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Site Specificity of Short-Term and Long-Term Habituation in the Tail-Elicited Siphon Withdrawal Reflex of *Aplysia*

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The study of habituation in animals with relatively simple nervous systems has contributed significantly to the understanding of mechanisms underlying learning and memory. Using the tail-elicited siphon withdrawal reflex of *Aplysia*, which is mediated in part by bilaterally symmetrical clusters of tail sensory neurons, we found that both short-term and long-term habituation can be restricted laterally, such that habituation produced by stimulation of one side of the tail does not generalize to the other side. Further experiments in this preparation revealed that long-term, laterally restricted habituation is sensitive to the temporal pattern with which stimuli are presented. We also determined that both short-term and long-term habituation can take place in a reduced behavioral preparation, and that short-term habituation can be restricted within relatively small stimulation sites located on the same side of the tail. These results provide insights into the cellular organization of habituation, and they provide a useful preparation for a cellular analysis of this basic form of learning.

Key words: Aplysia*; habituation; specificity; long-term; learning; tail; siphon*

The capacity for learning and memory provides great advantages for animals living in complex, changing environments. One ubiquitous form of learning, habituation, is characterized by the waning of a behavioral response that is elicited by repeated, innocuous stimulation. Habituation broadly influences the interaction of an organism with the world, preventing recurrent, nonthreatening environmental stimuli from endlessly distracting the animal from potentially meaningful stimuli of behavioral significance.

For many years, habituation has been an attractive model for the study of mechanisms contributing to learning and memory. Habituation is easy to induce in the laboratory as well as in the field, it is widely observed in species ranging from the simplest animals to humans, and it does not involve the generation of new behaviors but only the reduction of an existing behavior. Additionally, habituation occurs in both short-term and long-term forms (for reviews, see Harris, 1943; Thompson and Spencer, 1966; Carew and Sahley, 1986).

To examine the cellular mechanisms contributing to a behavioral modification such as habituation, it is necessary to link behavioral and cellular levels of investigation. Neither level alone is sufficient for a mechanistic analysis, because it is usually not possible to deduce cellular mechanisms that underlie behavioral change by observing the behavior alone, and on the other hand, ambiguity can result from performing a mechanistic analysis on isolated neural systems in which the behavioral significance of the observed cellular changes is unknown. One productive strategy to achieve a mechanistic analysis of a form of learning such as habituation is to develop a preparation in which, using identical stimulus conditions, habituation can be analyzed at several levels. The first steps in such an analysis are to demonstrate that (1) a particular stimulus regime will induce habituation in a freely moving, behaving animal and (2) the same stimulus conditions will similarly induce habituation in a reduced preparation in which the effect of habituation on behavior can be assessed while, at the same time, a cellular analysis can be carried out. The experiments presented in this paper satisfy these behavioral requirements for both short-term and long-term habituation in the marine mollusk *Aplysia*.

Habituation was examined in the siphon withdrawal reflex elicited by mild stimulation of the tail in *Aplysia.* This tail-induced reflex, previously used to study forms of learning involving response augmentation such as that occurring in sensitization (Walters, 1987a,b; Scholz and Byrne, 1987), is easy to elicit and to quantify, and some of the underlying neural circuitry is known (Walters et al., 1983; Cleary and Byrne, 1993; Fang and Clark, 1993). In addition, the sensory organization of the tail offers the advantage of bilateral symmetry: duplicate copies of the sensory components exist on both sides of the midline of the animal. Such bilateral sensory representation enables a useful form of within-animal experimental design, because training can be arranged so as to differentially activate anatomically separate sensory clusters by delivering different patterns of tactile stimulation to separate sides of the tail of the animal (Walters et al., 1983). This is particularly helpful for studies of long-term learning in which nonspecific experimental variables can become especially prevalent. In this paper we show that in response to identical stimuli and stimulus patterns, both intact animals and reduced preparations exhibit side-specific short-term and long-term habituation. In addition to providing a detailed behavioral description of habituation in this system, the side-specificity of habituation provides insight into its underlying cellular organization. The present work thus provides a useful framework for an analysis of cellular mechanisms underlying habituation [Stopfer and Carew, 1994, 1996 (companion paper)].

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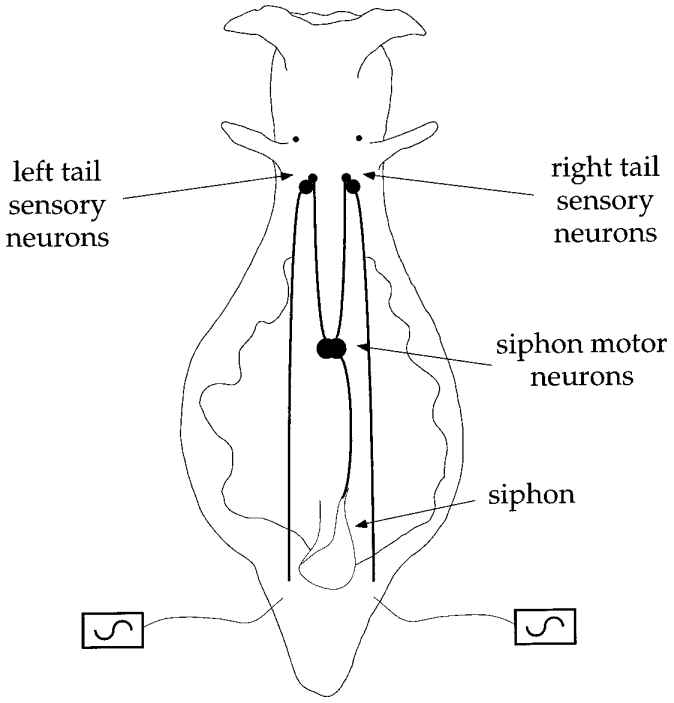

Figure 1. Anatomical components of the tail-elicited siphon withdrawal reflex. Two separate sensory input pathways (left and right sides of the tail) activate bilaterally symmetric sensory neuron clusters in the pleural ganglia. These pathways converge on a common motor output (the siphon motor neurons in the abdominal ganglion), providing a controlled behavioral assay for habituation. The two pathways can be activated independently via electrodes implanted on either side of the tail.

Some of the results described in this paper have been reported previously in abstract form (Stopfer and Carew, 1994).

MATERIALS AND METHODS

Intact animals. Adult *Aplysia californica* weighing 75–150 gm were collected and shipped by commercial suppliers (Marinus Supplies and Marine Specimens Unlimited). Animals were housed and tested individually in floating plastic colanders in an 1140 l aquarium filled with circulating, cooled (15°C), aerated artificial seawater (Instant Ocean, Aquarium Systems).

