Long-Term Habituation of a Defensive Withdrawal Reflex in Aplysia

Abstract. *A tactile stimulus to the siphon of* Aplysia *produces a defensive withdrawal reflex consisting of contraction of the siphon, the gill, and the mantle shelf. We studied long-term habituation of this reflex using two types of preparations, one focusing on the siphon component and the other on the gill component of the reflex. Siphon withdrawal, studied in unrestrained animals, showed marked habituation within a single ten-trial training session. Five daily training sessions produced habituaton that built up across days and lasted for at least 3 weeks. Furthermore, spaced training produced significantly longer lasting habituation than massed training. Gill withdrawal, studied in a restrained animal, also showed long-term retention of habituation. Since the neural circuitry of gill withdrawal is relatively well understood, it may be possible to study the cellular mechanisms underlying a long-term behavioral modification.*

The neural mechanisms of simple behaviors and their modifications can be investigated in several invertebrate preparations (*1*). In one of these preparations, the marine mollusk *Aplysia*, the cellular mechanisms of short-term habituation, dishabituation, and sensitization of a defensive withdrawal reflex have been studied (*2*, *3*). The behavioral modifications so far demonstrated in the gill-withdrawal reflex typically last only minutes or hours (*4*). The behavioral modifications characteristic of higher animals, however, can last days and may even be permanent. In an attempt to extend its usefulness for the study of learning, we examined the ability of *Aplysia californica* to undergo more prolonged behavioral changes. We now describe long-term habituation of the defensive withdrawal reflex. This behavioral modification resembles higher learning in two ways: (i) it lasts for and least 3 weeks and (ii) it shows differential sensitivity to temporal patterning of training trials.

In *Aplysia* the mantle cavity, which houses the gill, is covered by a mantle shelf that extends posteriorly to form a siphon, which normally protrudes out of the cavity between the parapodia. The siphon serves both as an exhalent funnel for fluid and particulate matter entering the mantle cavity and also as a receptive sheet for tactile sensory input. Direct tactile stimulation of the siphon by a weak or moderate strength jet of seawater elicits a complex defensive reflex consisting of the withdrawal of the siphon into the mantle cavity, and the concomitant contraction of the gill and mantle shelf. If repeatedly elicited, this reflex habituates; the siphon, the gill, and the mantle shelf will withraw for increasingly shorter periods and finally can stop withdrawing altogether (*2*). We studied long-term habituation in two different preparations, one focusing on the siphon component and the other on the gill

