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Cellular Analysis of Long-Term Habituation of the Gill-Withdrawal Reflex of *Aplysia californica*

Abstract. *Long-term habituation training in* Aplysia californica *produces a profound depression in the efficacy of synaptic transmission between mechanoreceptor neurons and gill motor neurons. This depression persists for more than 3 weeks. Thus a critical synaptic site for plasticity underlying long-term habituation is the same as that for short-term habituation. For this simple form of learning, short- and long-term memory share a common locus and aspects of a common mechanism: synaptic depression.*

An unresolved question in the study of learning is the relation between short- and long-term memory. Some behavioral and pharmacological studies suggest that short- and long-term memory are two different processes having different loci and different neural mechanisms (*1*). Other studies suggest that memory consists of a single trace which changes in character with time and with processing [for a review, see (*2*)]. This question can be directly approached by examining the loci and cellular mechanisms of both short- and long-term memory in a given behavioral system and by determining the interrelation between them. To accomplish this end, we have studied the retention of habituation of the gill-withdrawal reflex in *Aplysia californica*; this reflex involves both short- and long-term memory in habituation training (*3, 4*).

The neural circuit of the reflex to stimuli of weak and moderate intensity is well understood in terms of individual nerve cells and their interconnections (*5, 6*). We have found that in this reflex, short- and long-term habituation share a common locus: the synapses made by the sensory neurons on their central target cells. Moreover, both the short-term and long-term memory involve aspects of a common mechanism: homosynaptic

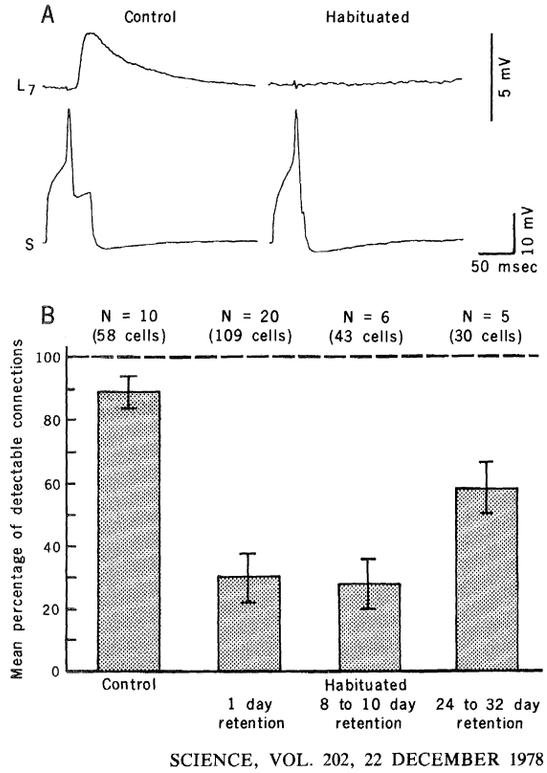

Fig. 1. Detectable and nondetectable connections. (A) Detectable EPSP from a control abdominal ganglion, and a nondetectable EPSP from an experimental animal that received long-term habituation training and whose synaptic connections were examined 24 hours after the last training session. The sensory neuron (S) was depolarized intracellularly to trigger a single action potential and to evoke a monosynaptic EPSP in the gill motor neuron L7. (B) Summary of the first experiment in which the ratio of the number of detectable EPSP's over the total number of connections sampled was determined in control animals ($N = 10$) and in habituated animals 1 day ($N = 20$), 8 to 10 days ($N = 6$), and 24 to 32 days ($N = 5$) after their last training session. Each animal contributed one ratio, and the graph indicates the mean percentage (± standard error) of detectable connections.