When the animals arrived in the laboratory, they were anesthetized by immersion in cold (3–5°C) artificial seawater and partially parapodectomized to reveal the siphon more fully . Thin, Teflon-insulated silver wires (0.005 inch diameter; Medwire) with 3 mm uninsulated tips were used as electrodes to elicit the siphon withdrawal reflex with highly repeatable stimuli. Electrodes were implanted into the thin muscle layer on both sides of the midline of the tail, generally about halfway between the tip of the tail and the insertion of the parapodia, and about halfway between the median of the tail and its lateral extent (Fig. 1). The other end of the electrode wire was attached to a miniature gold plug (Wire Pro) affixed to a small Styrofoam float. Electrodes would remain in place for many days, trailing securely behind the animal as it locomoted in its holding pan. The electrodes did not cause any obvious impediment to the behavior of the animal.

Animals were not handled at any point after the initial surgery; training and testing were conducted in the home pans of the animals. Just before an experiment, the gold electrode plugs were connected to the stimulus apparatus (see Training and Testing) (Fig. 2). During the training phase, the switch determining the active electrode was hidden; the experimenter timing siphon movement during training therefore did not know which side of the tail was receiving training stimulation.

Reduced preparations. Experiments began when a naive animal was anesthetized by injecting a quantity of magnesium chloride sufficient to cause a noticeable inflation of the hydroskeleton; this was generally ~120 ml. The animal was then placed dorsal-side up in a wax-bottom dissection tray. The posterior portions of the parapodia were removed to make the siphon more visible. Next, the animal was placed ventral-side up, and an incision was made along most of the length of the foot. The body wall was pinned back to reveal the digestive tract and the nervous system. The digestive tract was removed, leaving the CNS plainly visible.

All peripheral nerves were transsected except for the siphon nerve and the two P9 nerves, which separately innervate the left and right sides of the tail. The buccal ganglia (which do not participate in the tail-elicited siphon withdrawal reflex) were dissected away, but the rest of the CNS (cerebral, pleural, pedal, and abdominal ganglia) remained intact (see Fig. 7).

The CNS was drawn toward the tail, and then most of the body anterior to the mantle cavity was dissected away. The remaining tail, mantle organs, and CNS were then transferred to a specially constructed chamber (see below). The tail was pinned loosely in place, and a catheter was inserted into the tail. Next, a catheter was also inserted into the aorta and secured with 6–0 ethilon (Ethicon) surgical thread. In some experiments, a very fine, lacquer-insulated steel wire (0.001 inch diameter; California Fine Wire) was inserted into the siphon for use by an automated movement transducer, described below. Reduced preparations were left to rest for 1 to 2 hr before experiments began.

Reduced preparation experiments were carried out in a round chamber (25 cm diameter) with a Sylgard (Dow Corning, Corning, NY) bottom. Chilled water circulated through a coil in the dish, maintaining the bath at 15–18°C. Fresh, aerated, cool Ringer's solution was perfused through tail and aorta catheters to flush out the remaining magnesium chloride, to

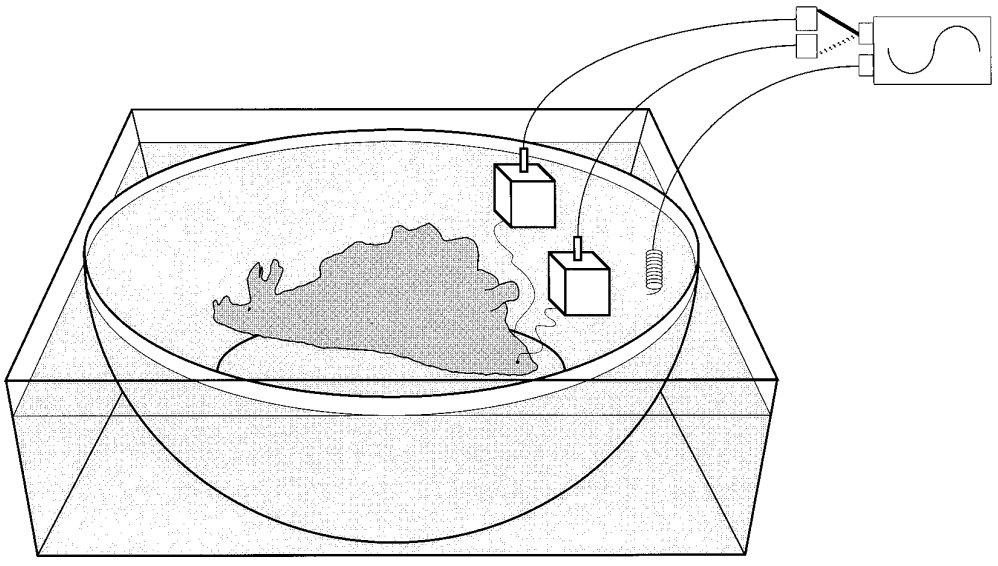

Figure 2. Apparatus for studying habituation in freely moving animals. Stimulator (*right*) can deliver quantifiable, mild shock to left or right side electrodes implanted in the tail. The electrode leads, buoyed on Styrofoam floats, trail behind the animal.

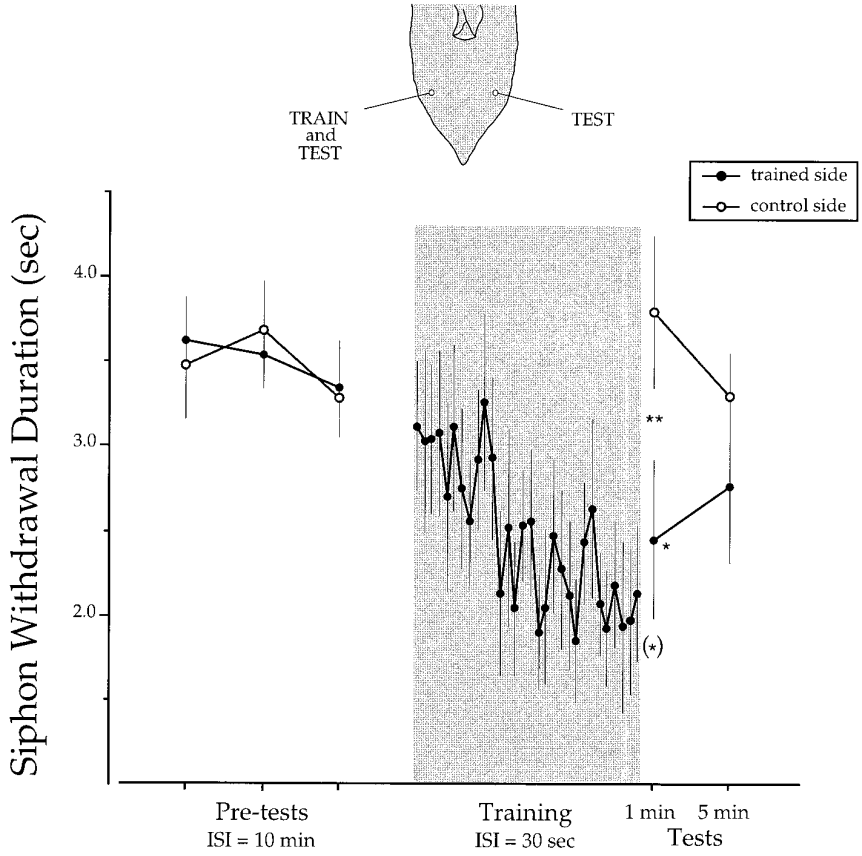

Figure 3. Short-term habituation of tail-elicited siphon withdrawal. The drawing at the *top* illustrates electrode implantation sites on the tail. Training (*shaded area* on *graph*) causes habituation of responses elicited only on the trained side of the tail; responses elicited at the control side remain comparable to pretest. Statistical significance ($p < 0.05$) in this and subsequent figures is indicated by these symbols: *, within-group significance (data point is significantly different from its own pretest); **, between-group significance (data points *above* and *below symbol* are significantly different from each other); (*), aggregate significance (the series of data points to the *left* of the *symbol* are significantly different, overall, from one another).

help sustain the viability of the preparation, and to supply hydrostatic pressure to maximize siphon mobility. A vacuum line maintained a constant water level in the dish.

