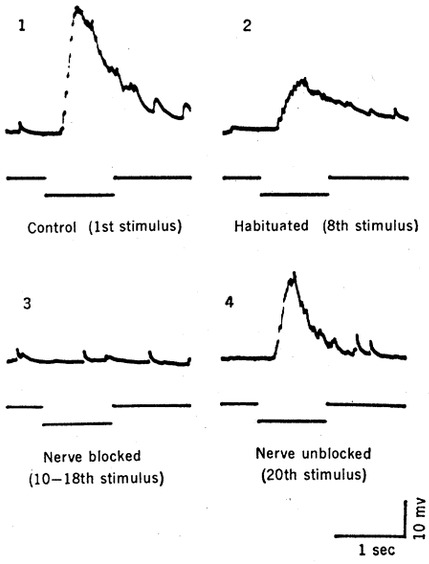

Fig. 3. Intracellular record from gill motor neuron (L7). The cell was hyperpolarized to prevent spiking. The square pulse indicates the duration of stimuli applied to the siphon every 2 minutes. Following the eighth stimulus, a segment of the nerve was bathed in tris-chloride, and complete block of 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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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.
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7. We thank K. Hilten for help in preparing the illustrations. Supported by PHS grants MH 15980, NB-07621, and NB-05980, by career scientist award K5-MH 18558 to E.R.K., career development award K1-MH 12240 to I.K., and a Canadian Medical Research Council Fellowship to V.C.

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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

capable of undergoing functional modification (*1–3*). However, the relevance of synaptic plasticity to a specific instance of behavioral modification has never been demonstrated. We have described behavioral parameters of habituation and dishabituation of the gill-withdrawal reflex in the intact *Aplysia* (*4*), and we have examined their cellular correlates in a semi-intact preparation (*5*). We now describe experiments in the isolated ganglion in which we have simplified the neural circuit of the reflex in order to investigate the cellular mechanisms. Our data indicate that both habituation and dishabituation of the gill-withdrawal reflex in *Aplysia* involve changes in the effectiveness of a specific set of central excitatory synapses between the sensory neuron and the motor neuron. These plastic changes result from homosynaptic depression and heterosynaptic facilitation, respectively.

The abdominal ganglion was isolated except for a strand of the siphon nerve which remained attached to a small piece of skin from the tactile receptive field of the gill-withdrawal reflex (Fig. 1A). A localized tactile or electrical stimulus was applied to the skin, and a double barrel microelectrode was inserted into one of the identified motor neurons (usually L7, Fig. 1A, part 2) (*6*) for recording and for measuring the membrane resistance. In some experiments we also impaled the cell bodies of the mechanoreceptor neurons of the gill-withdrawal reflex.

In experiments in the intact and semi-intact preparation (*4*, *5*), we used a jet of seawater that lasted from 500 to 800 msec as the tactile stimulus to elicit the gill reflex. To facilitate the analysis in the isolated ganglion, we have used brief (5 msec) mechanical or electrical stimuli to the skin. These brief stimuli produced complex excitatory postsynaptic potentials (EPSP's) in the gill motor neurons and, as in the semi-intact preparation, repeated stimulation led to a progressive decrement of the postsynaptic potential and recovery occurred after rest. To control for changes in the afferent input to the isolated ganglion, we stimulated the siphon nerve directly (Fig. 1B). Even with brief electrical stimuli to the nerve, we observed the six parametric features characteristic of habituation and dishabituation of the gill-withdrawal reflex in the intact preparation (*4*).

A decrement of the postsynaptic potential in the motor neuron could be produced either by a decrease in excitatory synaptic input or by an increase in an underlying inhibitory postsynaptic potential (IPSP) masked by the EPSP. The role of incrementing postsynaptic inhibition in the motor neuron could be excluded because similar EPSP decrement occurred when the membrane potential was hyperpolarized well beyond the equilibrium potential for the spontaneous IPSP's in the motor neuron.

The decrement in EPSP amplitude could in turn be produced by a decrease in the input resistance of the motor neuron. We tested this possibility by measuring the resistance of the motor neuron with intracellular hyperpolarizing pulses and found that the decrement of the complex PSP was not associated with a change in the input resistance of the motor neuron (Fig. 1B). These findings cannot rule out resistance changes at a site remote from the microelectrode in the cell body, but they do rule out gross changes in input resistance and suggest that the PSP alterations are due to changes in the synaptic input to the motor neuron.