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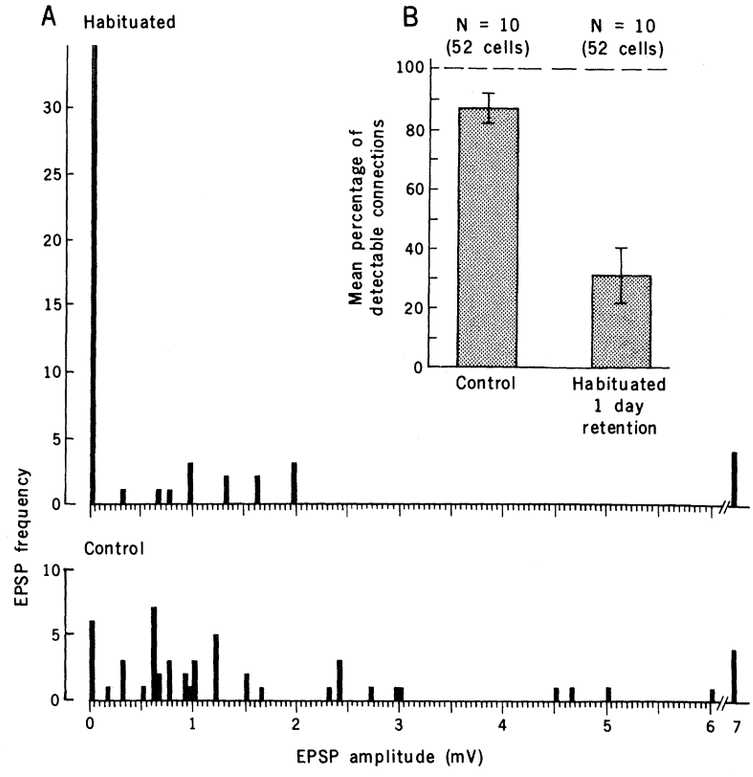

Fig. 2. Summary of the second experiment in which a blind procedure was used. (A) Histogram of EPSP amplitudes from control (52 cells from ten preparations) and experimental animals (52 cells from ten preparations). All sampled connections are included. Detectable EPSP's include all potentials above 50 μV. There was a clear trend toward nondetectable connections in animals receiving long-term habituation training. Histograms of EPSP amplitudes for control and experimental animals (1-day retention) from the experiments shown in Fig. 1 were virtually identical. (B) Ratios of detectable EPSP's over the total number of connections sampled. The percentage of detectable connections was significantly greater in control (N = 10) compared to experimental (N = 10; $P < .005$) animals.

depression of excitatory synaptic transmission.

After a single training session in which 10 to 15 tactile stimuli are applied to the siphon skin, habituation is retained for only a few minutes to a few hours (*3*). But, if several (four or more) training sessions are given at intervals of 1.5 or 24 hours, habituation is retained for days or weeks (*4*).

A first attempt to analyze the mechanism underlying long-term habituation revealed that repeated electrical stimulation of the siphon nerve, at rates that produce long-term habituation in the intact animal, could produce a profound and long-lasting (at least 24 hours) depression of the complex excitatory postsynaptic potential (EPSP) in one major motor neuron of the reflex, L7 (*7*). Since the monosynaptic EPSP produced by a single sensory neuron connecting with the same motor neuron can also be dramatically depressed with repeated stimulation (*6*), the same synapses might be the locus of both long-term and short-term habituation. We tested this hypothesis by analyzing the synaptic connection between the sensory neurons and gill motor neuron L7 in control animals and in trained animals that exhibited long-term memory of habituation.

We used 61 *Aplysia californica*, each weighing 100 to 250 g. After a minimum of 5 days of individual housing [see (*4*)], the reflex responsiveness of the animals was assessed by delivering two tactile stimuli (jets of seawater) to the siphon by means of a Water Pik (*3, 4*). Unresponsive animals were rejected (*8*); the scores of the accepted animals were ranked and animals were alternately assigned to control and experimental groups.

In the first experiment, intact animals were given habituation training and the sensory-to-motor connections were examined in isolated abdominal ganglia, either 1 day (N = 20), 1 week (8 to 10 days; N = 6), or more than 3 weeks (24 to 32 days; N = 5) after training. During a habituation training session, ten tactile stimuli (interstimulus interval, 30 seconds) were applied to the siphon skin with a Water Pik; all animals received at least 5 days of training (*9*). All 31 animals showed significant habituation when the score on the last day of training was compared with the score on day 1 (*10*).

