Neuronal Correlates of Habituation and Dishabituation of the Gill-Withdrawal Reflex in Aplysia

Abstract. *We have examined the neural correlates of habituation and dishabituation of the gill-withdrawal reflex in* Aplysia. *We obtained intracellular recordings from identified gill motor neurons in the abdominal ganglion of a semi-intact preparation of* Aplysia *while we simultaneously recorded behavior responses of the gill. Habituation and dishabituation were not due to peripheral changes in either the sensory receptors or the gill musculature but were caused by changes in the amplitude of the excitatory synaptic potentials produced at the gill motor neurons.*

As a result of the great complexity of the nervous systems of most animals, it has generally not been possible to explain specific behaviors in terms of neuronal mechanisms. For this reason, studies of neural correlates of learning and similar behavioral modifications have not been able to distinguish those correlates that are causally related to the behavioral change from those that are not. The numerical simplicity of the abdominal ganglion of the marine mollusk *Aplysia* makes it possible to relate the function of individual cells in this ganglion to several behaviors (*1*). One of these behaviors, the gill-withdrawal reflex, undergoes short-term habituation and dishabituation (*2*). Since the neural circuit of this reflex has been largely specified, it should be possible to relate modifications of this behavior to specific changes in the nervous system. We now show that habituation and dishabituation are causally related to alterations of excitatory synaptic potentials at gill motor neurons.

For the present studies we have used two types of semi-intact preparations to record from and to stimulate single identified motor cells while we monitored movements of the gill during habituation and dishabituation. One type of preparation consisted of the "headless" animal, in which all of the nervous system except the abdominal ganglion was removed (*1*). To approximate more closely conditions in the intact animal, we have also used a preparation (Fig. 1A) in which the animal was immobilized in a small aquarium and stimulated by jets of seawater, as described for behavioral experiments (*2*). A small slit was made in the body wall at the posterior end of the seminal groove, and the abdominal ganglion was externalized. A lucite stage covered with a layer of wax was inserted under the ganglion, which was then firmly pinned in place, with its nerves and connectives intact. Gill motor neurons were impaled with double barrel microelectrodes for intracellular recording and for changing the membrane potential. The neural circuit of the gill reflex consists of at least four motor neurons (Fig. 1B) that appear to receive both poly- and monosynaptic excitatory connections from mechanoreceptors in the skin (Fig. 1C). All of these motor neurons were examined and showed similar results, but most