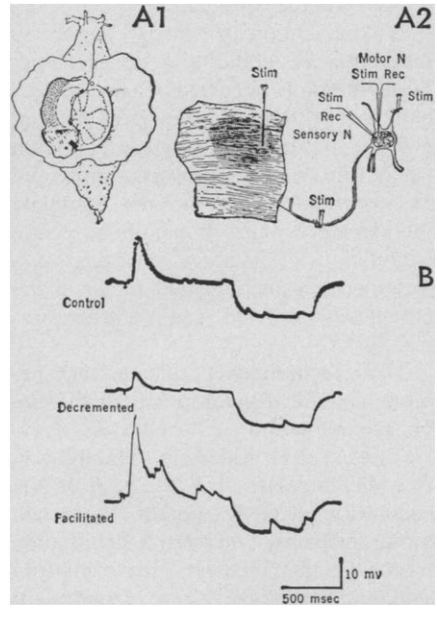

Fig. 1. (A) The isolated ganglion preparation. Part 1, the whole animal and a typical region (indicated in black) of the tactile receptive field which was used in the isolated ganglion experiment. Part 2, composite set-up for the isolated ganglion preparation. The ganglion was removed from the animal with a piece of skin from the tactile receptive field still attached to a strand of the siphon nerve. The region innervated by such a strand, when tested with tactile stimuli, is indicated by the stipling. The receptive field of individual sensory neurons was usually smaller. Electrical stimulation of the right or left connective provided extrastimuli for facilitation. In some experiments intracellular microelectrodes were inserted into sensory neurons as well as into the motor neuron (L7). (B) Complex evoked EPSP in cell L7. The PSP produced by electrical stimulation of the siphon nerve shows decrement with repetition of the stimulus and facilitation following a train of electrical stimuli to the left connective (6 per second for 4 seconds). Hyperpolarizing electrotonic potentials, produced by injecting current intracellularly, were used to measure the input resistance of the motor neuron. (Ten-second intervals between stimuli.)

A decrease in the excitatory synaptic input in the motor neuron could be caused either by an increase in inhibition of excitatory interneurons that contribute to the complex EPSP, or by a decrease in synaptic efficacy of individual afferent excitatory elements. A demonstration of decrement in an elementary monosynaptic excitatory input to the motor neuron would provide evidence for the latter possibility. We therefore simplified the afferent limb of the reflex pathway by examining, in L7, unitary and presumably monosynaptic EPSP's produced by localized stimulation of mechanoreceptors in the skin. We found a responsive region of the skin by using a tactile stimulus, and we then applied a weak electrical stimulus to this portion of the skin. We established the elementary nature of the EPSP's in these experiments by showing that the threshold for the PSP was all-or-none. The EPSP's appeared to be monosynaptic since they had a fixed shape and constant latency and were not abolished in solutions high in calcium content which tend to block polysynaptic inputs by raising the threshold of interneurons.

The elementary EPSP was clearly decreased with repeated stimulation (Fig. 2A). Recovery after rest is shown in Fig. 2B, and subsequent decrement is illustrated in the remaining records of this row. Decrement of a unitary PSP was observed with intervals between stimuli that ranged from 10 seconds to 5 minutes, and most of the decrement occurred within the first ten stimuli. Following 5 to 15 stimuli, complete recovery was usually obtained when the stimuli were withheld for 20 minutes.

Since the stimulus that produced the elementary, presumably monosynaptic,

EPSP could also activate other mechanoreceptor fibers, the decrement in the EPSP could have been due to presynaptic inhibition from parallel afferent fibers. Alternatively, the decrement could result from a homosynaptic change in the effectiveness of the EPSP. To test these possibilities it was necessary to stimulate an individual mechanoreceptor fiber in isolation. This test was made possible by the finding that cell bodies of primary mechanoreceptor neurons in *Aplysia* are located within the central ganglion, as has been demonstrated by Nicholls and Baylor (*7*) in the leech. A cluster of small cells near the motor neurons send their axons out the siphon nerve. Normally silent, these cells are excited by tactile stimulation of small regions of the skin of the siphon and appear to be primary mechanoreceptor neurons of the gill-withdrawal reflex. Upon direct stimulation, each spike in the sensory neuron produced a unitary EPSP in the gill motor neuron, similar to that produced by localized stimulation of the skin. In addition, the EPSP produced by intracellular stimulation of these cells had a short and constant latency which was not affected by high-calcium solutions. These findings provide further evidence that the mechanoreceptor neurons make monosynaptic connections with L7. This direct EPSP also showed decrement with repeated stimulation (Fig. 2D) and recovery with rest. As was the case with stimulation of the skin, the EPSP produced by direct stimulation of the sensory neuron sometimes diminished so markedly that after a few stimuli it was barely visible (Fig. 2D). These data rule out presynaptic inhibition and suggest that decrement of the EPSP is due to a change in excitatory synaptic efficacy resulting from either a decrease of transmitter release per unit impulse or a decrease in the sensitivity of the postsynaptic receptor. A prolonged decrement has been previously encountered in other synapses in *Aplysia* (*2, 3*) and has been studied in detail by Bruner and Tauc (*3*).

