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Classical conditioning in *Aplysia californica*

(associative learning/locomotion)

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ABSTRACT **A form of aversive classical conditioning is described in which a chemosensory conditioned stimulus rapidly acquires the ability to modulate a defensive response (escape locomotion). Because *Aplysia* show both sensitization and classical conditioning, it is now possible to begin to examine the relationship between nonassociative and associative learning on behavioral and cellular levels.**

A central problem in the study of behavior is the analysis of the mechanisms underlying the various forms of learning. What are the cellular mechanisms of associative learning and how do they relate to those of nonassociative learning? Are different forms of learning governed by separate mechanisms or by variations on a common mechanism? Answers to these questions are beginning to emerge. They have come primarily from the use of simple experimental systems in which the animals are capable of various forms of learning and are accessible to analysis on the cellular level (1–7).

Because of the relative simplicity of its nervous system and the detailed knowledge available on the biophysical, biochemical, and morphological properties of its neurons (8), the marine snail *Aplysia* has been useful for analyzing the mechanisms of two forms of nonassociative learning: habituation and sensitization. Each of these forms has been shown to be due to a change in strength at a specific set of synaptic connections (2, 9), and in each case the change in synaptic strength results from an alteration in the calcium current controlling transmitter release at the presynaptic terminals (10, 11).

A level of complexity beyond habituation and sensitization is associative conditioning. A major limitation to the further study of the mechanisms of learning in *Aplysia* has been the failure to demonstrate associative learning in this animal (12–14). We here describe a powerful form of classical conditioning in *Aplysia*.

The paradigm we have used was based upon classical defensive or aversive conditioning (15). In this paradigm one stimulus, the conditioned stimulus (CS), is repeatedly paired with an aversive unconditioned stimulus (US). This training commonly gives rise to a set of conditioned responses, which can be studied in two different ways. First, overt motor responses to the CS sometimes develop and they can be examined directly (5, 15). A second approach—which we have used here—is to examine the ability of the conditioned stimulus to modulate other (test) behaviors not involved in the original conditioning procedures (16, 17). The test behavior we have used is escape locomotion.

Despite its technical advantages and theoretical interest, this second approach, commonly used in vertebrates, has not been directly explored in invertebrates. This approach is useful for two reasons. (*i*) Examination of conditioned modulatory effects allows investigation of additional dimensions of learning not previously explored in invertebrates, dimensions that may illustrate new parallels between more complex aspects of learning in vertebrates and invertebrates. Such parallels could encourage the use of simple systems not only for mechanistic analysis but also for the behavioral study of fundamental psychological issues, such as the distinction between learning and performance (18). (*ii*) This protocol permits the use of a test system that survives restraint of the animal and surgical exposure of its central nervous system, a prerequisite for cellular analysis.

Our behavioral index of aversive classical conditioning was the modulation by the CS of escape locomotion (Fig. 1). This response offers several advantages for the study of learning in *Aplysia* on both behavioral and cellular levels: (*i*) Locomotion is easy to quantify because the individual steps are discrete and readily identifiable. (*ii*). Locomotion displays short-term and long-term sensitization (ref. 19 and unpublished data). (*iii*) The central program for locomotion is located within the circumesophageal ganglia and can be measured reliably in the patterned activity of both peripheral nerves and identified motor neurons in simplified preparations (20–22).

RESULTS

Conditioning Procedures. The experimental procedures we used are summarized in Fig. 2. All animals underwent the pretest on day 1 to assess baseline locomotor responsiveness before training. The test stimulus was a train of weak pulsed electric shock applied across the tail with spanning Ag/AgCl electrodes. The latency of the first step and the number of steps taken within a 5-min period after the shock were recorded by using criteria described in the legend of Fig. 1.