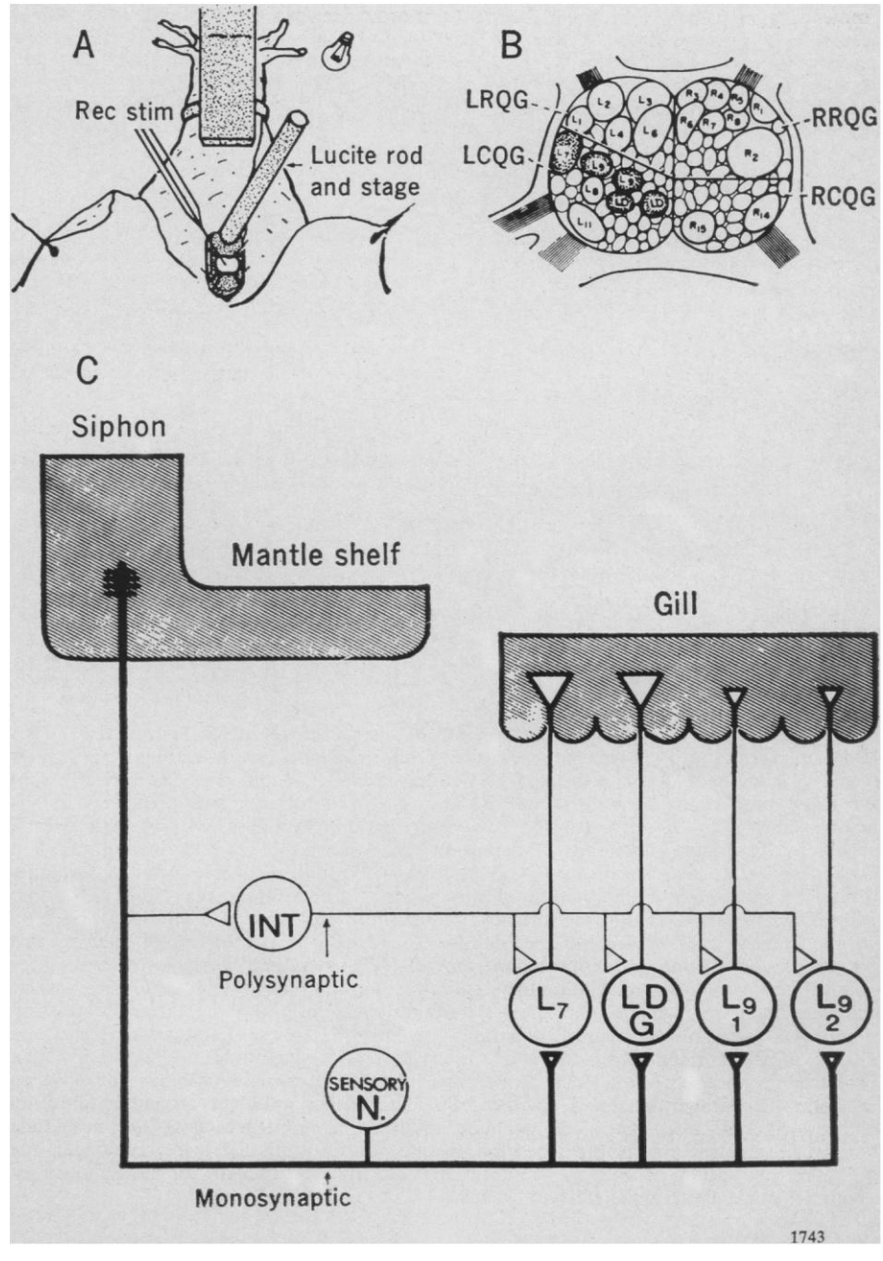

Fig. 1. (A) Top view of anterior portion of semi-intact preparation. The abdominal ganglion was externalized through a small slit made in the skin above the ganglion. The lucite rod and stage acted as a light guide, so that a strong light directed at the top of the rod transilluminated the ganglion. The body wall had a tendency to tighten around the lucite stage, thereby slowing blood loss. (B) Dorsal view of the abdominal ganglion showing identified cells. Motor neurons (shaded) innervating the gill and siphon are all located in the left caudal quarter ganglion (LCQG). (C) Proposed schematic diagram of circuit controlling defensive withdrawal reflex of the gill. All of the motor neurons receive excitatory input from tactile receptors in the skin, presumably via both mono- and polysynaptic pathways. Except for the monosynaptic input to L7 which has been directly demonstrated (*6*), all other indicated mono- and polysynaptic inputs have been inferred from less direct data.

of the work was done on cell L7.

The results of behavioral experiments (*2*) suggested that habituation of the gill reflex could not be explained by changes either at the neuromuscular junction or in the muscle itself. These factors were directly investigated in the semi-intact preparation. We found that contractions of the gill produced by intracellular stimulation of gill motor neurons or by electrical stimulation of efferent nerves did not substantially decrease in magnitude when evoked at intervals (1 to 5 minutes) that were effective in producing habituation (*3*). We also found that the size of a gill response produced by intracellular stimulation of gill motor neurons was the same before and during maximum habituation of the reflex (Fig. 2B). Similarly, following a dishabituatory stimulus, the reflexly evoked gill contraction increased in amplitude, whereas a directly evoked gill contraction remained unchanged. Thus, peripheral motor factors cannot explain either habituation or dishabituation of the gill reflex.

