Classical conditioning of the Aplysia siphon-withdrawal reflex exhibits response specificity

(conditioned response/beta conditioning/activity-dependent facilitation)

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ABSTRACT The gill- and siphon-withdrawal reflex of Aplysia undergoes classical conditioning of its amplitude and duration when siphon stimulation (the conditioned stimulus, CS) is paired with tail or mantle shock (the unconditioned stimulus, US). This conditioning of a preexisting response exhibits both temporal and stimulus specificities, which can be accounted for by activity-dependent enhancement of presynaptic facilitation of the siphon sensory neurons. To test whether conditioning of the reflex also exhibits response specificity (development of a new type of response to the CS that often resembles the response to the US), we measured the direction of siphon withdrawal in response to siphon stimulation (the CS) with tail or mantle shock as the US. The unlearned response to siphon stimulation is straight contraction, the response to tail shock is backward bending, and the response to mantle shock is forward bending. In the first experiment, we trained different animals with the tail or mantle US paired or unpaired with the CS; in a second experiment, we trained each animal with two CSs, one of which was paired with the US; in a third experiment, we varied US intensity; and in a fourth experiment, we trained each animal with two USs, one of which was paired with the CS. There was a significant, pairing-specific tendency for the direction of the response to the CS to resemble the response to the US after training in each experiment, demonstrating response specificity in conditioning of the withdrawal reflex. This feature of conditioning could in principle be accounted for by an elaboration of activity-dependent facilitation.

In classical conditioning, the response to the conditioned stimulus (CS) changes as a result of temporal pairing and correlation with the unconditioned stimulus (US). Frequently, the response to the CS comes to resemble the response to the US (1–5). This observation has led to protracted and still unresolved debates about what determines the learned response and also about what the animal actually learns to associate in conditioning (for review, see ref. 6). For example, does the animal learn to associate the CS with the US, with the response to the US, or with a central motivational state produced by the US? One way to gain insights into these behavioral questions is by examining the underlying neural mechanisms. Such a cellular analysis of associative learning has begun to be possible in several invertebrate preparations, including locust, Drosophila, Pleurobranchaea, Hermisenda, and Aplysia (for reviews, see refs. 7–9). Previous experiments have shown that the gill- and siphon-withdrawal reflex of Aplysia undergoes classical conditioning of its amplitude and duration when siphon stimulation (the CS) is paired with tail or mantle shock (the US) (10, 11). This conditioning exhibits both stimulus and temporal specificities (12, 13), which can be accounted for by a known neural mechanism, activity-dependent enhancement of presynaptic facilitation of the siphon sensory neurons (14–16). The recent observation that different types of stimuli produce different types of siphon responses (17) provided the opportunity to test whether conditioning of the siphon withdrawal reflex also exhibits response specificity—that is, whether the animal learns to make a new type of response to the CS that resembles its response to the US. We find that conditioning of this simple reflex can exhibit response specificity. Thus, Aplysia learn not only to strengthen the magnitude of a previously existing reflex response (alpha conditioning), they also can learn to develop a new type of response to the CS (beta conditioning).

MATERIALS AND METHODS

Aplysia californica (150–300 g) were obtained from Sea Life Supply (Sand City, CA) or Marinus (Long Beach, CA). Animals had their parapodia surgically removed to permit visualization of the entire siphon 1 or more days after arrival and were housed in individual perforated circular pans in a 200-gallon aquarium for 2 or more days before an experiment was begun (for details, see ref. 10). The CS was a light tactile stimulus applied to the inner surface of the left side of the siphon with a nylon bristle, and the US was an electric shock (60 Hz ac, 1.0 sec duration) delivered through bipolar capillary electrodes pressed against the tail or mantle shelf. During paired training, the CS onset preceded the US onset by 0.5 sec; during unpaired training, the interval was 2.5 min. The intertrial interval was 5 min. Both the CS and US were delivered by hand, with the timing of the stimuli guided by electronically controlled audio signals.