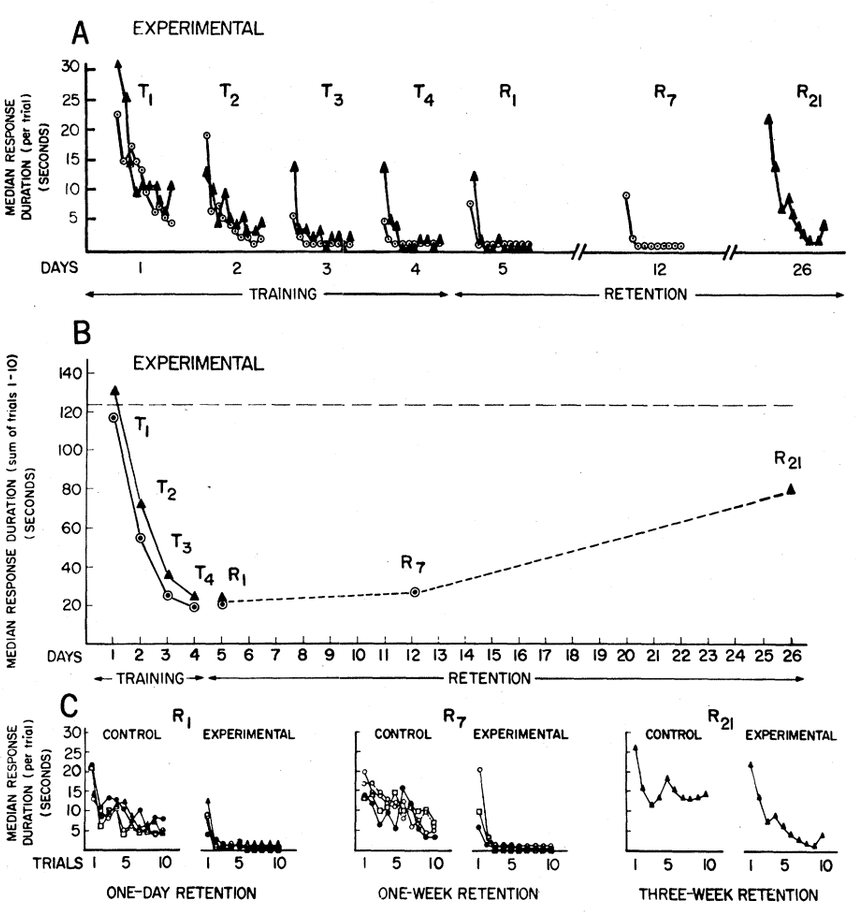

Fig. 1. (A) Long-term habituation. Build-up of habituation during training for 4 days (T_1 to T_4) and retention after 24 hours (R_1), 1 week (R_7), and 3 weeks (R_{21}). Data from three experiments are presented: two independent, identical replications in which retention was tested at 24 hours and 1 week have been pooled (⊙——⊙); in the third experiment, retention was tested at 24 hours and 3 weeks (▲——▲). Each data point is the median duration of siphon withdrawal for each of ten habituation trials. (B) Time course of habituation. Habituation within each daily session is expressed as a single score, the sum of ten trials (the net amount of time spent responding during the entire habituation training session). Compare retention tested at 24 hours and 1 week (⊙——⊙) and retention at 24 hours and 3 weeks (▲——▲) with control (day 1) habituation (upper dashed line). (C) Comparison of experimental and control habituation on each critical retention day. Retention days 1 (R_1) and 7 (R_7) are based on data from two independent replications, which were pooled in (A), and are presented separately [(●——●): experimental, $n = 9$; control, $n = 10$ and (○——○): experimental, $n = 10$; control, $n = 10$]. An additional control [(○- - -○), $n = 7$] received no habituation training until the 1-week retention test. A third independent replication is also shown [(□——□): experimental spaced training, $n = 5$; control, $n = 5$] and forms part of the massed as compared to spaced-training data (see Fig. 2). Three-week retention is based on 14 experimental and 14 control animals (▲——▲) in which retention was tested at 1 day and 3 weeks.

component of the defensive withdrawal reflex.

In the first series of experiments, habituation training was carried out in unrestrained animals, and long-term retention of habituation of the siphon-withdrawal component was measured. This could be accomplished without restraining the animal because the siphon extends out of the mantle cavity and can be visually monitored. In the second series of experiments unrestrained animals received identical siphon stimulation and then long-term retention of habituation of the gill-withdrawal component was assessed by restraining the animal and retracting the parapodia and mantle shelf to expose the gill.

A total of 132 *Aplysia californica* (100 to 400 g) were used in the two series of experiments. Three days before the beginning of any experiment, animals were housed in individual aquariums and not handled again throughout the experiment. The siphon-withdrawal reflex was elicited by delivering an 800-mesc jet of seawater to the siphon at a "variable" interstimulus interval of 30 seconds (*5*). The duration of the response was timed (from the offset of the stimulus until the siphon reappeared between the parapodia), by an observer using either a stopwatch or an electric timer. A "blind" experimental procedure was used in all retention tests.

We first examined the effects of four consecutive days of habituation training on retention of siphon withdrawal, tested 1, 7, and 21 days later. Animals were randomly assigned to an experimental or control group. The experimental group ($n = 33$) received ten habituation trials per day for days 1 to 4 and showed both within-session habituation and a buildup of habituation across days (Fig. 1A). On day 5 this experimental group and a control group ($n = 34$) were coded, were randomly mixed, and were given ten habituation trials using the blind procedure. The sum of ten trials (the net amount of time spent responding during the entire habituation training session) was then used as a single score for each animal in the statistical analysis (Fig. 1B) (*6*). Both experimental and control animals exhibited within-session habituation on day 5. However, the experimental animals showed significantly greater habituation (lower net response tendency) than the control group ($P < .001$), whereas the control group was not statistically different from the experimental group on day 1. These data indicate that the habituation produced across days 1 to 4 in the experimental group lasted for at least 24 hours (Fig. 1C, R_1). The experimental and control animals were then divided into two subgroups that were tested for retention of habituation after 7 and 21 days.