Animals were then matched on the basis of their pretest scores and assigned to one of three training groups. The "untrained" group received no further treatment during the 2-day training period. The "unpaired" group received training with the shrimp CS and the head shock US explicitly unpaired. The "paired" group was trained with specific temporal pairing of the CS and US (see Fig. 2). Three training trials per day (intertrial interval, 3 hr) were given for 2 days. The training procedure in each trial was similar to that of Mpitsos and Collins (5). Animals in the paired group received the CS applied over the anterior head region; 60 sec after onset of the CS the US was applied via spanning Ag/AgCl electrodes across the front of the head. The unpaired group was trained with the same CS and US as the paired animals, but received them specifically unpaired; on each trial the CS was delivered 90 min after the US. Eighteen hours after the last training trial all animals were tested, using a blind procedure (by an observer who did not know the animals' individual training histories). In the test session each animal received the CS for 60 sec and then, with

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Abbreviations: CS, conditioned stimulus; US, unconditioned stimulus.

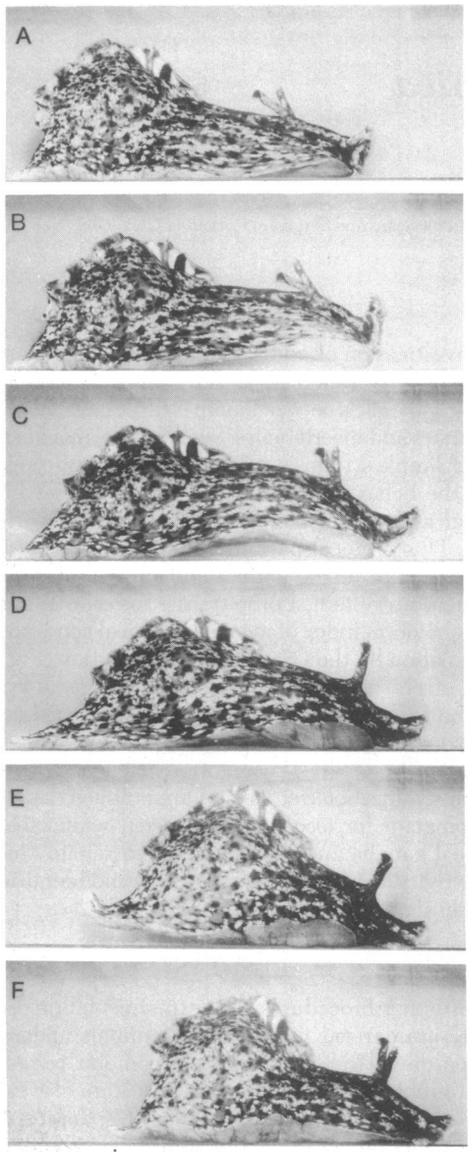

FIG. 1. One complete step in the locomotor sequence of *Aplysia*. (¼ life size.) (*A* and *B*) Elevation and extension of the head; (*C* and *D*) arching of the midbody; (*E* and *F*) retraction of the tail. Steps were counted as the number of tail retractions because these are discrete and easily observed.

the CS still present, the same test stimulus used in the pretest (weak tail shock) was delivered. The latency and number of steps taken within 5 min were monitored for each animal during the test session. These training procedures resulted in a form of conditioning characterized by (*i*) temporal specificity, (*ii*) a requirement of CS presence for the learning to be expressed, and (*iii*) rapid acquisition. Each of these characteristics will be described in turn.