Animals were assigned to training conditions in such a way as to balance their pretest scores in each replication, and the results of several replications were pooled. The pretest score was subtracted from each of the subsequent scores for that animal to provide a measure of change in responding. The normalized data were analyzed by analysis of variance with a within-animal factor (test). If there was a significant main effect or interaction involving pairing, planned comparisons were made at each individual test trial to assess the time course of the paired effect (18).

RESULTS

To examine response specificity, we measured the direction of siphon withdrawal in response to siphon stimulation (the CS) with tail or mantle shock as the US. The unlearned response to siphon stimulation is straight contraction of the siphon, the response to tail shock includes backward bending

Abbreviations: CS, conditioned stimulus; US, unconditioned stimulus.
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of the siphon, and the response to mantle shock includes forward bending (Fig. 1A). In experiment 1, we trained four different groups of animals with either tail shock (50 mA) or mantle shock (25 or 35 mA) as the US, either paired or unpaired with the CS. Training consisted of two blocks of 10 trials each, with ≈45 min between blocks. The direction of the response to the CS (backward bending, neutral, or forward bending) was scored blind before training (pretest), 30 min after each block of trials, and again 24 hr after the end of training. The response to the CS was neutral for 90% of the animals before training. During and after training, there was a significant tendency for the direction of the response to the CS to resemble the response to the US with which it was trained (n = 15 per group; F1,56 = 47.78; P < 0.01 for the main effect of US) (Fig. 1B). The response to the CS became more tail-like than it had been before training in animals that were trained with the tail US (F1,56 = 12.08; P < 0.01) and became more mantle-like in animals that were trained with the mantle US (F1,56 = 4.77; P < 0.05). There was no evidence for an associative effect during training but there was when the response was retested 24 hr later. That is, there was a significantly greater tendency for the direction of the response to the US 24 hr after paired than after unpaired training. The associative effect was indicated by a significant US × pairing × test interaction (F2,112 = 4.14; P < 0.05), which planned comparisons showed was due in part to a difference between paired and unpaired training 24 hr after training. This associative effect was larger with the tail US (F1,112 = 13.99; P < 0.01) but was also marginally significant with the mantle US (F1,112 = 2.83; P < 0.05, one tail). These results indicate associative response specificity in classical conditioning of the siphon withdrawal reflex.

In the first experiment, different animals received paired and unpaired training. As another test of associative response specificity, we performed a second experiment using a within-animal comparison. That is, each animal received training with two different CSs, one of which was paired with the tail shock US. This design controls for a possible source of bias in experiment 1, inadvertent differences in effective US intensity during paired and unpaired training, since any variation in US intensity should affect the responses to the paired and unpaired CSs equally when they are tested in the same animal. The two CSs were tactile stimulation of the siphon and a weak electric shock (5 mA, 60 Hz ac, 0.5 sec duration) applied to the mantle shelf with bipolar capillary electrodes. During training, one CS (CS⁺) was presented paired with the US and the other CS (CS⁻) was presented specifically unpaired (2.5 min after the US). The siphon stimulus was the CS⁺ for half of the animals and the mantle stimulus was the CS⁻ for the other half. During testing, the direction of siphon withdrawal was scored first in response to the siphon CS and then in response to the mantle CS. Like siphon stimulation, weak mantle stimulation initially produced a straight siphon contraction in most animals. As in experiment 1, the direction of the response to the CS