Training and testing. Training and testing stimuli in both freely moving animals and reduced preparation experiments consisted of brief 60 Hz AC pulses to the tail electrodes. A bath electrode was provided for current return. The intensity of the stimulus was calibrated for each site to be slightly above the threshold for reliably eliciting siphon responses; this empirically determined intensity ranged from 1 to 10 mA. Stimuli were generated by an AC stimulator consisting of an isolation transformer and a variable transformer that allowed selection of the proper current intensity. The output of the AC stimulator was gated by timed current pulses from a DC stimulator (Grass S88, Grass Instruments). Brief, 100 msec stimuli were used to prevent the animal from having enough time during the stimulus to change the position of its tail and thereby gain any opportunity to affect the stimulus intensity. In terms of the elicited behavior, these electrical stimuli were equivalent to weak tactile stimuli, causing modest but clear siphon withdrawal reflexes, but no behavioral signs of a reaction appropriate to a noxious stimulus (such as prolonged withdrawal or inking).

Siphon withdrawal was timed by an observer using a stopwatch from the start of withdrawal movement to the first indication that the siphon was beginning to relax to its rest position. For some experiments a siphon movement transducer was used. This device enveloped the siphon in an electric field that could be detected by the siphon antenna wire. The device generated a voltage directly proportional to the position of the siphon within the electric field. This voltage could be recorded and graphed by a computer, thus maintaining an objective record of siphon movement. The extremely fine antenna wire caused no tension or pull on the siphon and did not seem to alter its behavior in any way. Siphon reflex responses that were monitored by computer were also timed by an observer. These human- and computer-generated records were compared later and were found to correlate extremely well, which aided in the development of automated analysis software for use in cellular experiments that are described elsewhere [Stopfer and Carew, 1994, 1996 (companion paper)].

Experiments made use of within-animal controls such that one site on the tail received a training protocol (pretests, training, and tests) and another site on the tail received a control protocol (only the pretests and tests). In all cases, experiments were conducted blind; the observer testing siphon movement did not know the group designation of either side of the animal. Stimulus intensity was calibrated individually for each stimulus site to elicit a small but clear siphon withdrawal.

Animals first received a series of three bilateral pretests at a nonhabituating (10 min) ISI; stimuli were delivered every 5 min to alternate sides of the tail. Siphon withdrawal was measured after each stimulus. To determine which side of each animal would receive habituation training, the pretest responses for each side of each animal were averaged and then counterbalanced between animals. Thus, the mean pretest response duration for trained sites was roughly the same as for control sites.

Training sets consisted of 20 or 30 stimuli delivered at a 30 sec ISI. Short-term training consisted of a single set; testing was conducted 5 min after the training set. In long-term experiments, training consisted of four additional sets with a 90 min interval between each set, delivered to preparations that had just been tested for short-term habituation. Long-term testing was conducted 24 hr after the final set. Some of the long-term training protocols were controlled by a computer timing program that could activate the stimulator.

For one long-term experiment, pairs of electrodes were implanted close together (within 3 mm of each other) on each side of the tail so that separate electrodes could be used for testing and training. For another long-term experiment, the trained side of the tail received the usual training protocol, whereas the control side received an equivalent number of stimuli but delivered at a constant, nonhabituating (10 min) ISI. The two protocols were timed to co-terminate, and testing began 24 hr later.

For a test of generalization of habituation, pairs of differently spaced electrodes were implanted on the same side or different sides of the tail. Generalization tests were conducted with ipsilateral electrodes placed either 1–2 or 2.5–3.5 cm apart. The preparation is illustrated in Figure 2.

Statistical analysis. Overall statistical significance was first determined by ANOVA. Subsequent individual planned comparisons were made by *t* tests. Multiple comparisons were made by Newman–Keuls tests. All probability values are two-tailed. All results are expressed as means ± SEM.