In the behavioral response of the intact animal, dishabituation occurs following the presentation of a strong stimulus to another part of the receptive field. Similarly, in the isolated ganglion, facilitation of the decremented complex EPSP (Fig. 1B, facilitated) and of the decremented unitary PSP (Fig. 2, C and D) occurred following a strong stimulus to the right or left connective. At times the facilitated PSP was even larger than the control (Fig. 2D, facilitated).

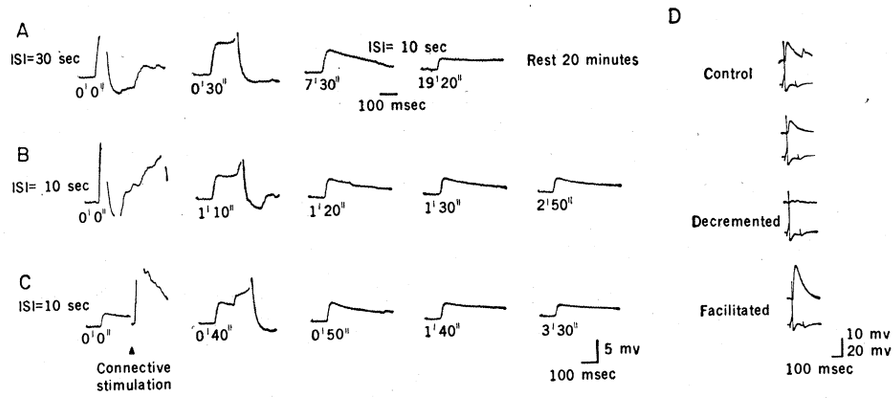

Fig. 2. Response decrement and facilitation of elementary, presumably monosynaptic, EPSP's. The tops of the spikes have been cut off in the first two traces of A, B, and C. (A) Decrement of an EPSP in L7 produced by electrical stimulation of the skin (30-second or 10-second intervals between stimuli, ISI). After 19 minutes the ISI was shortened from 30 seconds to 10 seconds. (B) Spontaneous recovery (after rest of 20 minutes) and subsequent decrement. (C) Facilitation of the decremented EPSP following a train of stimuli to the left connective (9 per second for 2 seconds). (D) Decrement and facilitation of a unitary EPSP produced by a single spike elicited by intracellular stimulation of a sensory neuron (interval between stimuli, 10 seconds). Top trace of each pair is from the motor neuron (L7), lower trace from the sensory neuron. First three pairs of traces illustrate consecutive stimuli. Following the third evoked EPSP a strong stimulus was applied to the left connective (7 per second for 5 seconds). The fourth pair of traces shows the facilitated EPSP 30 seconds after connective stimulation.

The facilitation of the PSP did not result from a change in the resistance of the extrasynaptic membrane of the motor neuron (Fig. 1B, facilitated). In some instances a large depolarization produced by the facilitating stimulus brought the resting membrane potential into the range of anomalous rectification (*8*), and this increase in input resistance contributed slightly to the increments of the first few responses after the extrastimulus. However, facilitation still occurred when changes in membrane potential were compensated for. Similarly, the facilitation of the EPSP was not a consequence of the action potentials produced in the motor neurons by the facilitatory stimulus. These action potentials did not affect the presynaptic terminals of the afferent pathway since directly firing the motor neuron, even at high frequencies, produced no facilitation.

The facilitation occurred in an elementary PSP produced either by stimulation of the skin or by direct stimulation of the cell body of a single mechanoreceptor neuron (Fig. 2, C and D). In studies of the mechanoreceptor neurons it was possible to show that facilitation occurred without a change in the frequency of firing of the sensory neuron, thereby excluding posttetanic potentiation as a mechanism for the facilitation. These data suggest that this heterosynaptic facilitation involves a presynaptic mechanism.

As was the case for the complex EPSP and for gill-withdrawal reflex in the intact animal (*4*), the elementary monosynaptic EPSP showed (i) decrement with repeated stimulation, (ii) recovery with rest, (iii) greater decrement with short rather than with long interstimulus intervals, (iv) facilitation following a strong stimulus to another pathway, and (v) decrement of the facilitatory effect with repetition. We have not examined the effects of stimulus intensity on the elementary EPSP.