Fig. 1. Response specificity in conditioning of the siphon withdrawal reflex. (A) In naive animals, siphon stimulation causes straight contraction of the siphon, tail stimulation causes backward bending, and mantle stimulation causes forward bending. During testing, straight contraction was scored as 0, backward bending was scored as +1, and forward bending was scored as -1. (B) Experiment 1. Different animals received training with siphon stimulation as the CS either paired (P) or unpaired (UP) with tail or mantle shock as the US. The data points indicate the mean direction of siphon withdrawal in response to the CS at each test, and the error bars indicate the SEM. The horizontal dashed line shows the average direction of responding in the pretest (PRE). Significant differences between paired and unpaired training are indicated by * (P < 0.05) or † (P < 0.05, one tailed). (C) Experiment 2. Each animal received training with two CSs (siphon and mantle stimulation), one of which (CS⁺) was paired with the tail US. (D) Experiment 3. Different animals received training with the siphon CS either paired or unpaired with a weaker tail US. (E) Experiment 4. Each animal received training with two USs (tail and mantle shock), one of which was paired with the siphon CS.
changed to resemble the response to the US after training, and this effect was significantly greater for the CS+ than for the CS− when tested 24 hr after training (Fig. 1C). This associative effect was indicated by a significant pairing × test interaction (n = 12 per group; F_{2,46} = 4.48; P < 0.05), which planned comparisons showed was due in part to a difference between the responses to the paired and unpaired CSs 24 hr after training (F_{1,46} = 11.24; P < 0.01). These results thus replicated the results of experiment 1 with a within-animal design.

In both experiments 1 and 2, we observed an associative effect 24 hr after training but not during training. One possible explanation of this result is that the US produced a strong nonassociative effect that masked the expression of the associative effect during training. To test this possibility, we carried out a third experiment in which we varied the US intensity and also tested more frequently. We trained four different groups of animals with either a 40- or 60-mA tail shock as the US, either paired or unpaired with the siphon CS, and tested 15 min after 5, 10, 15, and 20 trials, and again 24 and 48 hr after training. There were different patterns of results with the two US intensities, as indicated by significant US intensity × pairing (n = 16 per group; F_{1,60} = 6.16; P < 0.05) and US intensity × pairing × test (F_{2,300} = 2.25; P < 0.05) interactions. With the stronger US, the pattern was similar to that seen in experiments 1 and 2, with a significant difference between paired and unpaired training only 48 hr after training (F_{1,300} = 5.15; P < 0.05) (data not shown). With the weaker US, there were significant differences between paired and unpaired training after 10 trials, 15 trials, and 20 trials, and 24 hr but not 48 hr after training (P < 0.05 in each case) (Fig. 1D). These results suggest that the failures to observe an associative effect during training in experiments 1 and 2 (and with the stronger US in experiment 3) were due to masking by the nonassociative effect.

As a more demanding test of response specificity, we performed a fourth experiment in which we trained each animal with two different USs (50-mA tail shock and 25- or 35-mA mantle shock), one of which was paired with the siphon CS. We maintained the same total number of shocks (n = 20) and intershock interval (5 min) so that each animal received 10 pairings of the CS with one US, alternating with 10 presentations of the other US alone. The animals were tested after 10 shocks, 20 shocks, and overnight. The response to the siphon CS became more tail-like in animals that were trained with the tail US paired and did not change in animals that were trained with the mantle US paired (Fig. 1E). This difference is indicated by a significant paired US × test interaction (n = 30 per group; F_{2,116} = 3.12; P < 0.05), which planned comparisons showed was due in part to a significant difference between training with the tail US paired and training with the mantle US paired after 20 shocks (F_{1,116} = 6.89; P < 0.01). These results show that an animal trained with more than one US can develop a response to the CS that is appropriate to the US with which it is paired.

**DISCUSSION**

Hull (19) distinguished between alpha conditioning, in which there is an increase in the amplitude of a preexisting response to the CS, and beta conditioning, in which an animal learns to make a new type of response to the CS that frequently resembles its response to the US. Previous studies of conditioning of the *Aplysia* siphon-withdrawal reflex have demonstrated alpha conditioning (10–13). We have now shown that the withdrawal reflex also undergoes beta conditioning. Like alpha conditioning, beta conditioning of the withdrawal reflex has a nonassociative component ("sensitization" in the case of alpha conditioning and "pseudoconditioning" in the case of beta conditioning) and an associative component that depends on temporal pairing of the CS and US during training. Walters and Erickson have independently obtained similar results on both nonassociative (21) and associative (22) changes in the type of siphon response with different training and testing procedures, suggesting that the results may have wider generality.