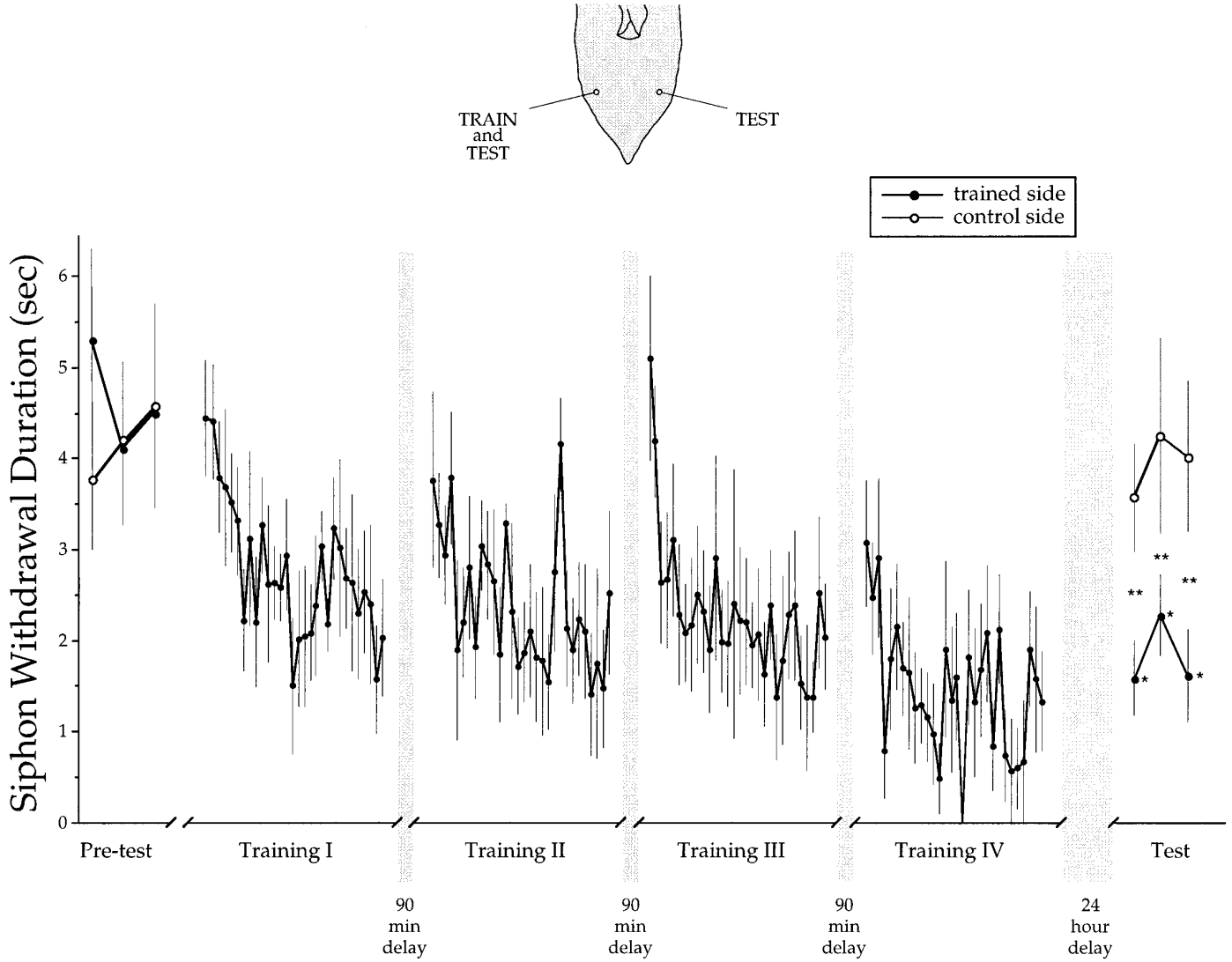

Figure 4. Long-term habituation of tail-elicited siphon withdrawal. *Vertical gray bars* represent intervals between training sessions (see Materials and Methods for details of training). Responses elicited at trained stimulation sites showed significant long-term habituation and savings. Control sites showed no change. See legend to Figure 3 for statistical significance indicated by *asterisks*.

RESULTS

Intact animals

Intact animals exhibit side-specific short-term habituation

After pretesting, each animal received a series of 30 stimuli delivered at a 30 sec ISI to one side of the tail only. Twelve animals were used in this experiment. Results are illustrated in Figure 3. The siphon withdrawal response exhibited significant habituation during the course of training (ANOVA: $F_{(29,319)} = 2.52$, $p < 0.001$).

Bilateral tests were then conducted after the conclusion of training at ~1 and 5 min. Trained- and control-side tests were conducted 30 sec apart; thus, half of the 1 min tests were actually conducted 1.5 min after training, and half of the 5 min tests were conducted 5.5 min after training. In half of the experiments, the trained side was tested first, and in the other half, the control side was tested first (decided at random).

Overall, control-side responses were not significantly different from their own mean pretest scores (ANOVA: $F_{(4,88)} = 0.64$, NS). Trained-side responses, however, were reduced significantly relative to their own mean prescores (ANOVA: $F_{(4,88)} = 3.11$, $p < 0.05$). At the 1 min test, trained-side responses were reduced significantly, relative to their prescores (dif = -1.04 ± 0.47 sec; Newman–Keuls test, $p < 0.05$). At the 5 min test, the trained-side response had begun to recover toward its mean baseline level (dif = -0.75 ± 0.45 sec; Newman–Keuls test, NS).

These results demonstrate that the tail-elicited siphon withdrawal reflex exhibits short-term habituation and that habituation can be restricted to the trained side of the animal.

Intact animals exhibit side-specific long-term habituation

Pretests and 24 hr tests (three each) were conducted at a 10 min ISI. Ten animals were used in these experiments; the results are illustrated in Figure 4. An ANOVA for the pretests and tests indicated an overall significant effect of training ($F_{(5,90)} = 5.55$, $p < 0.001$), and a significant interaction between subjects and treatment ($F_{(5,90)} = 3.75$, $p < 0.005$). Moreover, control-side responses did not change significantly over the course of training ($F_{(5,45)} = 0.49$, NS). Subsequent comparisons showed that after

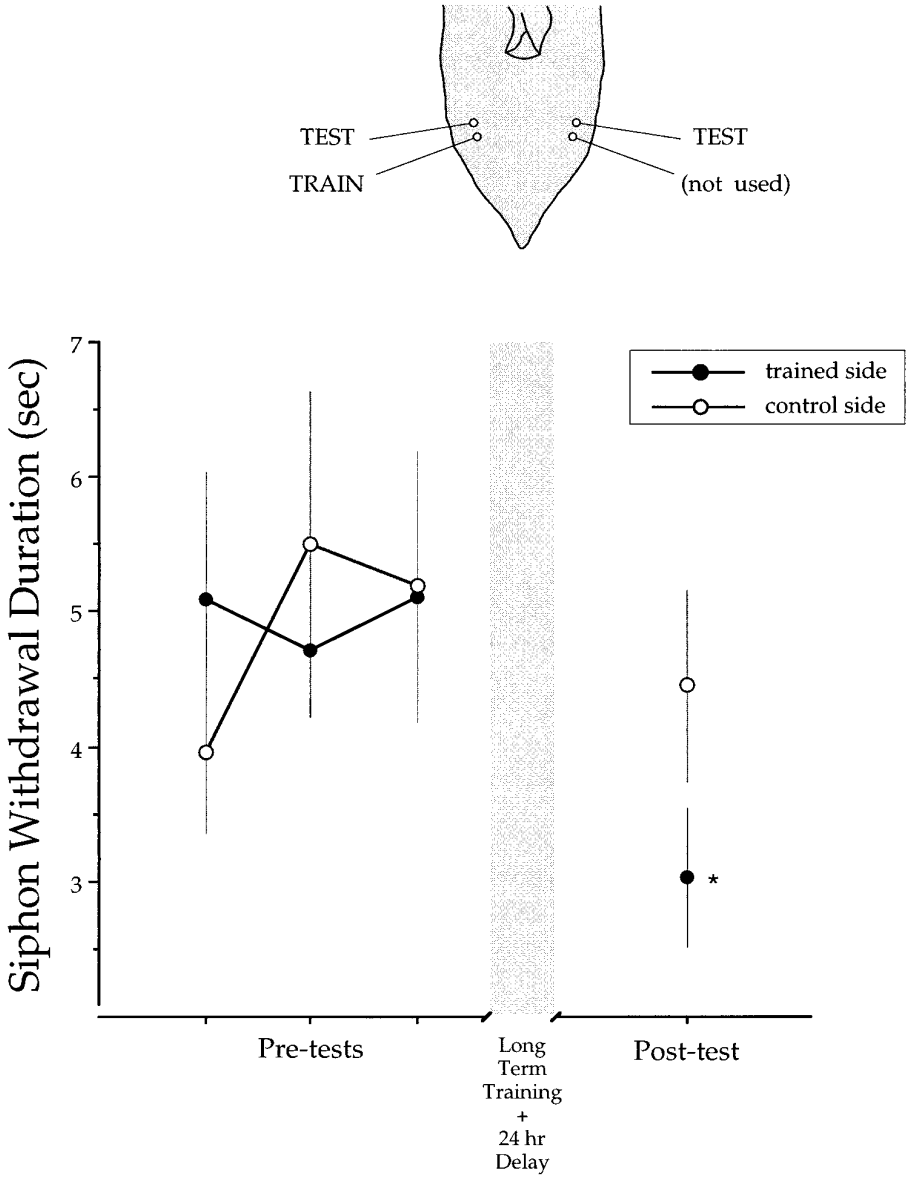

Figure 5. Response decrement of tail-elicited siphon withdrawal is not caused by a peripheral effect secondary to differential electrode activation. Pretest and post-test electrodes were activated an equal number of times. Only the trained side of the tail showed significant long-term habituation. See legend to Figure 3 for statistical significance indicated by *asterisk*.