To maximize the difference between experimental and control animals, we initially used experimental animals that exhibited pronounced long-term habituation, and control animals if they showed a brisk reflex response to siphon stimulation 1 hour after dissection. Subsequent experiments showed that this selection procedure was unnecessary and had no effect on the experimental results.

We measured the amplitude of the monosynaptic EPSP's between sensory neurons and the gill motor neuron L7 in controls and in the three groups of habituated animals. In control animals, almost all of the connections sampled revealed detectable (> 50 μV) monosynaptic EPSP's of short latency (less than 10 msec), and constant shape (*11*). In contrast, trained animals displayed a marked depression in the amplitude of the EPSP, such that the occurrence of detectable monosynaptic EPSP's in motor neuron L7 was significantly reduced (Fig. 1).

In view of the magnitude of this depression, we analyzed our data using the ratio of detectable EPSP's to the total number of sampled connections in each animal (Fig. 1B). Three to ten connections were sampled in a given experiment and each animal contributed a single ratio. In control animals (N = 10) the mean percentage of detectable connections was 89 ± 5 percent (± standard error). By contrast, in habituated animals tested, 1 day and 8 to 10 days after their last training, the incidence of detectable connections was only 30 ± 8 and 28 ± 8 percent, respectively. From 24 to 32 days after training the effect had diminished somewhat; 58 ± 8 percent of the cells showed detectable connections. An analysis of variance showed that there was a significant difference in the percentage of detectable connections among the groups ($F_{3,37}$ = 10.7; $P < .01$). Subsequent planned com-

parisons showed that there was a significantly greater percentage of detectable connections in control animals compared to the percentage of connections of experimental animals tested 24 hours after training ($P < .005$), 8 to 10 days after training ($P < .005$), and 24 to 32 days after training ($P < .01$).

To assure that the selection procedures imposed in the first experiment did not influence the outcome, we repeated the first experiment by examining synaptic connections 24 hours after training, using no selection criteria. All habituated animals ($N = 10$) and all controls ($N = 10$) were used and were matched in terms of the length of time they were kept in aquaria. In addition, to assure that experimenter bias was not a factor, we also used a blind procedure (*12*). The results of this experiment were essentially identical to those of the first experiment (Fig. 2B). The percentage of detectable connections was significantly greater in the control, 87 ± 5 percent, compared to experimental, 31 ± 9 percent, animals ($P < .005$).

In both experiments (Figs. 1B and 2B), the major change was in the incidence of detectable EPSP's; there was no significant difference in the amplitude of those EPSP's that were detectable. The rates of depression of the detectable EPSP's from control and experimental animals were also similar. However, in the histogram of EPSP's, the amplitudes of all sampled connections, detectable and nondetectable, there was a clear trend toward nondetectable connections in animals receiving long-term habituation training (Fig. 2A). These data suggest that, as habituation training is extended from short- to long-term, the connections of most sensory neurons are progressively depressed until they finally become undetectable. On the other hand, the connections of a few neurons are not significantly affected. We do not know whether the residual connections represent neurons whose connections are resistant to long-term habituation training or neurons whose receptive fields are not activated by the tactile stimuli used in our training procedure.

Our data indicate that the locus of plasticity which underlies short-term habituation of the gill-withdrawal reflex is the same as that which underlies long-term habituation: the monosynaptic connection between the mechanoreceptor neurons innervating the siphon skin and their central target cells. There is a striking parallel between long-term depression of synaptic efficacy as indicated by the incidence of detectable connections and long-term habituation of the reflex response (*13*).

These results also lead to two other conclusions. (i) These findings provide what we believe is the first evidence that an instance of long-term memory can be accounted for by long-term changes in the effectiveness of previously existing synaptic connections. (ii) In critical synapses of pathways capable of being modified by learning, relatively few stimuli are required to produce a rather profound plastic change.