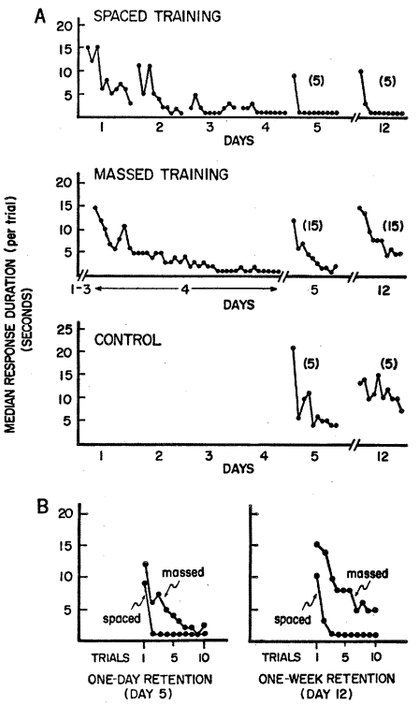

Fig. 2. (A) Spaced as compared to massed habituation training. Two groups of experimental animals were given either 4 days of habituation training (ten trials per day, spaced training) or 1 day of habituation training (40 trials, massed training). Both groups as well as a control group, which had received no training on days 1 to 4, were tested for retention after 24 hours (day 5) and 1 week (day 12). The spaced-training group was significantly lower than the massed-training group at both 24 hours ($P < .01$) and 1 week ($P < .01$), despite the fact that both groups had received the same number of training trials. The massed-training group was not significantly different from controls in either retention test. Animals in the massed-training group do, however, show a significantly lower response tendency on day 5 than they do on day 4 (trials 1 to 10) (intragroup comparison, $P < .01$). In the 1-week retention test (day 12) the massed-training group was no longer significantly lower than their initial performance (day 4, trials 1 to 10) indicating that this group showed no retention of training after 1 week while the spaced-training group still exhibited significant retention of habituation. The number of animals in each group is indicated in parentheses. (B) Comparison of spaced as compared to massed habituation on each retention day.

Seven days later, on day 12, the first subgroup of the experimental and control animals ($n = 19$ and 20, respectively) were again coded, and all animals, including an additional control group ($n = 7$) that had received no previous stimulation, were given ten habituation trials. The experimental group still exhibited significantly greater habituation ($P < .01$) than either control group (Fig. 1C, R_7). The two control groups were neither statistically different from each other nor from the experimental group on day 1. An intragroup analysis revealed that the (greater) habituation exhibited by the experimental subgroup on day 5 was unchanged on day 12. The control subgroup exhibited the same habituation on both day 5 and day 12 (in each case comparable to experimental day 1).

Twenty-one days later, on day 26, the remaining subgroups of experimental and control animals ($n = 14$ and 14, respectively) were again tested. The experimental animals still exhibited significantly greater habituation ($P < .01$) than the controls (Fig. 1C, R_{21}). An intragroup analysis revealed that the experimental subgroups showed significantly less habituation on day 26 than they did on day 5 ($P < .005$) even though they still showed significantly greater habituation than they exhibited on day 1 ($P < .005$). Therefore, these animals exhibited partial recovery of reflex responsiveness compared to their performance on day 5, but they still exhibited significant retention of habituation compared to their initial performance on day 1. The control subgroup showed habituation on day 26 comparable to that on day 5.

Long-term habituation of the siphon-withdrawal reflex can therefore be produced by 4 days of habituation training. This behavioral modification persists unchanged when tested after 7 days and is only partially recovered after 3 weeks (Fig. 1B). Since, in its time course, long-term habituation in *Aplysia* resembles behavioral modifications characteristic of higher animals, we next examined whether long-term habituation shared other features with more complex learning.