the training phase, in the 24 hr test, none of the control-side responses showed any significant change from their mean baseline responses (test 1: dif = -0.63 ± 0.68 sec; test 2: dif = -0.05 ± 0.72 sec; test 3: dif = 0.45 ± 0.78 sec; Newman–Keuls test for each, NS).

Training, however, had a significant effect on the trained-side responses, revealed by a within-group ANOVA ($F_{(5,45)} = 8.84$, $p < 0.001$). Further planned comparisons showed that relative to their own mean baselines, all three trained-side test responses were habituated significantly (test 1: dif = -3.13 ± 0.36 sec; test 2: dif = -2.44 ± 0.25 sec; test 3: dif = -3.10 ± 0.50 sec; Newman–Keuls test for each: $p < 0.05$). Furthermore, for all three tests, trained-side responses were significantly reduced compared with control-side responses (test 1: dif = -1.98 ± 0.15 sec, $t_{(9)} = 3.88$, $p < 0.004$; test 2: dif = -1.97 ± 0.33 sec, $t_{(9)} = 2.27$, $p < 0.05$; test 3: dif = -2.13 ± 0.39 sec, $t_{(9)} = 2.82$, $p < 0.05$). As was the case for short-term habituation, there was no significant generalization of long-term habituation to the control site.

Long-term response decrement can be attributed to habituation

In both the short-term and long-term experiments, trained-side electrodes (through which both testing and training stimuli were delivered) were activated more times than control-side electrodes (which were activated only for tests). This raised the possibility that the trained-side response decrement observed could result, at least in part, from peripheral factors arising from differential electrode activation (e.g., electrode plating, tissue alteration, etc.). To rule out this possibility, another experiment was performed in which testing and training were conducted through independent electrodes that were separated slightly, implanted ~3 mm apart. A pair of electrodes with comparable spacing was also implanted on the control side. In this way, identically positioned trained-side test electrodes and control-side test electrodes were activated an equal number of times. In all other respects, this experiment was identical to the previous long-term study (Fig. 4). Ten animals were used. Results are presented in Figure 5.

An ANOVA revealed an overall significant effect of training ($F_{(1,18)} = 7.56$, $p < 0.013$). Control-side responses were not

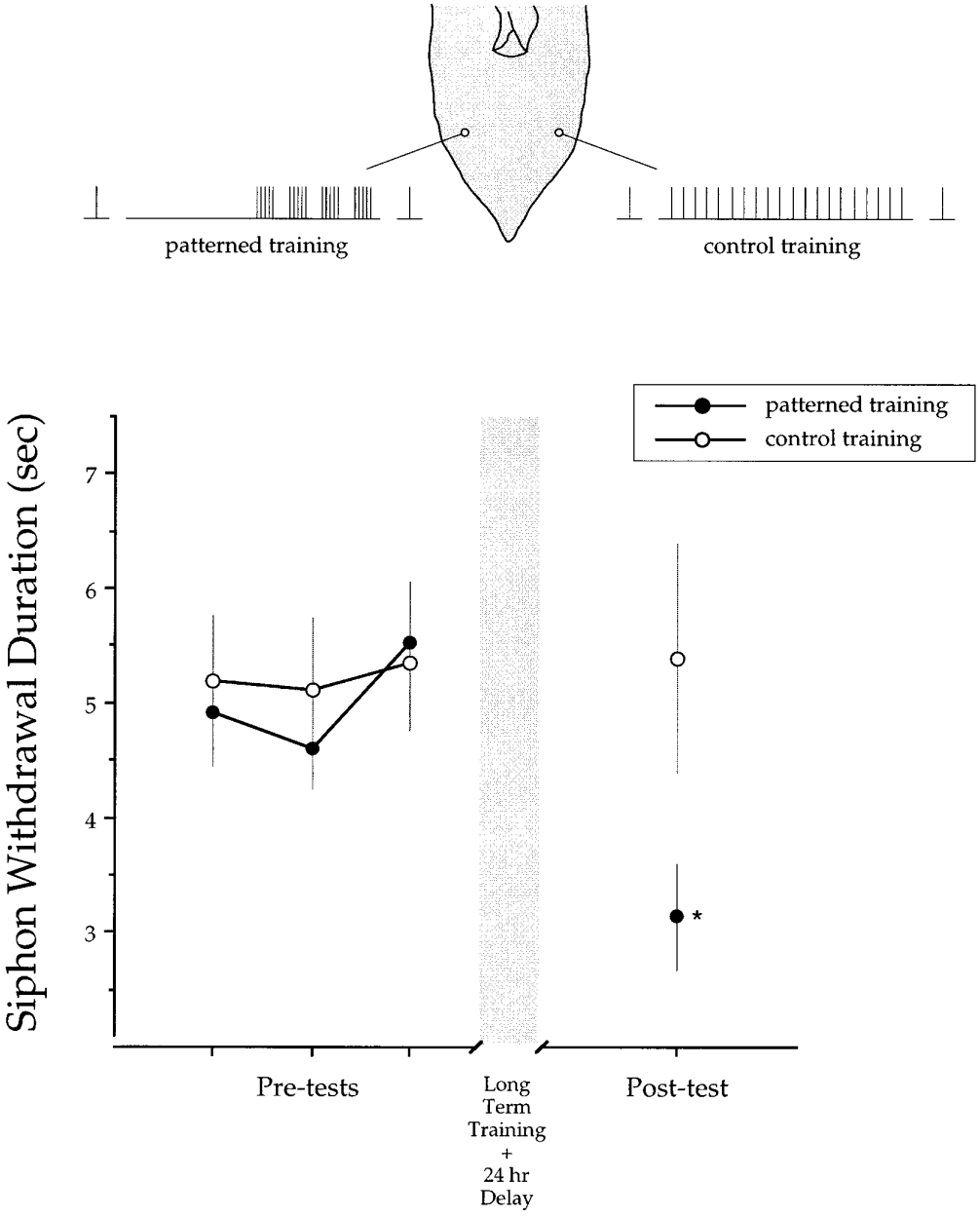

Figure 6. Long-term habituation is sensitive to the stimulus training pattern. An equal number of stimuli (120) was delivered to both sides of the tail. Only the side receiving patterned stimuli (see Fig. 4) exhibited significant long-term habituation. See legend to Figure 3 for statistical significance indicated by *asterisk*.

affected by training, as indicated by a planned comparison (dif = -0.43 ± 0.73 sec, $t_{(9)} = 0.60$, NS). Trained-side responses, however, were habituated significantly compared with their own mean pretest responses, shown by a planned comparison (dif = -1.95 ± 0.47 sec, $t_{(9)} = 4.13$, $p < 0.003$).

