11. H. P. Zeigler, H. J. Karten, H. L. Green, *Psychonom. Sci.* **15**, 156 (1969); H. P. Zeigler and H. J. Karten, *J. Comp. Neurol.*, in press; H. P. Zeigler, in *The Bird: Its Brain and Behavior*, I. Goodman and M. Schein, Eds. (Academic Press, New York, in press).
12. D. S. Farner, in *Biology and Comparative Physiology of Birds*, A. J. Marshall, Ed. (Academic Press, New York, 1960), vol. 1, pp. 411–467; B. R. Wolin, in *Contemporary Research in Operant Behavior*, A. C. Catania, Ed. (Scott, Foresman, Glenview, Ill., 1958), p. 286.
13. C. Pfaffman, in *Nebraska Symposium on Motivation*, M. R. Jones, Ed. (Univ. of Nebraska Press, Lincoln, 1961), pp. 71–108; A. N. Epstein, in *The Chemical Senses and Nutrition*, M. R. Kare and O. Maller, Eds. (Johns Hopkins Univ. Press, Baltimore, 1967), pp. 263–280.
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Acquisition and Retention of Long-Term Habituation in Aplysia: Correlation of Behavioral and Cellular Processes

Abstract. *To examine the cellular mechanisms responsible for transition from a short-term to a long-term behavioral modification, a rapid training procedure was developed for producing long-term habituation of the defensive withdrawal of gill and siphon in* Aplysia. *Four ten-trial training sessions, with 1½-hour intersession intervals, produced habituation that was retained for more than 1 week. This 5-hour procedure could be applied to a test system in the isolated abdominal ganglion where the cellular changes accompanying the acquisition of long-term habituation can be examined. During acquisition, intracellular recordings were obtained from L7, a major gill and siphon motor neuron, and the pattern of stimulation used in the behavioral experiments was applied to an afferent nerve. Acquisition was associated with a progressive decrease in the complex excitatory synaptic potential produced in L7 by afferent nerve stimulation. When retention was tested 24 hours later, the synaptic decrement was still evident. Thus, a behaviorally meaningful stimulus sequence, consisting of only 40 patterned stimuli, leads to changes in synaptic effectiveness lasting one or more days in a neural pathway involved in short-term habituation of this reflex.*

Cellular studies in several invertebrates have shown that short-term habituation and dishabituation involve changes in the synaptic effectiveness of excitatory synaptic connections [(*1–3*); for vertebrates, see (*4*)]. For example, in the marine mollusc *Aplysia*, habituation of the defensive gill-withdrawal reflex involves a change in effectiveness of the excitatory synapses made by mechanoreceptor sensory neurons onto motor neurons and interneurons (*1*). A similar locus and mechanism have been found in studies of habituation of escape responses in crayfish (*2*) and may also be involved in the cockroach (*3*).

The short-term behavioral modifications so far analyzed last several minutes to several hours (*1–4*). Whether similar synaptic changes characterize long-term behavioral modifications that endure days and weeks is not known. Recently, Carew *et al.* (*5*) described long-term habituation in *Aplysia*. Four consecutive days of siphon stimulation led to the acquisition of long-term habituation of both gill and siphon withdrawal that persisted for more than 3 weeks. We now report that, in the isolated abdominal ganglion, acquisition of long-term habituation is correlated with a pronounced decrease in excitatory synaptic input to a major motor neuron, L7, which persists for at least 24 hours. This neurophysiological investigation was possible because of a new behavioral procedure for habituation training that produces, within a few hours, long-term habituation that lasts more than a week.

Carew *et al.* (*5*) found that the time course of retention of long-term habituation depended on the pattern of stimulation: Massing 40 trials in one session was not as effective as spacing 40 trials (10 per day) over four daily sessions. However, it was not determined whether training sessions separated by less than 24 hours might also be effective. Since this would be technically advantageous for cellular neurophysiological studies, we first examined whether long-term habituation could be produced in a single day.