Temporal Specificity. A critical feature of associative learning is temporal specificity. In both classical and instrumental learning the subject displays a change in behavior due to specific temporal relationships between events. A common interpretation (23) is that the subject changes its behavior as it learns that one event (the CS in classical conditioning and the operant response in instrumental conditioning) comes to predict the occurrence of another (the US or reinforcement). The first question we examined was whether training with a pattern of temporally paired CS and US presentations produces a different outcome than training with a pattern of explicitly unpaired

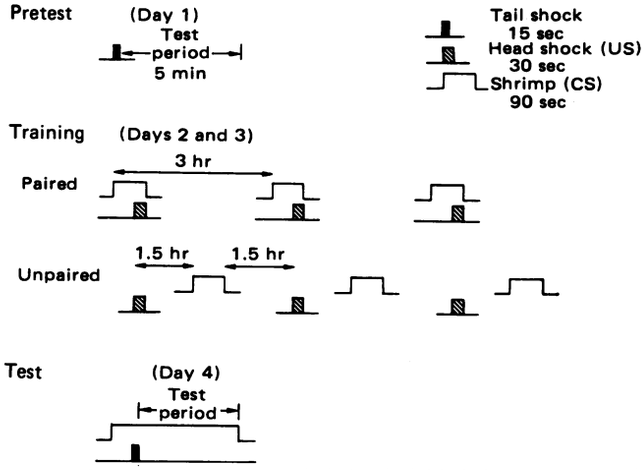

FIG. 2. Behavioral protocol. Animals (150–350 g) were housed individually in perforated circular pans (28-cm diameter, 7.6-cm depth) that were suspended in a 750-liter tank of aerated artificial sea water (15°C). The test stimulus was a 15-sec train of 50-mA ac pulses (1.5 sec each), 0.33 Hz, to the tail. The US was a head shock, 30 sec of 400-mA ac pulses (1.5 sec), 0.33 Hz. Large currents were necessary for effective stimulation because of the seawater shunt between the electrodes and skin. The CS was 1.5 ml of crude shrimp extract. Immediately prior to CS onset a plastic bag was placed around each pan to separate its contents from the water in the tank. CS offset was accomplished by siphoning all the water out of the pan and then removing the bag. These procedures were identical for paired and unpaired groups.

presentations or no training at all (Fig. 3). Before training there were no marked differences among the three groups in the amount of locomotion elicited by tail shock (the test stimulus). After training, a one-way analysis of variance indicated there was an overall significant difference ($F_{2,45} = 27.4$; $P < 0.01$) among the groups in their escape locomotor responses to the test stimulus in the presence of the shrimp CS. Paired comparisons showed that the number of steps taken by the untrained group was not significantly different from that of the unpaired group, but the animals in the paired group walked more than those in either control group ($P < 0.005$ in each case; Fig. 3*A*). A direct

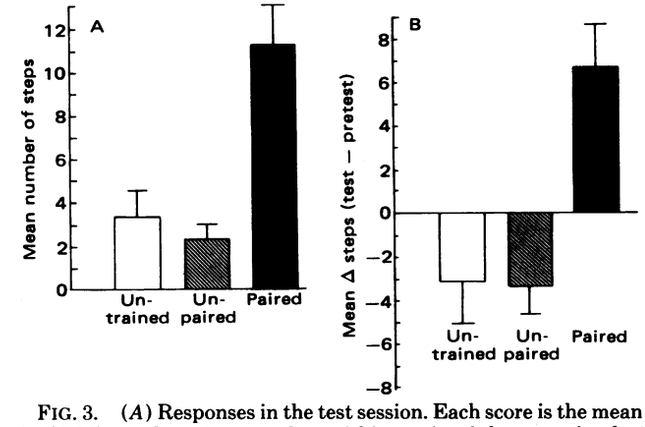

FIG. 3. (*A*) Responses in the test session. Each score is the mean (± SEM) number of steps taken within 5 min of the test stimulus. Paired animals ($n = 18$) walked significantly more than untrained animals ($n = 12$) or unpaired animals ($n = 18$), $P < 0.005$ in each case. All paired comparisons were by t tests for independent groups; all probability values are two-tailed. (*B*) Differences between test and pretest scores (from same experiment as *A*). Paired animals walked significantly more in the test than in the pretest ($P < 0.005$, t for correlated means). The decrease in locomotion of the untrained group is not statistically significant, whereas the decrease of the unpaired group is ($P < 0.025$). Such decreases are often, but not invariably, observed in control groups.