These results demonstrate that differential response decrement of tail-elicited siphon withdrawal is not caused by a peripheral effect secondary to differential electrode activation, because pretest and test electrodes were activated an equal number of times. Rather, the response decrement is attributable to habituation. This result also confirms the side-specific, long-term habituation revealed in the previous experiment (Fig. 4).

Long-term habituation is sensitive to the stimulus training pattern

One characteristic of long-term habituation is that some patterns of stimulation are more effective than others. For example, Carew et al. (1972) found that four widely spaced sets of training trials were much more effective in producing long-term habituation than were massed training trials. To examine the effect of stimulus pattern, two different training protocols were delivered to different sides of the same animal. One side received the habituation protocol described above (Fig. 4), consisting of 30 stimuli in each of four patterned training sets; the other side received a nonhabituating, distributed protocol consisting of an equivalent number of stimuli, delivered at a consistent 10 min ISI (*top* of Fig. 6).

Fourteen animals were used; the results are shown in Figure 6. An overall ANOVA revealed a significant effect of training pattern ($F_{(1,26)} = 1.26$, $p < 0.02$). Furthermore, the patterned training-side test score was significantly lower than its mean pretest score (dif = -2.84 ± 0.65 sec, $t_{(13)} = 4.40$, $p < 0.001$), whereas the control training-side test score was not changed significantly from its own prescore (dif = 0.61 ± 1.12, $t_{(13)} = 0.54$, NS). Thus, as in siphon-elicited siphon withdrawal (Carew et al., 1972), long-term habituation of tail-elicited siphon withdrawal is

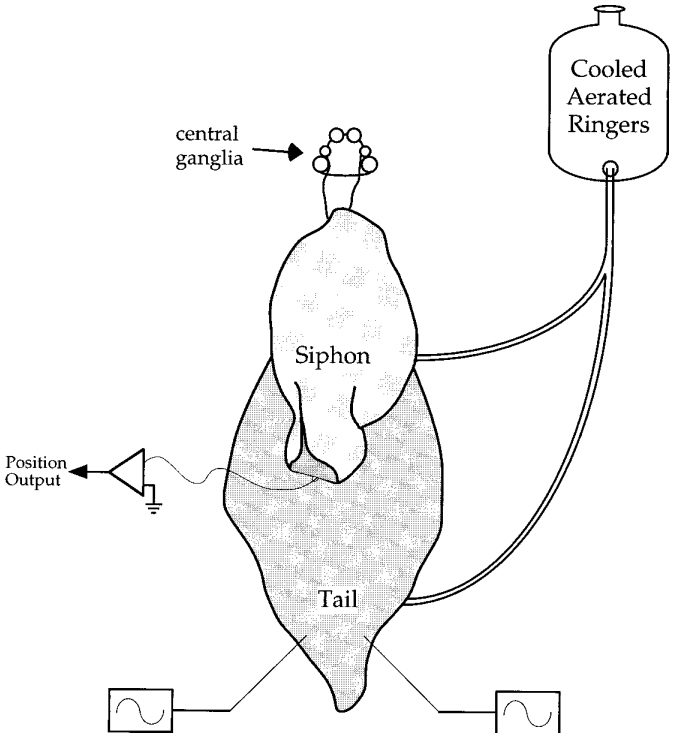

Figure 7. The reduced preparation. As in the freely moving animal, both sides of the tail were stimulated by weak electric pulses delivered by implanted electrodes. Siphon withdrawal response duration was monitored by an observer, and response amplitude was recorded by an automated movement transducer. Perfusion lines were placed in the tail and the aorta.

sensitive to the pattern of stimulation that is used to produce the learning.

Reduced preparation

Reduced preparations exhibit side-specific short-term and long-term habituation

To study the cellular mechanisms of habituation (including its acquisition, which requires a reduced preparation), it is important to produce the response decrement using behavioral stimuli and response measures identical to those used in the intact animal. To achieve this, we next examined habituation using stimuli identical to those used in the previous studies but now in a reduced preparation (see Materials and Methods), illustrated in Figure 7. Thirteen of these preparations were used. All stimuli, as well as all training and testing procedures, were essentially identical to those used in intact animals. Using these preparations we examined both short-term and long-term habituation. The results are shown in Figure 8.

During training, the siphon withdrawal reflex showed significant short-term habituation, as indicated by an ANOVA ($F_{(19,228)} = 13.94, p < 0.001$). An overall ANOVA comparing pretest and test scores showed that training had a significant effect on response duration ($F_{(2,48)} = 3.31, p < 0.05$). Control-side scores were not significantly affected by training, as indicated by a planned comparison (dif = 0.68 ± 0.44 sec, $t_{(12)} = 1.55$, NS); however, trained-side responses were significantly reduced (dif = −1.09 ± 0.25 sec, $t_{(12)} = 4.46, p < 0.001$). These results indicate that the response decrement was not attributable to nonspecific rundown of the preparation but rather to habituation induced on the trained side. Habituation endured for at least 5 min; however, there was a significant amount of spontaneous recovery during the 5 min between the last training stimulus and the test stimulus (dif = 0.71 ± 0.14 sec, $t_{(12)} = 4.99, p < 0.001$).

Further training (four spaced blocks, each consisting of 30 stimuli delivered at a 30 sec ISI; training responses not shown) also produced long-term habituation in the reduced preparation (Fig. 8). Control-side responses remained similar to their own pretest responses despite the 24 hr delay (dif = 0.06 ± 0.41 sec, $t_{(12)} = 0.15$, NS). Trained-side responses, however, were still significantly decremented (dif = −0.97 ± 0.18 sec, $t_{(12)} = 2.89, p < 0.02$). As with short-term habituation, these data demonstrate that the long-term response decrement was not caused by rundown of the preparation but rather was attributable to the side-specific induction of habituation.

Habituation can be site-specific even on the same side of the tail

The previous studies in both intact animals and reduced preparations showed that habituation can be restricted to a single side of the tail, presumably because of the independent, bilateral representation of sensory input beginning with the two sensory neuron clusters (Fig. 1). In a final set of experiments, we wished to characterize further the site specificity of habituation by examining the effects of two training sites on the *same* sides of the tail. These experiments were conducted using the reduced preparation. The first group ($n = 5$) received training similar to that of the short-term group described above (Fig. 8) and was intended as a replication to illustrate the lack of contralateral generalization. In the second group ($n = 9$), electrodes were placed unilaterally at a short distance (1–2 cm) apart. In the third group ($n = 7$), both electrodes were again placed on the same side of the tail, but at a greater distance (2.5–3.5 cm) apart.