We used the training procedures of Carew *et al.* (*5*) with one modification: Four habituation training sessions were presented as before, but successive sessions were separated by 1½ hours rather than by 24 hours, so that all the training sessions necessary for acquisition could be given in 1 day. The withdrawal reflex was produced by an 800-msec jet of seawater to the siphon of an unrestrained animal (*6*). The duration of the siphon component of the withdrawal reflex (the time during which the siphon was completely hidden between the parapodia) was measured by an observer using an electric timer. A single training session consisted of ten trials, with an intertrial interval of 30 seconds. All animals were maintained in individual aquariums for at least a week before the

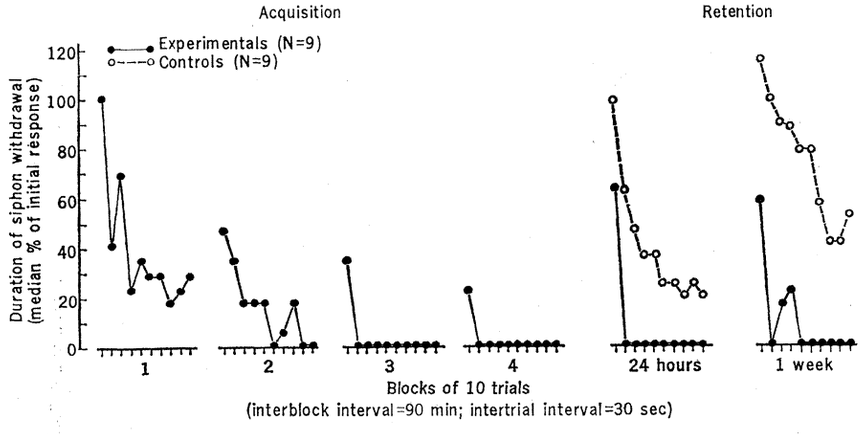

Fig. 1. Acquisition and retention of long-term habituation of siphon withdrawal. Data are expressed as percentage of the median of each group initial response (block 1, trial 1), which was 17 seconds for the experimentals and 19 seconds for the controls. For statistical analysis, the duration of siphon withdrawal for each animal was summed for trials 1 to 10; this measures the total time an animal spent responding in the habituation session. Intergroup statistical comparisons were made by means of Mann-Whitney U tests, and intragroup comparisons by means of Wilcoxon matched-pairs, signed-ranks tests. After four blocks of siphon-habituation training (acquisition), experimentals exhibited significantly greater habituation than controls in retention testing after both 24 hours and 1 week ($P < .001$ for both tests).

experiment and were then assigned to either an experimental group that received habituation training or to a control group that received no training. At 24 hours and again at 1 week after the last training session, all animals were coded and retention of habituation was measured by a blind procedure.

During acquisition, experimental animals showed habituation during the first ten-trial session and a buildup of habituation across sessions (Fig. 1). In retention tests, the experimental animals exhibited significantly greater habituation (lower net response tendency) than controls after both 24 hours ($P < .001$) and 1 week ($P < .001$) (Fig. 1). Furthermore, an intragroup analysis revealed that the experimental animals showed significantly greater habituation in both the 1-day and 1-week retention tests ($P < .005$ in both) than they did in the first ten-trial session of acquisition (*7*).

The finding that four ten-trial training sessions within a 6-hour period can lead to the acquisition of long-term habituation made it feasible to examine the cellular mechanism of acquisition and 24-hour retention (*8*). Tactile stimulation leads to habituation of both the siphon withdrawal and gill withdrawal components of the reflex (*5, 6*). Of the two, the neural circuit of the gill-withdrawal component is better understood (*1, 9*), and the neural correlates of its modification can be examined in the isolated ganglion (*1, 10*), which can be maintained in culture one or more days (*11*). In the test system, an afferent nerve (either the siphon or branchial nerve) was electrically stimulated, and the synaptic input produced was intracellularly recorded in L7, one of the major motor neurons for the gill (*12*). We chose stimulus parameters for both nerves (2-msec biphasic pulses) that produce a complex postsynaptic potential (PSP) in L7 comparable to that produced by an 800-msec jet of seawater to the siphon (*10*). This synaptic potential decreases in amplitude when the nerve is stimulated at rates that produce habituation (*1, 10*). In the intact animal and in the isolated ganglion, the response decrement is limited to the stimulated pathway. Habituation of the gill-withdrawal reflex after stimulation of the siphon does not alter reflex responsiveness to stimulation of the mantle shelf. Similarly, decrement of the PSP produced by repeated stimulation of the nerve from the siphon (siphon nerve) does not alter the synaptic responsiveness of the pathway from the mantle shelf (branchial nerve). Thus, each ganglion carries with it its own control; this is essential for distinguishing true decrease in synaptic effectiveness due to repeated stimulation from deterioration of the preparation due to isolation.