One feature of complex learning is that temporally spaced training usually produces better learning than massed training (*7*). To investigate the effects of massed as compared to spaced ha-

bituation training on long-term retention, we randomly divided 25 animals into three groups: a spaced-training group ($n = 5$), a massed-training group ($n = 15$), and a control group ($n = 5$). Animals in the spaced-training group were given ten habituation trials per day for days 1 to 4 (as in previous experiments). Animals in the massed-training group were given no stimulation on days 1 to 3 and were given 40 consecutive habituation trials on day 4 (Fig. 2A). On days 5 and 12 all animals, including the controls, were coded and were given ten habituation trials. The findings of the previous experiments were confirmed; the animals receiving spaced training exhibited significantly greater habituation than did control animals on days 5 ($P < .001$) and 12 ($P < .001$) (Fig. 2B). Moreover, the animals receiving spaced training exhibited significantly greater habituation on days 5 and 12 than did the animals receiving massed training ($P < .01$), despite the fact that both groups had received the same number of training trials (Fig. 2). Indeed, the animals that received massed training were not statistically different from controls and were comparable on day 5 to the animals in the spaced-training group on day 2. These data indicate that habituation training that is spaced across days is much more effective in producing long-term habituation than is masssed habituation training in a single day.

The defensive withdrawal reflex is controlled by the abdominal ganglion, and many of the neural components of the reflex have been identified (*2*, *3*). Since more is known about the gill component than about the siphon component of the reflex, we undertook a second series of experiments on the gill-withdrawal reflex in order to develop a preparation for studying long-term habituation on the cellular level.

Animals were randomly divided into a 1-day experimental group ($n = 6$), a 1-day control group ($n = 6$), a 7-day experimental group ($n = 11$), or a 7-day control group ($n = 10$). Experimental animals received daily habituation training (siphon stimulation) for a minimum of 5 days. Either 24 hours or 7 days after training, the experimental animals were mixed with controls (*8*), and the gill-withdrawal reflex of both groups was measured (by a blind procedure) with a photocell placed under the gill, by means of a modification of the restrained preparation described by Pinsker and co-workers (*2*). In the testing procedure, as in the training procedure, habituation was produced by ten trials of siphon stimulation. Both 1-day and 7-day experimental animals exhibited significantly greater habituation of gill withdrawal than controls ($P < .001$) (Fig. 3A). Moreover, a trial analysis revealed that 1- and 7-day experimental animals exhibited a significantly decremented gill-withdrawal response, compared to controls, on nine out of ten trials and eight out of ten trials, respectively (*9*) (Fig. 3B).

Whereas the reflex responsiveness to siphon stimulation was significantly reduced in experimental animals, there was no statistical difference between gill-withdrawal reflexes produced by purple gland stimulation in both experimental and control animals (Fig. 3A). These findings illustrate that, as in short-term habituation (*3*), long-term habituation does not generalize from the siphon to nonstimulated parts of the receptive field, but is restricted specifically to the stimulated afferent pathway.

Long-term habituation in *Aplysia* can last several weeks and shows sensitivity to the temporal patterning of training trials, two features that also characterize