The results of the contralateral electrode group were similar to those of the previous short-term experiment and are illustrated in Figure 9*A*. As above, the three pretest responses were collapsed into a single average score and compared with the 5 min test response. After training, responses elicited by the contralateral control electrode were similar to their own pretest responses (dif = −0.30 ± 0.27 sec, $t_{(4)} = 0.5$, NS), despite significant habituation at the trained side of the tail (dif = −3.5 ± 0.04 sec, $t_{(4)} = 2.80, p < 0.05$). Furthermore, there was a significant difference between the two test results (dif = 3.2 ± 0.09, $t_{(4)} = 2.66, p < 0.05$). Thus, confirming the previous experiment, habituation readily occurred in the reduced preparation, endured for at least 5 min, and did not generalize to a test site across the midline of the tail.

The results for the second group (close ipsilateral electrodes) are shown in Figure 9*B*. As consistently observed, habituation training caused a significant decrease in response duration at the trained site, measured 5 min after training (dif = −1.90 ± 0.12 sec, $t_{(8)} = 3.70, p < 0.01$). Unlike the contralateral case (Fig. 9*A*), however, habituation generalized to the near ipsilateral test site, where similar, significant response decrement was observed (dif = −2.00 ± 0.20 sec, $t_{(8)} = 2.50, p < 0.04$). These data demonstrate that habituation will generalize ipsilaterally to sites within 2 cm of the training site.

The results of the third group (distant ipsilateral electrodes) are shown in Figure 9*C*. Again, the trained-site stimulus elicited significantly habituated responses 5 min after training compared with its own pretest response level (dif = −2.20 ± 0.16 sec, $t_{(6)} = 2.48, p < 0.05$). Habituation, however, did not generalize appreciably to the distant test site; no significant response decrement

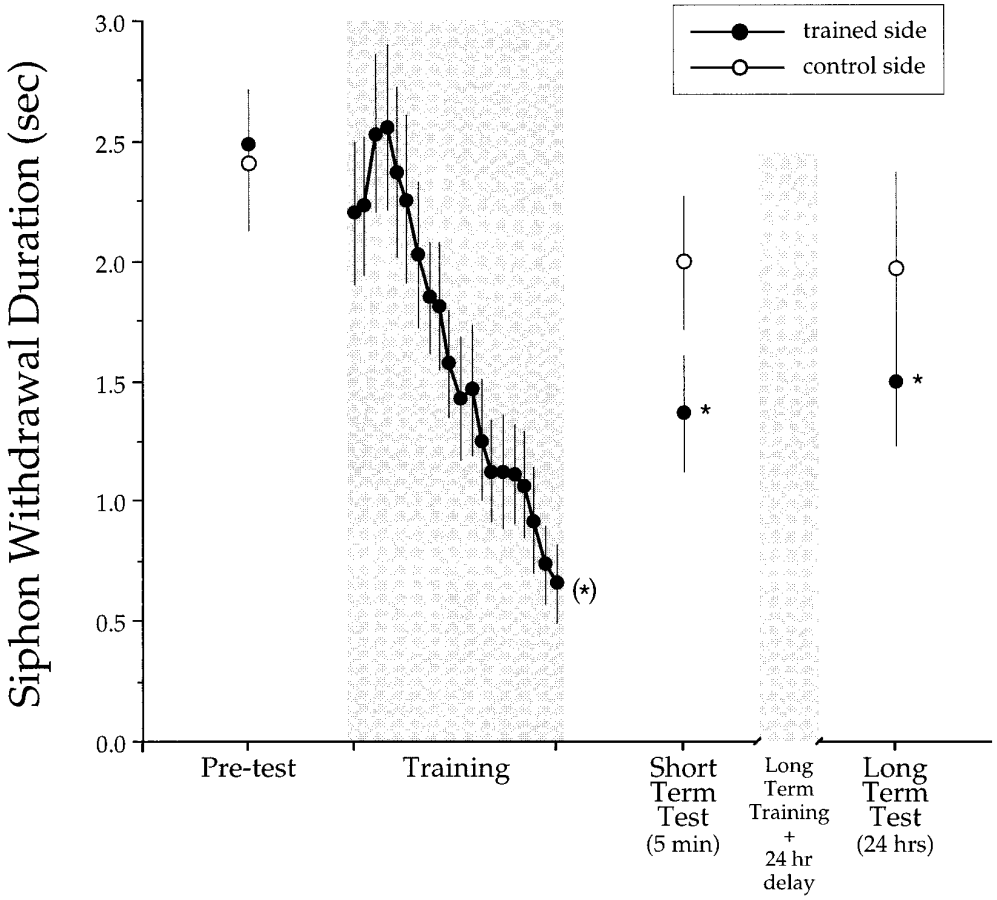

Figure 8. The reduced preparation exhibits both short- and long-term habituation. Only stimuli elicited on trained sides of the tail showed short-term and long-term habituation; stimuli elicited at control sides elicited stable responses over the 24 hr period. See legend to Figure 3 for statistical significance indicated by *asterisks*.

was observed when stimuli were delivered there (dif = -0.60 ± 0.18 sec, $t_{(6)} = 0.79$, NS). Thus, the site specificity of habituation is limited to areas on the tail separated by at least 2.5 cm.

DISCUSSION

Our results demonstrate that both short-term and long-term habituation readily occur in the tail-elicited siphon withdrawal reflex in freely moving animals and in reduced preparations. The within-animal training control provides a powerful means of assuring that the response decrement observed after repeated stimulation is in fact attributable to habituation rather than to some nonspecific general effect such as fatigue or rundown of the preparation. This control is particularly important for reduced preparations in which run-down over time could potentially confound the analysis, especially of long-term habituation.

Short-term habituation endures for a few minutes and then recovers spontaneously back to baseline. Extending short-term to long-term habituation requires more stimuli in a spaced pattern: long-term habituation occurs when four such training sets are delivered, each set separated by a 90 min rest. This observation is consistent with the results of Carew et al. (1972), who examined siphon-elicited siphon withdrawal, and illustrates a common characteristic of habituation: repeated, patterned stimulation with intervening periods of rest is often highly effective in producing long-term habituation (also see Woodworth and Schlosberg, 1964). In similar fashion, recent results in *Drosophila* show that distributed patterning of training in a Pavlovian conditioning paradigm is highly effective in producing long-term retention of a learned odor avoidance behavior (Tully et al., 1994).

Site specificity for different forms of tail-induced behavioral plasticity and their neuronal correlates in *Aplysia* was first described by Walters (1987a,b), who showed that both tail-elicited siphon and tail withdrawal reflexes would exhibit long-term sensitization only when test stimuli were applied to trained sites on the tail and not when similar test stimuli were delivered to bilaterally symmetric control sites. Moreover, Scholz and Byrne (1987) identified specific biophysical correlates of site-specific long-term sensitization in the tail-elicited siphon withdrawal reflex.