Short-term habituation is due to a presynaptic mechanism: less transmitter is released per impulse in the terminals of the sensory cells (*6*). Our results do not allow us to distinguish between a pre- and postsynaptic mechanism for long-term habituation, but the question can be readily examined. If the change is also presynaptic, it would be helpful to know if the change in synaptic efficacy at the sensory-to-motor synapse during long-term habituation is accompanied by any observable ultrastructural changes, such as a change in the volume of the synaptic terminals of the sensory neurons, in the distribution of the synaptic vesicles in relation to active release sites, or in the number of active sites. Electron microscopic and morphometric analysis of labeled sensory neuron terminals (*14*) could answer these questions.

V. F. CASTELLUCCI
T. J. CAREW
E. R. KANDEL
Division of Neurobiology and Behavior, Departments of Physiology and *Psychiatry, College of Physicians and Surgeons, Columbia University, New York, 10032*

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8. Animals were accepted if the mean duration of their two test responses (interstimulus interval, 30 seconds) was 10 seconds or larger. Duration was measured as the time elapsed between the onset of the stimulus and the reappearance of the siphon between the parapodia. In addition, any animal giving a zero score for either of the two stimuli was excluded. During the experiments, animals were rejected if they did not eat the daily amount of seaweed given to them, or if they showed development of scar tissue or a damaged gill or siphon. There was no difference between experimental and control animals with regard to these factors.
9. All groups received a single ten-trial training session per day. One group of animals ($N = 20$) was trained for at least 5 days (median number of days of training, 9 days; range, 5 to 27 days) and the efficacy of the monosynaptic connection between the sensory cells and motor neuron L7 was examined 24 hours after the last training session. Another group of animals ($N = 6$) was trained for 6 days and synaptic efficacy was examined 8 to 10 days after the last training session. In the final group of animals ($N = 5$) trained for 5 days, synaptic efficacy was examined 24 to 32 days later.
10. The score of a daily session for one animal is defined as the summation of duration of the ten responses for that day.
11. The procedure to measure the efficacy of synaptic connections was the following: animals were injected with isotonic $MgCl_2$ (about half of body weight) to block synaptic activity and minimize the possibility of further habituating or, more likely, sensitizing the reflex, during surgery. The mantle shelf, siphon, and gill with the abdominal ganglion were excised and transferred to a chamber containing a solution of high Mg^{2+} (220 m*M*) and low Ca^{2+} (1 m*M*) content in order to block synaptic transmission during desheathing of the ganglion capsule. After being desheathed, the ganglion was bathed in artificial seawater with normal content of Ca^{2+} and Mg^{2+} (10 m*M* and 55 m*M*, respectively). A minimum of 30 minutes of rest was allowed before testing began. Only motor neuron L7 was used as the follower cell. L7, a major motor neuron which was used as a prototype for other motor neurons, was identified by the characteristic gill movement it causes, its location, and its spontaneous activity. Individual sensory cells were identified by their size, location, absence of spontaneous activity, and the antidromic spike evoked in them by stimulation of the siphon nerve. As many sensory cells as possible were sampled in each ganglion. Sampling consisted of producing a single spike in a sensory cell by brief depolarizing current pulses and determining the presence or absence of detectable EPSP's in L7. Amplitude of first evoked EPSP was measured and, when possible, five successive EPSP's were evoked at 30-second intervals in order to assess their kinetics of depression. If penetration of the sensory cell caused a burst of spikes, a minimum of 4 minutes of rest was given and then the first EPSP was evoked. There was no difference in the incidence or magnitude of injury discharge in control and experimental preparations. Before sampling the next sensory cell, we further identified the impaled sensory cell by its antidromic response after brief electrical stimulation of the siphon nerve.
12. In this series, animals were probed, ranked, and distributed either to the control group, with no training, or to the habituated group, which received at least 5 days of training (median number of days, 8; range, 5 to 12). Each day, two experimental and two control animals were coded by one person while a different person distributed the animals to be tested. Control and experimental animals were thus used in a parallel fashion.
13. See figure 1B in (*4*).
14. C. H. Bailey, E. B. Thompson, V. Castellucci, E. R. Kandel, *J. Neurocytol.*, in press.
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