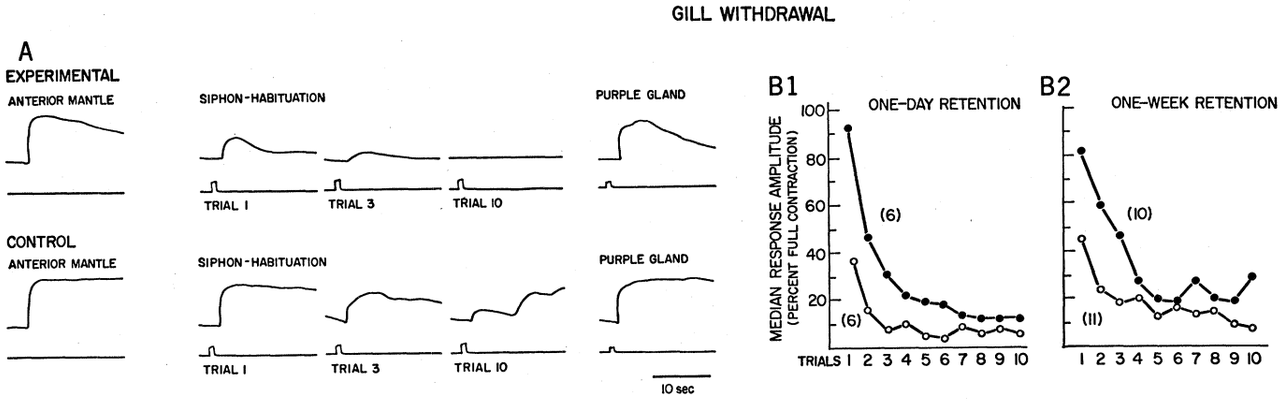

Fig. 3. (A) Long-term habituation of gill withdrawal. Gill-withdrawal reflex of experimental and control animals was recorded with a photocell. One week previously, experimental animals had exhibited significant 24-hour retention of habituation of siphon withdrawal as compared to controls. Seven days later, retention of habituation of gill withdrawal (produced by the previous siphon habituation training) was measured. A single test gill-contraction was first produced by vigorous tactile stimulation of the anterior mantle region. After a brief period of rest, habituation of gill withdrawal was produced by repetitive siphon stimulation with a jet of seawater (ten trials; interstimulus interval, 30 seconds), and was followed by another single test stimulus to the purple gland. Anterior mantle and purple gland stimulation produced comparable contractions in both experimental and control animals. However, siphon stimulation produced a significantly reduced initial gill withdrawal in experimental animals and significantly greater habituation of the gill-withdrawal reflex in experimental than in control animals (compare trials 1, 3, and 10 of both experimental and control records). Time calibration, 10 seconds [see (*9*)]. (B) Comparison of experimental and control habituation of gill withdrawal at 1 day and 1 week. Median amplitude of gill responses expressed as a percentage of a full contraction. The number of animals in each group is indicated in parentheses. (B1) Gill withdrawal 24 hours after siphon habituation training. Experimental animals (○——○) exhibited significantly greater habituation ($P < .001$) of gill withdrawal (sum of trials 1 to 10) than controls (●——●) and were significantly lower than controls on nine out of ten trials. (B2) Gill withdrawal 7 days after siphon habituation training. Experimental animals again exhibited significantly greater habituation ($P < .001$) and were significantly lower than controls on eight out of ten trials [see (*9*)]. These data indicate that long-term habituation of siphon withdrawal also produces long-term habituation of gill withdrawal.

higher forms of learning. An understanding of the neural processes underlying long-term habituation in *Aplysia* may therefore provide insights into neural mechanisms of more complex long-term memory.

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4. Behavioral modifications of a feeding response lasting 1 to 4 days have previously been described in *Aplysia* by M. E. Lickey, *J. Comp. Physiol. Psych.* **66**, 712 (1968); and by I. Kupfermann and H. Pinsker, *Comm. Behav. Biol.* **2** (Part A) 13 (1968).
5. A "variable" interstimulus interval was determined by the duration of siphon withdrawal, that is, the next stimulus of a series would be delivered 30 seconds after the termination of the previous response.
6. The performance of independent groups was compared by means of Mann-Whitney U tests (intergroup comparisons) and of related groups by means of Wilcoxon matched-pairs signed-ranks tests (intragroup comparisons). In cases where more than two groups were involved, a preliminary analysis of variance (Kruskal-Wallis for intergroup and Friedman two-way for intragroup) was performed to establish an overall significant difference.
7. R. S. Woodworth and H. Schlosberg, *Experimental Psychology* (Holt, Rinehart and Winston, New York, 1964), pp. 786–794.
8. Two different control groups were used: the 24-hour controls received no previous siphon stimulation, whereas the 7-day controls received ten trials of siphon stimulation on day 5 of the experiment and then, 1 week later, gill withdrawal was measured for both experimental animals and controls.
9. Gill withdrawal for experimental and control animals was compared by expressing each gill withdrawal elicited by siphon stimulation as a percentage of a "complete" gill withdrawal, which previously had been produced by vigorous stimulation of the anterior mantle region. Both 24-hour and 7-day control animals exhibited an initial gill withdrawal on trial 1 (produced by siphon stimulation) that was comparable to their "complete" response, whereas both experimental groups exhibited a significantly decremented initial gill withdrawal compared to controls. Twenty-four-hour groups: experimental, 37 percent; control, 93.5 percent ($P < .001$). Seven-day groups: experimental, 43 percent; control, 91 percent ($P < .001$). Furthermore, as in the previous siphon habituation studies, the net response tendency of gill withdrawal (sum of percent full contraction for trials one to ten) of the experimental animals was significantly lower than controls ($P < .001$ at both 24 hours and 7 days).
10. We thank B. Jahan-Parwar for his suggesting that handling may confound behavioral studies; K. Hilten for her assistance in preparing the illustrations; V. Castellucci, I. Kupfermann, and W. Alden Spencer for their thoughtful criticism of the manuscript; and W. Hening for his help in the independent replication of some of the experiments. Supported by PHS grants MH 19795 and NS09361. Additional support was provided to T.J.C. by fellowship from postdoctral program in biological psychiatry to New York University School of Medicine and by career scientist award MH18,558 to E.R.K.