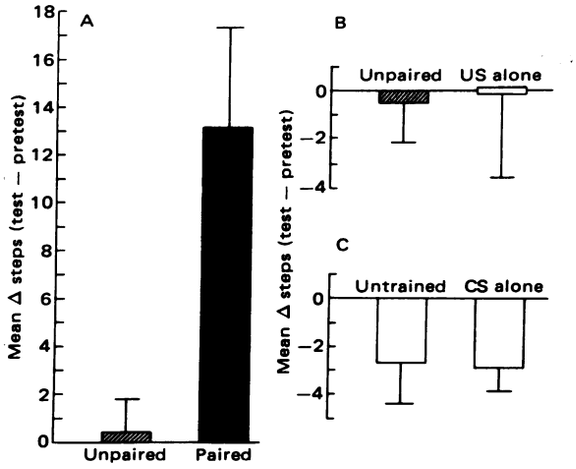

FIG. 4. (*A*) Stimulus site control. Differences (mean ± SEM) between test and pretest scores. US delivery during training was with seawater-filled capillary electrodes (0.5-cm diameter, 0.2-cm separation) to obtain repeatable, restricted stimulation of the anterior head. Paired animals ($n = 8$) showed significantly greater locomotion ($P < 0.005$) than unpaired animals ($n = 8$). (*B*) Differences between test and pretest scores of animals trained with CS and US unpaired ($n = 8$) or with US alone ($n = 8$). There was no significant difference between these groups. (*C*) Differences between test and pretest scores of untrained animals ($n = 8$) and animals trained with CS alone ($n = 8$). There was also no significant difference between these groups.

comparison of the difference in locomotion exhibited by the different groups before and after training is shown in Fig. 3*B*. The data presented in this way clearly show that the paired group is different not only in the amount of escape locomotion exhibited in the test session but also in the direction of the effect: Both control groups showed a decrease in locomotion in the test session whereas the paired group showed an increase. No significant differences were found in the mean latencies to the first step among these groups in the test or pretest.

Although the testing in this experiment was conducted blind, the training was not. Thus the possibility existed that trainer bias could have contributed to the differences observed. To control for this and related artifacts in the training procedure, we carried out several control experiments. We first replicated the experiment but used a blind training procedure as well as a blind testing procedure. Training was done by a person who knew neither the design nor the purpose of the study. As in the previous experiment, animals in the paired group ($n = 8$) exhibited significantly more escape locomotion during the test session ($P < 0.005$) than did the unpaired control group ($n = 8$).

Even when the training was carried out blind, the difference in training procedures for paired and unpaired groups may have produced an unintentional bias on the part of the trainer (for example, he may have delivered a stronger US to one group or another by direct contact with the spanning electrodes). Thus, a functionally stronger or weaker US delivered to one group might have contributed to the differential effect we observed. To test this possibility we examined the two conceivable US intensity differences by giving unpaired training with (*i*) a functionally stronger US and (*ii*) a functionally weaker US than that previously used. The weak US was a shock with half the current (200 mA) normally used. The strong US consisted of the same current as normal (400 mA), but the electrodes, instead of being held near the skin, were brought into direct contact with the skin. Separate pilot experiments had indicated that such contact causes considerably more sensitization of a variety of responses than does shock from noncontacting electrodes. After training these two groups ($n = 8$ in each group) were not significantly different from each other (mean steps ± SEM for strong US group = 2.25 ± 2.25, and for weak US group = 3.00 ± 2.27) and appeared similar to the unpaired groups receiving the standard US.