The simplified circuit diagram (Fig. 3) illustrates the suggested locus and mechanism for these plastic changes. Only the motor neuron (L7) on which the work with the elementary EPSP was done is shown. Repetitive homosynaptic stimulation of tactile receptors leads to a plastic change at the indicated synapse between the afferent fibers and the motor neuron. The exact mechanism of the synaptic change is uncertain because we cannot exclude a change in receptor sensitivity, although this mechanism seems somewhat unlikely in view of the ease with which

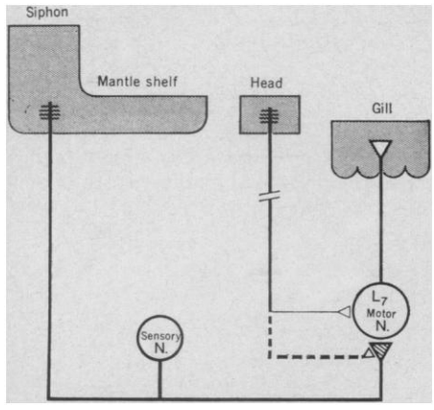

Fig. 3. Schematic wiring diagram to indicate the locus of the postulated plastic changes underlying habituation and dishabituation of the gill-withdrawal reflex. Habituation is due to a decrement in excitatory transmission at the crosshatched synapse. Dishabituation is due to a heterosynaptic facilitation of the same synapse. The dashed line indicates a hypothetical pathway which synapses on the presynaptic terminals of the sensory fibers and mediates the proposed presynaptic facilitation. The dishabituatory stimulus also produces an excitatory input to the motor neuron. Dishabituation can be produced by a strong stimulus to most parts of the animal's body surface although only the head is indicated in the diagram. The exact neural pathway from the head, indicated by the interrupted line, has not yet been worked out.

heterosynaptic facilitation occurs. By analogy to the brief low-frequency synaptic depression that has been analyzed in detail at the vertebrate and at the crayfish neuromuscular junction (*9*), we suggest that the decrement of the elementary EPSP represents a decrease in the release of excitatory transmitter from the presynaptic terminal. However, unlike the brief low-frequency depression evident at many synapses (*10*), the synapses in the habituating pathway show a remarkably large and prolonged decrement. This quantitative difference from the usual forms of low-frequency depression suggests that additional features may be operative in synapses of the habituating pathway. The heterosynaptic facilitation of the EPSP appears to be due to presynaptic facilitation (*2*) at the same synapse, perhaps this results from an enhanced release of transmitter substance.

It should be emphasized that we have used the monosynaptic pathway between the mechanoreceptor neurons and L7, one of the two major motor neurons of the reflex, as a model for studying the total reflex. Comparable experiments with monosynaptic inputs need to be done on other gill motor neurons, particularly on LD-G (*6*), the other major motor neuron, and on the polysynaptic pathway. However, the undecremented elementary EPSP's produced by a single spike in individual afferent neurons are relatively large (1 to 10 mv) and often trigger a spike in the motor neuron. In addition, there are at least ten such units which synapse on L7. It therefore seems likely that a substantial portion of the complex EPSP in the gill motor neuron L7 is due to the monosynaptic EPSP's from mechanoreceptor neurons. Furthermore, the spike activity of L7 contributes a substantial part to the total gill contraction (*6*). Since changes in the spike activity of L7 are directly produced by changes in EPSP amplitude (*5*), a substantial part of the habituation and dishabituation of the early component of the withdrawal reflex (*4*) can be explained by alterations in the efficacy of excitatory synapses between the sensory and motor neurons. Indeed, if similar processes occur on the other motor neurons and on the interneurons, these mechanisms could explain all of the habituation and dishabituation.

These data also lead to some other, more general, conclusions. First, the data indicate that habituation and dishabituation both involve a change in the functional effectiveness of previously existing excitatory connections. Thus, at least in these simple cases, it seems unnecessary to explain the behavioral modifications by invoking electrical or chemical fields or a unique statistical distribution of activity in a neural aggregate. The capability for behavioral modification seems to be built directly into the neural architecture of the behavioral reflex (*5, 6*). Second, although a number of investigators have postulated on the basis of indirect evidence that habituation involves active inhibition (*11*), in *Aplysia*, where a major component of the synaptic mechanism of habituation can be studied directly, neither pre- nor postsynaptic inhibition appear to be critically involved. Third, these experiments illustrate that habituation and dishabituation are separate processes and support the evidence (*12*) that dishabituation is not merely a removal of the decrementing process but is an independent facilitatory process superimposed upon the decrement. Seen in this perspective, dishabituation is essentially a special case of quasi-conditioning (sensitization), the process whereby a strong stimulus enhances other responses. Although habituation and dishabituation are independent, they do not involve different neurons with overlapping fields of action. Rather, the two processes appear to represent two independent regulatory mechanisms acting at the same synapse.

Finally, these studies strengthen the assumption, underlying some current cellular neurophysiological approaches to learning, that a prerequisite for studying behavioral modification is the analysis of the wiring diagram underlying the behavior (*13*). We have indeed found that once the wiring diagram of the behavior is known, the analysis of its modifications becomes greatly simplified. Thus, although this analysis pertains to only relatively simple and short-term behavioral modifications, a similar approach may perhaps also be applied to more complex as well as longer lasting learning processes.

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