The finding that habituation was not due to fatigue of peripheral muscle suggested that it must be associated with some change in either the sensory input or its central processing. In either case, habituation would be associated with changes that would be reflected in the gill motor neurons. This inference could be tested by recording from the major motor neurons of the gill reflex. Tactile stimulation within the receptive field (siphon and mantle shelf) of the gill-withdrawal reflex produced large excitatory postsynaptic potentials (EPSP's) in all the identified motor neurons of the gill (*1*). When the tactile stimulus was repeated, the resulting EPSP gradually decreased in size (Fig. 3, first line) and the number and frequency of the evoked spikes correspondingly decreased (Fig. 2A, first line). Restoration of reflex responsiveness produced either by rest (Fig. 2A, second line) or by a dishabituatory stimulus (Fig. 2A, third line) was associated with an increase of the evoked EPSP and an increase in the number and frequency of spikes in the gill motor neurons.

Since the reflexly evoked gill contraction is determined by the output of several motor neurons, there was not a perfect correlation between the spiking in any one motor neuron and the magnitude of the gill contraction. However, the correlation was reasonably close, particularly for the initial second of the spike discharge that followed a tactile stimulus. A decrease of one or two spikes within the initial discharge was usually associated with a measurable decrease of the gill contraction. The sensitivity of gill contraction to small changes in firing rate or to total number of spikes in gill motor neurons could be demonstrated independently by means of intracellular stimulation of single gill motor neurons. Variation of a few spikes in a 1-second train of directly evoked spikes in cell L7 caused clear variation in the amount of gill contraction (Fig. 2C). In some circumstances, as reported (*1*), an individual spike in cell L7 or LD-G produced a visible twitch of the gill. These results indicate that relatively small changes in EPSP amplitude at gill motor neurons will lead to a change in the magnitude of the gill contraction.

The decrease in the evoked EPSP during habituation was not necessarily due to a central process, since it might have been the result of adaptation of sensory receptors. We examined this possibility in three types of experiments. First we found that reflex habituation still occurred when the receptors were circumvented by electrical stimulation of an afferent nerve. This showed that the central nervous system could mediate habituation, but it did not rule out the possibility of receptor adaptation during natural stimulation of the skin. We therefore recorded from afferent units in filaments of peripheral nerves (*4*). During a series of 5 to 10 tactile stimuli, presented every 1 or 2 minutes, there was no systematic decrease in the afferent vol-