The abdominal ganglion with the siphon and branchial nerves still attached was removed from the animal and pinned to the wax floor of a Lucite chamber (*1, 10*). The nerves were pinned securely around capped pairs of Ag-AgCl electrodes. The chamber was constantly perfused at 15°C with artificial seawater (Instant Ocean) buffered with 0.01*M* tris(hydroxymethyl)-aminomethane (tris) HCl (*p*H 7.6) and containing streptomycin (0.1 mg/ml), penicillin (200 unit/ml), and glucose (0.1 percent). The motor neuron (L7) was impaled with a double-barrel microelectrode and hyperpolar-

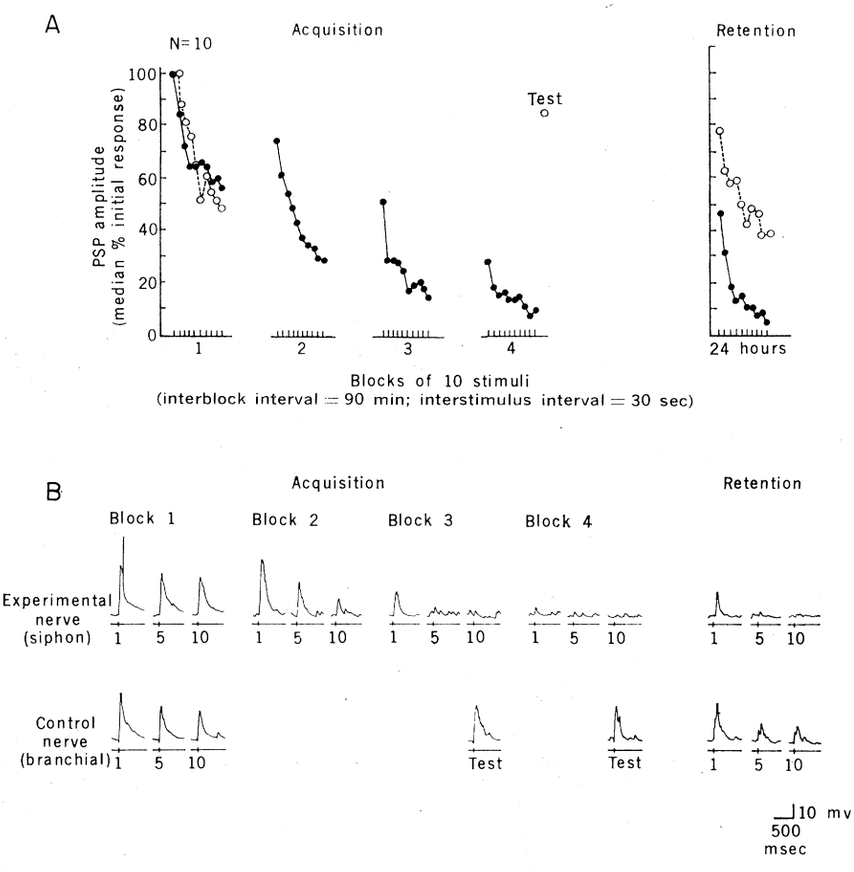

Fig. 2. (A) Acquisition and retention of long-term synaptic decrement. The amplitudes of the excitatory synaptic potential produced by stimulating experimental (●) and control (○) nerves are expressed as a percentage of the initial amplitude. In acquisition, two afferent nerves, the branchial and siphon, were alternated, one serving as experimental (six siphon, four branchial), the other as control. In block 1, ten stimuli were first applied to the experimental nerve and then to the control nerve. Stimuli to each nerve produced comparable synaptic decrement in L7. Repeated blocks of stimuli to the experimental nerve produced a progressive increase in synaptic decrement. A single stimulus to the control nerve after block 4 produced a synaptic potential substantially recovered from the last control potential of block 1, and was significantly greater than the first experimental synaptic potential of block 4 ($P < .001$). In retention testing, the cell was reimpaled 24 hours later and repolarized to approximately the same membrane potential maintained during acquisition. The retention ordinate is redrawn to indicate that the repolarization was not exact, although it could be closely approximated. The first experimental synaptic potential was significantly less than the first control potential ($P < .01$), and the overall synaptic responsiveness from repeated stimulation of the experimental nerve (sum of synaptic potential amplitudes for trials 1 to 10) was significantly less than from the same number of stimuli to the control nerve ($P < .001$). (B) Sample experiment from data summarized in (A). Stimuli 1, 5, and 10 from each block are shown. During acquisition, the experimental nerve received ten stimuli in each block; the control nerve received ten stimuli only in block 1. A single test stimulus was delivered to the control nerve after blocks 3 and 4 to assess the condition of the preparation. In retention testing (24 hours after block 4 of acquisition), both experimental and control synaptic potentials have partially recovered. However, the experimental synaptic potential showed greater and more rapid decrement than did the control.

ized to prevent spiking. One of the nerves was chosen as experimental, the other as control. Stimulus parameters that produced PSP's of comparable amplitude in L7 were selected for both nerves. The parameters were then held constant throughout acquisition and retention (*13*). In most experiments, the input resistance of the motor cell was monitored continuously by injecting hyperpolarizing current pulses into the soma.