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Operant Behavior Changes Norepinephrine Metabolism in Rat Brain

Abstract. *Rats performing a lever-pressing response for water reward in an operant situation, when compared with control groups, showed an increase in brain norepinephrine metabolism. One control group included rats which were handled and deprived of water in the same way as the experimental group but were not trained to perform the operant task. We conclude that performance in an operant situation affects norepinephrine metabolism.*

We now report that performance in an operant situation increases the metabolism (*1*) of norepinephrine (NE) in the brain of the rat. Previous experiments (*2*, *3*) with drugs that affected both norepinephrine metabolism and behavior have demonstrated the opposite relationship, expressing changes in behavior as a function of NE metabolism. We have demonstrated that behavior itself can modify NE metabolism.

Previous experiments on changes in NE metabolism have tested the effects of aversive stimulation rather than the behavior of the animal. In general, acute aversive stimulation causes a reduction of the endogenous concentration of brain NE (*4*, *5*), whereas long-term aversive stimulation causes a rise (*6*). Increased "turnover" of NE is associated with aversive stimulation and increased motor activity (*5*, *7–9*). These experiments demonstrated the effects of aversive stimulation on NE metabolism, but they did not show that the change in NE metabolism is a function of the behavior (performance) of the animal.

Rats performing in an operant situation showed a greater decrease in total brain dopamine (DA) and NE than control rats, after both groups of animals were treated with α-methyltyrosine, an inhibitor of synthesis (*3*). That experiment suggested a relation between operant behavior and NE metabolism; however, interpretation of the relation was confounded by the drug. We now describe the effects of performance in an operant situation on NE metabolism in the rat brain; a tracer amount of tritium-labeled NE was used in order to avoid the effects of drugs or other variables.

Male, albino, Sprague-Dawley (Holtzman) rats, approximately 60 days old and weighing 250 g, were used. With the animals under ether anesthesia, we placed an indwelling cannula in the right lateral ventricle, a modification of the method described by Hayden *et al.* (*10*). Animals were housed in pairs and were randomly divided into three groups: (i) an ad lib (food and water) control group, (ii) a water-deprived control group, and (iii) a group trained to press a lever for water reward. The water-deprived group was included in order to test the effect of water-deprivation on NE metabolism; this condition was present in the trained group since a state of water-deprivation is necessary to maintain lever-pressing behavior reinforced with water. Animals were trained as in the experiment with α-methyltyrosine (*3*); a variable interval, 30-second (VI-30) schedule was used. On this schedule lever-pressing behavior is reinforced intermittently with an average interval between reinforcements of 30 seconds.

At the end of a 2-hour session, the trained rats were taken from the chambers and returned to their home cages; 15 minutes later they were watered for 5 minutes. The animals in the water-deprived control group were handled in the same way as the animals in the trained group; they were removed to empty cages for 2 hours each day, returned to their home cages, and 15 minutes later were watered for 8 minutes. By watering the animals in the water-deprived group for 3 minutes longer than the animals in the trained group, we were able to make sure that the amount of water and food consumption was the same in both groups, as evidenced by the fact that their body weights did not differ.

On the 15th day of the VI-30 schedule all rats were injected with 1.0 μc (10 μl) each of tritiated NE (New England Nuclear; specific activity 10.8 curie/mmole), dissolved in Merle's solution (*11*), with a 50-μl Hamilton syringe.