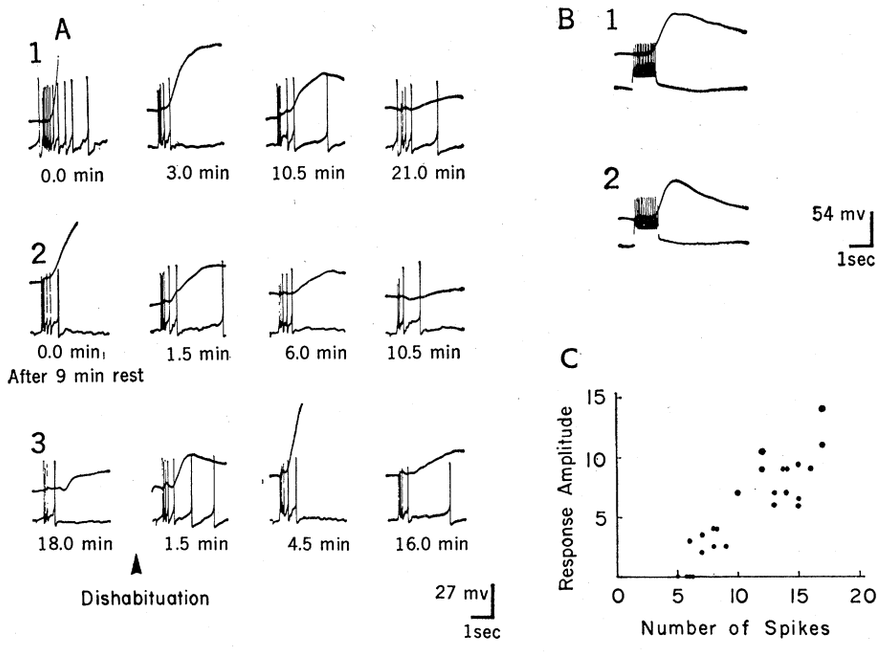

Fig. 2. Correlation of contraction of the gill and responses of motor neuron (L7). (A) Gill contractions (top traces of each line) and simultaneous intracellular recordings from an identified motor neuron, L7 (bottom traces). Sample records are all from the same preparation. Tactile stimuli (500 msec in duration) were presented to the mantle shelf every 90 seconds. Top row: Habituation. Stimuli were presented over a period of 21 minutes. Number of spikes in the 1-second interval following the first evoked spike in each trace: 9,6,6,4. Middle row: Partial recovery (after a 9-minute rest) and subsequent rehabituation of the reflex. Number of spikes: 7,6,5,3. Bottom row: Dishabituation. Following the last habituation trial shown in the first trace, a strong stimulus was applied to the siphon. The discharge of the motor neuron and the amplitude of the gill contraction progressively increased during the first three stimuli following the dishabituatory stimulus and remained elevated for several minutes. Number of spikes: 4,5,7,5. (B) Gill responses produced by intracellular stimulation of same motor neuron illustrated in Part A, before habituation (B1) and during maximal habituation (B2) of the reflex. (C) Magnitude of gill contraction produced by motor neuron (L7). Same experiment as in parts A and B. The abscissa indicates the number of spikes produced by depolarizing pulses, 1 second in duration and of different intensities. The ordinate indicates in arbitrary units the amplitude of the gill contraction as measured by the output of a photocell placed under the gill. The amplitude of the gill contraction was linearly related to the amount of photocell uncovered as the gill contracted.

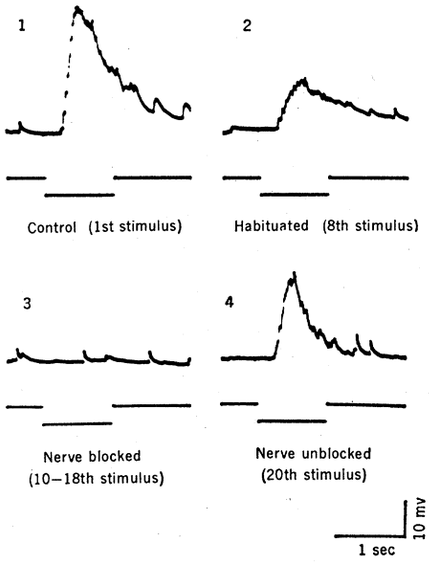

Fig. 3. Intracellular record from gill motor neuron (L7). The cell was hyperpolarized to prevent spiking. The square pulse indicates the duration of stimuli applied to the siphon every 2 minutes. Following the eighth stimulus, a segment of the nerve was bathed in tris-chloride, and complete block of the nerve occurred starting at the tenth stimulus. Following the 18th stimulus the tris-chloride was replaced with seawater. When the nerve conduction was restored after one stimulus, the response was larger than the fully habituated level before the nerve block.

ley, although the number of spikes evoked varied considerably from trial to trial (*3*). Since the gill reflex may be mediated by very small afferent fibers that could not be recorded in the nerve filaments, this experiment cannot completely rule out the possible contribution of sensory adaptation to the reflex habituation. However, the existence of an EPSP that correlated with behavioral habituation permitted a more definitive test of whether sensory adaptation might be involved in habituation. If the decline of the EPSP was due to a central process, then the decremented EPSP should start to recover, despite continued stimulation of the receptors, provided that input to the ganglion was blocked. For this study the abdominal ganglion was completely isolated except for an intact siphon nerve and siphon. The siphon nerve was led through a chamber that could be filled with solutions free of Na^+ and Ca^{2+} (*5*) so that nerve conduction could be reversibly blocked. Tactile stimuli were first presented to the siphon at intervals of 1 or 2 minutes with the nerve not blocked, until the EPSP recorded in motor neuron L7 decremented to less than half of its initial value (Fig. 3). Peripheral stimulation was then continued for another 10 to 20 minutes with the nerve blocked. Despite the continued peripheral stimulation, when the nerve was unblocked the EPSP showed recovery and was larger than the habituated response immediately before the conduction block. The amount of recovery for a given interval of peripheral stimulation with the nerve blocked was comparable to the recovery produced by an equal interval of complete rest. It therefore appears that sensory adaptation contributes little or nothing to the habituation.