Finally, we investigated the possibility that, even if US intensity differences did not account for the effect, differences in the *site* of US stimulation might contribute to the differences between paired and unpaired groups. We were specifically concerned that, when animals in the paired group withdrew their heads in response to the CS (as most animals do during training), the amount of head and neck surface they exposed to the US might be different, and therefore the paired group might have received a qualitatively different US than the unpaired group (which received the CS and US 90 min apart). We controlled for this possibility by replicating the basic experiment shown in Fig. 2 with the US delivered through seawater-filled glass capillary electrodes, which repeatedly provided precise contact to the middle of the anterior head (centered between the oral tentacles and the rhinophores) even when the animal's head became withdrawn. With this different method of US delivery the paired group still showed significantly more escape locomotion in the presence of the CS than the unpaired group ($P < 0.005$; Fig. 4*A*). These experiments demonstrate that training with the CS and US specifically paired endows the CS with properties not observed with unpaired presentation of the same stimuli.

We also found that training with either the US alone (Fig. 4*B*) or with the CS alone (Fig. 4*C*) failed to produce the con-

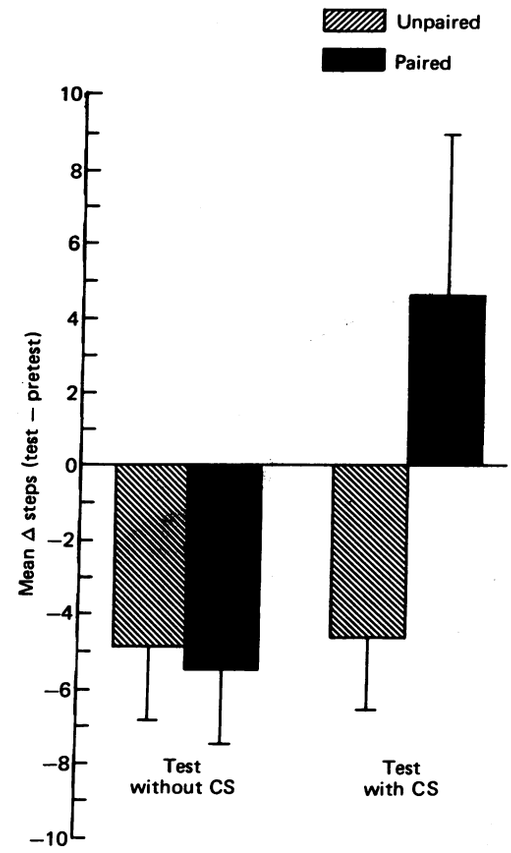

FIG. 5. Differences between test and pretest scores in the absence and (3 hr later) in the presence of the CS. Paired animals ($n = 8$) walked significantly more than unpaired animals ($n = 8$) in the presence of the CS ($P < 0.025$). The paired animals also walked significantly more than they had in the absence of the CS ($P < 0.01$, t for correlated means).