In our studies we show that intact, freely moving animals and reduced preparations both exhibit habituation that is side-specific. Thus, both short-term and long-term habituation can be restricted to a single side of the animal, corresponding to the separate, bilateral sensory neuron clusters located in the bilaterally paired pleural ganglia (Walters et al., 1983). This side specificity indicates that cellular sites of plasticity for both short- and long-term habituation must exist upstream from the motor neurons that receive common interneuronal input from both sides of the tail (Frost et al., 1988; and our unpublished observations), and it suggests the possibility that both short-term and long-term forms of plasticity may occur at the same or similar cellular loci.

Generalization experiments in reduced preparations also reveal that habituation can be restricted even to sites on the same side of the tail. Habituation will generalize to test sites within a distance of less than ~2.0 cm on one side of the tail, but not beyond 2.5 cm, or (as demonstrated in intact animals) to sites across the midline of the tail. The lack of generalization between two ipsilateral sites

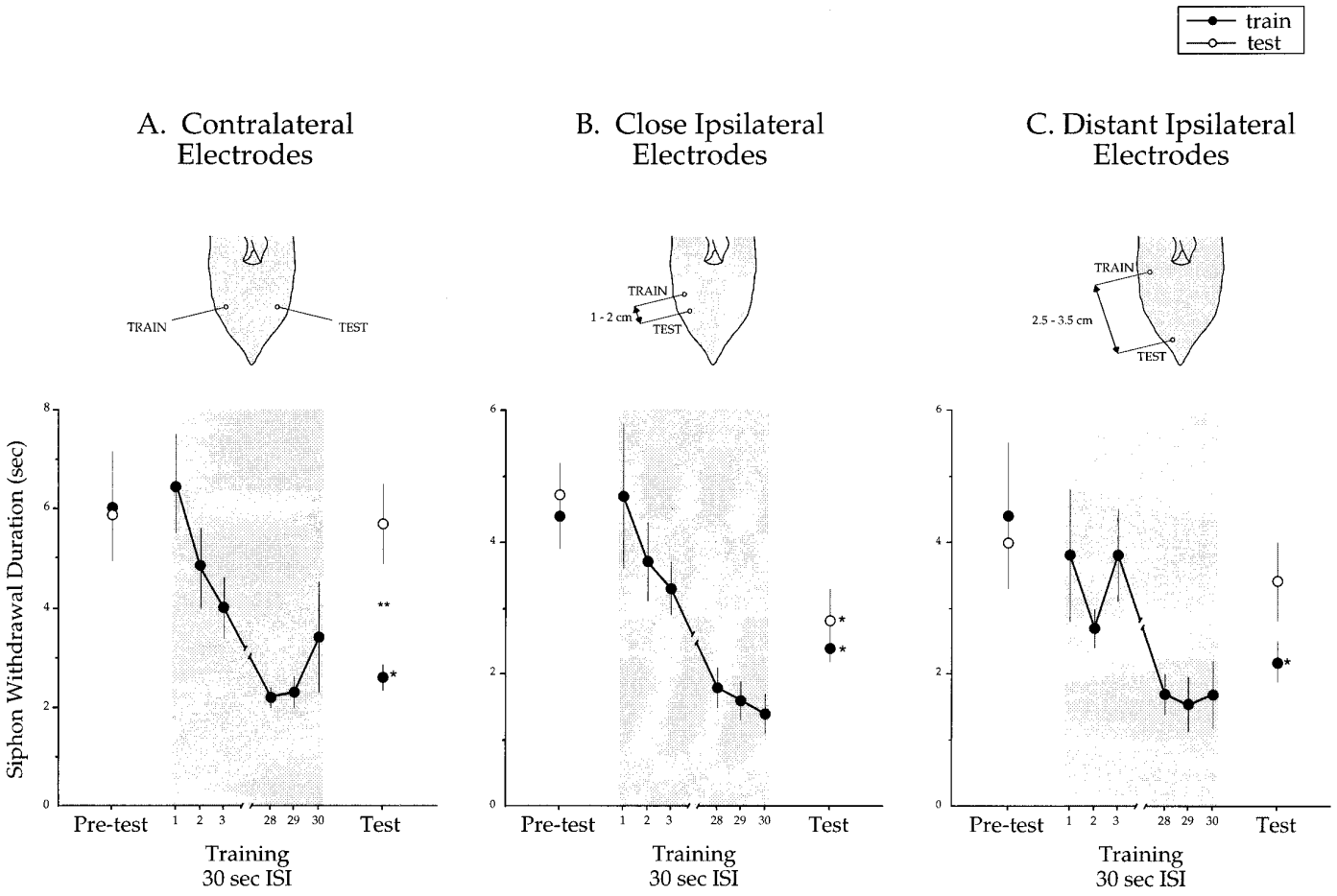

Figure 9. Habituation response generalization in the reduced preparation. *A*, Replicating a result from the previous experiment, short-term habituation does not generalize to sites across the midline of the tail. *B*, Generalization is revealed when test and training sites are spaced closely (within 2 cm) on one side of the tail. *C*, When test and training sites are spaced more widely (beyond 2.5 cm) on one side of the tail, generalization is not evident. Thus, habituation can be restricted to relatively small ipsilateral areas of the tail. See legend to Figure 3 for statistical significance indicated by *asterisks*.

suggests that the mechanisms underlying response decrement can be expressed even within a subset of sensory neurons (or interneurons) in ipsilateral neuronal circuits, providing further clues as to possible sites of neuronal plasticity underlying habituation. As mentioned above, motor neurons can be ruled out as potential sites of plasticity underlying habituation, because they provide the final common reflex pathway from both trained and nontrained sides of the animal. This observation is consistent with the findings of a wide variety of preparations in which motor neurons do not seem to be modified intrinsically by habituation training. These preparations include the spinal cat (Spencer et al., 1966), the frog spinal cord, (Farel et al., 1973), the crayfish escape reflex (Zucker, 1972), and the gill withdrawal reflex in *Aplysia* (Castellucci et al., 1970). Finally, the relative independence of sensory input (even on the same side of the animal) in producing habituation also provides the behavioral basis for a useful within-animal control for cellular experiments in which different sensory neurons, even within a single ganglion, can be used to study the mechanisms of habituation [Stopfer and Carew, 1994, 1996 (companion paper)].

Taken collectively, our behavioral results obtained in freely moving animals and in reduced preparations provide evidence restricting the potential cellular sites underlying habituation. These results also provide the necessary foundation for a cellular analysis of habituation. To further examine questions of mechanism, it is necessary to perform additional experiments using reduced preparations in which sensory and motor neuron activity can be examined directly while simultaneously measuring the habituation of a specific behavioral response (Stopfer and Carew, 1994). As described in the companion paper (Stopfer and Carew, 1996), in which such a combined behavioral and cellular analysis is used, the preparations described in this paper provide a useful system for examining the cellular basis of habituation, a fundamental form of learning observed throughout the animal kingdom.

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