A contribution of sensory factors to dishabituation could also be excluded. Dishabituation occurred when the dishabituatory stimulus was presented at a point on the body surface far from where the test stimuli were presented and consequently could not stimulate the same receptors. In addition, strong stimulation of a nerve produced restoration of a decremented EPSP in the completely isolated ganglion preparation (*6*).

These experiments indicate that habituation and dishabituation of the defensive gill-withdrawal reflex in *Aplysia* are central processes. Habituation is a direct result of a decrease of the excitatory synaptic potentials at gill motor neurons, whereas dishabituation is due to an increase of the excitatory synaptic potentials. These experiments cannot completely rule out a contribution due to peripheral changes, but the results of various control experiments suggest that if systematic peripheral changes are present, their contribution to the overall response decrement must be small relative to the contribution of central factors. Since the EPSP in the present experimental conditions is complex, containing mono- and polysynaptic excitatory inputs and perhaps even inhibitory components, it was not possible to determine the mechanism of the EPSP decrement. The experiments described in the last paper of this series were designed to provide this analysis (*6*).

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References and Notes

1. I. Kupfermann and E. R. Kandel, *Science* **164**, 847 (1969).
2. H. Pinsker, I. Kupfermann, V. Castellucci, E. R. Kandel, *ibid.*, this issue.
3. As the preparations aged, directly evoked gill contractions as well as afferent volley discharges to tactile stimuli gradually decreased in magnitude. Such examples of peripheral fatigue appeared to be limited to preparations that were physiologically deteriorated, as judged by the sluggishness of the contractions and by the failure of the fatigued responses to recover with prolonged rest.
4. The afferent volley was recorded with platinum electrodes and high gain a-c amplification. The nerve was soaked in a 0.5 percent trypsin solution (weight/volume) for a half hour, and small filaments were dissected out by means of steel insect pins that were electrolytically sharpened. The nerve with the siphon attached was placed in seawater, and the cut end of the nerve from which the filaments were dissected was immersed in a separate chamber filled with mineral oil. We thank Dr. B. Djahnparwar for describing the technique of dissecting single fibers in *Aplysia* nerves.
5. This technique is based upon one used by J. Bruner and J. Kehoe, (personal communication). Initial experiments were done with an isotonic sucrose solution. Later experiments utilized an isotonic tris solution, and this appeared to block conduction more quickly and completely than the sucrose solution.
6. V. Castellucci, H. Pinsker, I. Kupfermann, E. R. Kandel, *Science*, this issue.
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Neuronal Mechanisms of Habituation and Dishabituation of the Gill-Withdrawal Reflex in Aplysia

Abstract. *The cellular mechanisms of habituation and dishabituation of the gill-withdrawal reflex in* Aplysia *were studied with an isolated abdominal ganglion connected to a piece of skin from the tactile receptive field of the reflex. By obtaining simultaneous intracellular recordings from both the sensory neurons and one of the main identified motor neurons, we have been able to reduce the reflex to its monosynaptic components. The monosynaptic excitatory postsynaptic potentials showed a profound low-frequency depression when repeatedly elicited and showed heterosynaptic facilitation after application of a strong stimulus to another pathway. Thus, both habituation and dishabituation can be explained in part and perhaps entirely by changes in the efficacy of specific excitatory synapses.*

A plastic change in the functional effectiveness of synapses has often been suggested as a neuronal mechanism of behavioral modification. In vertebrates and invertebrates, certain synapses are