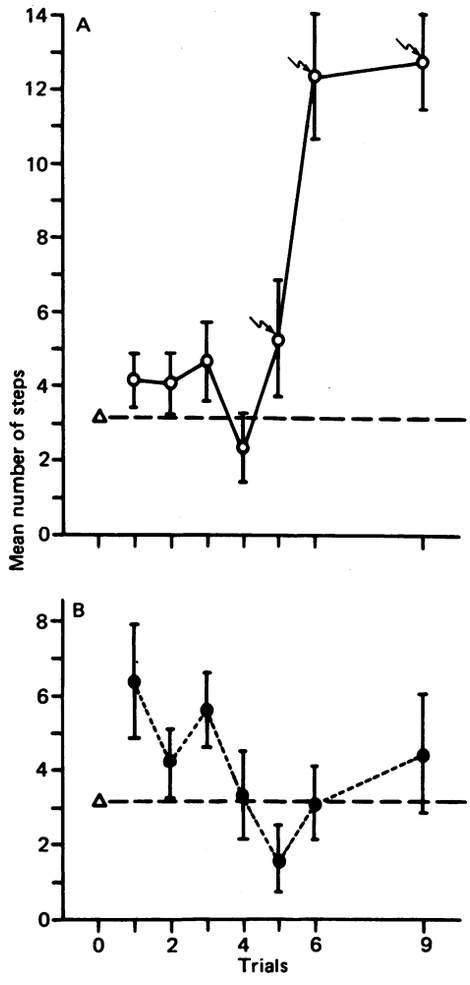

FIG. 6. Acquisition. Different groups were given from zero to nine training trials and then tested 18 hr after the last trial. Each point is the mean (± SEM) number of steps taken in the test session. Three conditions were examined: paired training (*A*, ○), unpaired training (*B*, ●), and no training (△ in *A* and *B*, each representing the same data). Numbers of animals per group were: untrained, $n = 27$; paired and unpaired (respectively), one trial, $n = 16$, $n = 16$; two trials $n = 24$, $n = 24$; three trials, $n = 24$, $n = 24$; four trials, $n = 16$, $n = 16$; five trials, $n = 16$, $n = 15$; six trials, $n = 26$, $n = 26$; nine trials, $n = 16$, $n = 16$. The horizontal broken lines indicate baseline (untrained) performance. Significant differences between paired and unpaired scores were found on trials 5, 6, and 9 (indicated by arrows, $P < 0.025$, $P < 0.005$, and $P < 0.005$, respectively).

ditioned facilitatory effect of the CS on escape. These findings further support the conclusion that the ability of the CS to enhance escape locomotion is dependent upon the specific temporal relationship between CS and US during conditioning.

Requirement of CS Presence. Associative learning is usually characterized by marked stimulus specificity (24). The presence of the CS is necessary for expression of the conditioned response: it is not elicited by other stimuli, except by those that are quite similar. We have begun to examine these features by testing for the requirement of the CS during the test session. If paired training had merely produced a general increase in responsiveness, one could predict that the test stimulus alone might elicit the conditioned effect in the absenee of the CS.

To test this possibility we first trained animals with the standard paired and unpaired protocols (Fig. 2). These groups were then tested twice, first in the absence of the CS, and then, 3 hr later, in the presence of the CS. After training neither group exhibited much locomotion in response to tail shock in the absence of the CS (Fig. 5). However, in the presence of the CS, the paired group exhibited significantly more escape locomotion in response to the test stimulus than did the unpaired group ($P < 0.025$), and, in addition, significantly more locomotion than it had shown in response to the same test stimulus in the absence of the CS ($P < 0.01$, t for correlated means). In the absence of the CS both groups showed a decrease in locomotion relative to the pretest scores. In the presence of the CS only the paired group showed an increase in locomotion relative to the pretest. These results indicate that the CS is required for the conditioned effect to be exhibited and suggest that the conditioning does not take the form of a nonspecific increase in responsiveness.

Acquisition. We next examined one aspect of the acquisition of the learned response by training different groups of animals with different numbers of trials. Each group was tested the morning after its last training trial. Thus animals receiving one to three trials trials were tested on day 3, while animals receiving four to six trials, as well as animals given zero trials (no training), were tested on day 4 (Fig. 2). Animals receiving nine trials were tested on day 5. In each case paired and unpaired groups were run and each point on the composite acquisition curve (Fig. 6) consisted of at least two experiments for each group. The composite curve for the paired groups (Fig. 6*A*) was sigmoid. In trials one through four there were no significant differences between the groups. However, after five training trials the paired group exhibited significantly more locomotion in response to the test stimulus than did the unpaired group ($P < 0.025$), in part due to an unexplained decrease in the control group. By trials six and nine there were consistently large and significant differences between paired and unpaired groups ($P < 0.005$ in each case).

Two aspects of the acquisition curve (Fig. 6) deserve mention. First, although the data were variable, unpaired groups tended to walk more than paired groups after receiving one to three trials. We are not sure how reliable this small trend is. Second, acquisition appears to be quite rapid in the paired groups after the fourth trial. Rapid acquisition is a common characteristic of aversive classical conditioning in vertebrates (see below).

