. In 31 of 38 regions, the predicted values were not statistically different from the observed values ($P > .05$). For a given region, three to seven quantal classes were used with a minimum of five predicted responses in each class. The predicted and observed distributions were compared in a chi-square table with 2 degrees of freedom fewer than the number of quantal classes used.
15. The value of q was obtained from the median value of the first peak of evoked responses after the peak of failures (Fig. 2A). To qualify as a peak we demanded (i) a minimum of five responses and (ii) that the two adjacent bins be lower by one or more units.
16. In three other experiments, we could not produce failures after 300 stimuli. We estimated q from the coefficient of variation (based on the assumption of a Poisson distribution). The equation used for the coefficient of variation technique was $m = \bar{E}^2/\sigma_E^2$, where σ_E^2 is the variance of the amplitudes of the EPSP's and $\bar{E}$ is their mean amplitude. Our estimates of q were lower (5 to 15 μv) than those (20 to 90 μv) obtained when transmission was lower and $m < 10$. During the peak of facilitation, average estimates of q decreased by as much as 50 percent while m (≥ 25) increased by more than five- to tenfold. Under conditions of high release ($m \geq 25$), the probability of release P may be high and the coefficient of variation estimates based on a Poisson distribution may underestimate the true value of q [for example, see X. Wernig, *J. Physiol. (London)* **244**, 207 (1975)]. This underestimate would be more pronounced after facilitation.
17. This work was supported by NIH research career development award 5K04-NS-70346-02 to V.C., NIH research scientist award MH-18-588 to E.R.K. and NIH grant MH-262102-01. We thank J. H. Schwartz for his comments and criticism.

13 January 1976; revised 10 September 1976

Synaptic Facilitation and Behavioral Sensitization in *Aplysia*: Possible Role of Serotonin and Cyclic AMP

Abstract. *The neural changes accompanying sensitization of the gill-withdrawal reflex in* Aplysia *are associated with presynaptic facilitation at monosynaptic connections between sensory neurons and motor cells. To analyze the molecular mechanisms underlying the facilitation, the pharmacological actions of serotonin, octopamine, and dopamine were examined. Only serotonin enhanced synaptic transmission between the sensory and the motor neurons. A serotonin antagonist, cinanserin, reversibly blocked the synaptic facilitation. The action of serotonin may be mediated by adenosine 3',5'-monophosphate (cyclic AMP). Exposing the ganglion to dibutyryl cyclic AMP or injecting cyclic AMP into the cell body enhances the synaptic action of a sensory neuron. The mechanism of presynaptic facilitation, therefore, may include activation of one or more serotonergic neurons, which enhance the release of a neurotransmitter by increasing the intracellular concentration of cyclic AMP in the terminals of the sensory neurons.*

Sensitization of the gill-withdrawal reflex in *Aplysia* involves a presynaptic facilitation of neurotransmitter output at the monosynaptic connections made by an identified group of 24 sensory neurons innervating the siphon skin and six identified gill motor cells (*1*). We have found that facilitation can be simulated by serotonin and that this action may be mediated by adenosine 3',5'-monophosphate (cyclic AMP).

Facilitation associated with short-term sensitization is not affected by prolonged inhibition of protein synthesis (*2*). This finding suggested to us that facilitation might result from a small molecule within the presynaptic terminal, perhaps an intracellular second messenger, regulating the mobilization of the transmitter packets from one storage compartment to another. This idea was supported by the finding that strong and prolonged electrical stimulation of the connectives from the head (or other pathways that produce the facilitation) leads to a prolonged, synaptically mediated increase in cyclic AMP in the abdominal ganglion (*3*). This action can be simulated by three biogenic amines: dopamine, octopamine, and serotonin (*4*). Exposure to one of these substances for 5 minutes increases cyclic AMP in the ganglion. The duration of this increase (up to 45 minutes) resembles that of presynaptic facilitation (and