Paralleling the behavioral experiments, four blocks of ten stimuli each (interstimulus interval, 30 seconds) were presented to the experimental nerve during acquisition, with 1½ hours separating successive blocks. In the first block, repeated stimulation of the experimental nerve invariably produced decrement of the complex PSP. After the first block of stimuli to the experimental nerve, ten stimuli were delivered to the control nerve. Since PSP decrement produced from stimulation of one nerve does not generalize to the other nerve (*10*), the ten stimuli delivered to the control nerve produced comparable decrement (Fig. 2A). In the second, third, and fourth blocks of ten stimuli, only the experimental nerve was stimulated, and the PSP decrement built up across blocks, reaching 90 percent (10 percent of initial experimental PSP) in the fourth block (Fig. 2A). After the fourth block, and occasionally after the third block, a single test stimulus was delivered to the control nerve to assess the condition of the preparation. The same statistical tests were used as in the behavioral experiments (Fig. 1). The first experimental PSP of the fourth block was significantly smaller than the test PSP from the control nerve ($P < .001$). Also, an intragroup comparison revealed that the net experimental PSP decrement (as measured by the sum of PSP amplitude for trials 1 to 10) in the fourth block was significantly greater than the net experimental PSP decrement in the first block ($P < .005$; Fig. 2).

Although some deterioration may have occurred during the experiments, the buildup of PSP decrement cannot be accounted for by deterioration. Test stimuli to the control nerve produced PSP's that were 84.5 percent of the initial control PSP. Some portion of even this 15.5 percent change in control PSP is probably caused, not by deterioration, but by the decrement produced by the ten stimuli delivered to the control nerve in the first block of acquisition (4.5 to 5 hours earlier). Also, in experiments in which input resistance of the neuron was monitored, it did not vary by more than 7 percent throughout the experiment. The buildup of PSP decrement was therefore chiefly caused by the four sessions of ten stimuli each to the experimental nerve.

At the end of acquisition, we removed the microelectrode from cell L7, maintained the ganglion for another 24 hours in organ culture (*11*), and then reimpaled the motor neuron to test for 1-day retention of PSP decrement (*14*). We hyperpolarized L7 to approximately the same membrane potential as during acquisition and stimulated the nerves as on the previous day (with identical stimulus parameters). Stimulating the experimental nerve on the first trial produced a PSP significantly smaller than that from the control nerve ($P < .01$). The ten repeated stimuli to the experimental nerve also always produced significantly greater PSP decrement than did ten stimuli to the control nerve ($P < .01$) (Fig. 2, A and B). The control nerve also showed a decrement, but it was much less profound. Therefore, the synaptic decrement produced by four blocks of ten stimuli to the experimental nerve persisted for at least 24 hours.