DISCUSSION

These experiments provide direct evidence that *Aplysia* can be classically conditioned, forming a powerful temporally specific association between a chemical CS and a noxious electrical US. The association is rapidly acquired and is dependent for its expression upon the presence of the CS. The features of temporal specificity, the requirement of the CS during testing, and rapid acquisition are characteristic of aversive classical conditioning in vertebrates (18). Moreover, in *Aplysia* as in vertebrates, aversive conditioning has the interesting feature that conditioning often occurs without overt changes in the external behavioral response to the CS. Rather, conditioning leads to a change in internal state that is manifest in the acquired ability of the CS to modulate a variety of behaviors (25, 26). For example, aversive classical conditioning can endow a CS with the capability of suppressing appetitively reinforced bar pressing (16), enhancing instrumental avoidance responding (27), facilitating an unconditioned startle response (17), and accelerating heart rate (28). These conditioned properties of the CS are specific to the temporal relationships of the CS and US during training as well as to the particular CS used. The acquisition of conditioned aversive responses as revealed by such tests is typically quite rapid in vertebrates, often peaking within 5–10 training trials (17, 29). The similarity of the effects described for *Aplysia* to those of aversive classical conditioning in vertebrates encourages the search for further similarities and suggests that mechanisms found in the study

of this form of associative learning in *Aplysia* may have general significance.

What is the learned response? The associative effect in our experiments is not simply the direct conditioning of a locomotor response because no locomotion is elicited when the shrimp extract is presented alone after conditioning. Two other possibilities for the conditioned effect are: (*i*) conditioning of another motor response (e.g., head withdrawal or a postural response) that itself facilitates locomotor responses to tail shock, and (*ii*) conditioning of a motivational or arousal state (perhaps analogous to what is called "fear" in vertebrates) that facilitates escape responses. At present we cannot distinguish between these possibilities. However, neither the withdrawals nor the postures of paired and unpaired animals are noticeably different during the test session. Thus, in contrast to aversive conditioning in *Pleurobranchaea*, in which conditioning leads to the development of an overt withdrawal response to the CS (5), in *Aplysia* the conditioning appears to take the form of a powerful change in internal state that becomes manifest in the modulation of a response not directly elicited by the CS. That a specific motor response is not conditioned is consistent with theoretical interpretations of aversive classical conditioning as a conditioned motivational state rather than a conditioned motor response (18, 25, 26). That *Aplysia* display well-defined appetitive and defensive motivational states (14, 30) further supports this possibility.

The hypothesis that *Aplysia* can learn conditioned motivational states has several interesting implications. One prediction is that a range of behaviors will be affected by the CS after conditioning and that these effects will be motivationally consistent; defensive responses should be enhanced and appetitive responses suppressed by the CS (25). A second implication is relevant to hypotheses about the relationship between sensitization and classical conditioning (8, 31, 32). Because sensitization is thought to be a component of motivational states (30), the conditioning of a motivational state would suggest that this form of associative learning is likely to involve, in part, aspects of sensitization. This notion is attractive because it suggests that different forms of learning may utilize various combinations of a restricted set of fundamental plastic mechanisms.

Learning in *Aplysia* persists after the animal has been restrained and the head ganglia have been surgically exposed. Moreover, we have recently observed neural correlates of aversive conditioning in identified pedal motor neurons (unpublished data). Three other forms of associative learning have also been demonstrated in related gastropod mollusks: avoidance conditioning in *Pleurobranchaea* (4, 5), bait-shyness in *Limax* (6), and intersensory associations in *Hermissenda* (7). In each of these animals neuronal correlates of associative learning have also been reported (33–35). Thus, in addition to its being possible to examine the relationship between nonassociative and associative learning within the same species, it may be possible to examine on the cellular level the relationships among different forms of associative learning across related species.

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