That such limited training (40 stimuli) should lead to such prolonged plastic changes in neuronal function is interesting in view of the generally reported difficulty in producing 24-hour synaptic changes in acute experiments, even after thousands of stimuli, in both vertebrates and invertebrates (*15*). The ease with which we produced prolonged synaptic changes is probably attributable to the selection of a stimulus pattern of known behavioral effectiveness and the use of a neuronal pathway known to be involved in a modifiable behavioral reflex.

In the test system, these synaptic changes occurred centrally, in the absence of sensory adaptation or motor fatigue. The synaptic alterations do not result from a change in the input resistance as recorded in the cell body of the motor neuron, although we cannot exclude more remote resistance changes. Thus these synaptic changes resemble those found with short-term habituation (*1, 10*). However, similar-appearing synaptic changes can be produced by several different cellular mechanisms. Once these changes are analyzed on an elementary level (*1*), one may be able to determine whether long-term habituation involves an extension of the processes underlying short-term habituation, or whether short- and long-term habituation result from two different cellular mechanisms.

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References and Notes

1. V. F. Castellucci, H. Pinsker, I. Kupfermann, E. R. Kandel, *Science* **167**, 1745 (1970); V. Castellucci and E. R. Kandel, in preparation.
2. R. S. Zucker, D. Kennedy, A. I. Selverston, *Science* **173**, 645 (1971).
3. J. J. Callec, J. C. Guillet, Y. Pichon, J. Boistel, *J. Exp. Biol.* **55**, 123 (1971).
4. W. A. Spencer, R. F. Thompson, D. R. Neilson, *J. Neurophysiol.* **29**, 253 (1966).
5. T. J. Carew, H. M. Pinsker, E. R. Kandel, *Science* **175**, 451 (1972).
6. Siphon stimulation elicits withdrawal of both siphon and gill components of the withdrawal reflex; long-term habituation of siphon withdrawal is accompanied by long-term habituation of gill withdrawal (*5*). Since the siphon component can be readily monitored in an unrestrained animal, it is easier to use as a behavioral index of long-term habituation.
7. We replicated this behavioral study with identical results. After training, experimentals ($N = 9$) showed significantly greater habituation than controls ($N = 8$), both after 24 hours ($P < .001$) and 1 week ($P < .01$).
8. It has not been possible to examine synaptic changes for longer than 24 hours after acquisition because the synaptic input from afferent nerve stimulation gradually deteriorates when the isolated ganglion has been in organ culture for more than 24 hours, even though the resting and action potentials are maintained much longer (*11*).
9. I. Kupfermann and E. R. Kandel, *Science* **164**, 847 (19[illegible] I. Kupfermann, H. Pinsker, V. Castellucci, E. R. Kandel, *ibid.* **174**, 1252 (1971).
10. T. J. Carew, V. F. Castellucci, E. R. Kandel, *Int. J. Neurosci.* **2**, 79 (1971).
11. F. Strumwasser, in *Invertebrate Nervous Systems, Their Significance for Mammalian Neurophysiology* (Conference on Invertebrate Nervous Systems, California Institute of Technology), C. A. G. Wiersma, Ed. (Univ. of Chicago Press, Chicago, 1967), pp. 291–319; J. H. Schwartz, V. F. Castellucci, E. R. Kandel, *J. Neurophysiol.* **34**, 939 (1971).
12. In this test system, we examined only a component of the total neural circuit of the gill-withdrawal reflex, the afferent input (sensory and interneuronal) onto the motorneuron L7.
13. In several control experiments, the stimulating current to both nerves was monitored continuously to ensure that it remained constant.
14. In some experiments, long-term retention could not be measured because of difficulty in reimpaling L7 or deterioration of the preparation between acquisition and retention. We have only presented acquisition data from experiments in which 24-hour retention could also be measured. However, in the other experiments in which retention could not be measured the acquisition data were identical to those in Fig. 2A.
15. J. C. Eccles, *Physiology of Synapses* (Springer-Verlag, Berlin, 1964); W. A. Spencer and R. S. April, in *Short-Term Changes in Neural Activity and Behavior*, G. Horn and R. A. Hinde, Eds. (Cambridge Univ. Press, London, 1970), pp. 433–474.
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