These results extend the range of similarities that have been observed between conditioning of the *Aplysia* gill- and siphon-withdrawal reflex and conditioning in vertebrates. These similarities include acquisition, retention, extinction, spontaneous recovery, differential conditioning, and effects of interstimulus interval, contingency, and context (10–13). An advantage of studying the *Aplysia* withdrawal reflex is that it is relatively amenable to cellular analysis. The circuit for the reflex includes a monosynaptic component, with identified siphon sensory neurons directly exciting identified gill and siphon motor neurons (23–25). Frost et al. (26) have recently found two types of motor neurons that cause forward and backward bending of the siphon. In addition, some interneurons mediating the polysynaptic component of the reflex have also been identified (27–29).

Previous studies have described a neuronal mechanism, activity-dependent enhancement of presynaptic facilitation, which contributes to classical conditioning of the withdrawal reflex (14–16). Briefly, the US causes presynaptic facilitation of all of the sensory neurons, but the facilitation is enhanced in those neurons in the CS pathway that are active just before the US is delivered. This mechanism can account for aspects of the stimulus and temporal specificities of conditioning and in principle can also account for many of the parametric and higher-order features of conditioning (30, 31). However, it cannot account for the response specificity of conditioning if the facilitation is assumed to be "cell-wide"; that is, if all of the synapses from an individual sensory neuron are facilitated equally (Fig. 2A).

Several types of neuronal mechanisms could in principle contribute to response specificity in conditioning of the withdrawal reflex. First, response specificity could be a consequence of Hebb-type synaptic plasticity (32), which depends on conjoint activity of pre- and postsynaptic neurons (Fig. 2B). However, Hebb-type plasticity has been tested at the synapses from sensory neurons to motor neurons in the circuit for the reflex and does not occur there (33). Second, selective increases in the excitability of motor neurons might contribute to a nonassociative tendency for the response to the CS to resemble the response to the US. Frost et al. (26) have found that the siphon motor neurons undergo a long-lasting heterosynaptic increase in excitability after tail shock, and they have suggested that this effect could contribute to response specificity if it were restricted to specific motor neurons. Such a restriction could arise either from specialized facilitator neurons (not illustrated) or from activity-dependent enhancement of the increase in motor neuron excitability (Fig. 2C). The molecular mechanism of the increase in excitability appears to be similar to that of presynaptic facilitation of the sensory neurons, and it might therefore also be activity dependent. Activity-dependent enhancement could lead to a greater increase in excitability in those motor neurons that are fired by the US, which would tend to make the response to the CS resemble the response to the US in a nonassociative fashion. Activity-dependent increases in the excitability of neurons in cat motor cortex (33) may similarly contribute to the response specificity of eye-blink conditioning in that preparation (35). If this mechanism were combined with activity-dependent facilitation of the sensory neurons (which acts to amplify the magnitude of the response to the CS if it is paired with the US), these mechanisms together could account for the associative results we obtained in experiments 1–3, in which each animal was trained with one US. However, they could not easily
account for the results of experiment 4, in which each animal was trained with two USs, because (i) the nonassociative effects of the 2 USs would tend to cancel out, and (ii) the CS was paired with a US for all animals in that experiment.

Clark and colleagues (36, 37) have suggested that a third neuronal mechanism, branch-specific facilitation, might contribute to response specificity in conditioning of the withdrawal reflex (Fig. 2D). It has generally been assumed that all of the synapses of a given sensory neuron are facilitated equally ("cell wide" facilitation; Fig. 2A). However, Clark and Kandel (36) have found that central and peripheral synapses of the same sensory neuron can be facilitated differentially, and this might also occur for different central synapses. This mechanism would require specialized facilitator neurons for the different responses that can be learned. Several facilitators for the gill- and siphon-withdrawal reflex have now been identified, and they show some degree of topographical specialization (20, 38). An activity-dependent form of branch-specific facilitation could account for all of our results, including those from experiment 4. Finally, plasticity in interneurons in the polysynaptic component of the reflex might also contribute to response specificity (26). Interneuronal effects could include either Hebb-type plasticity (which has not been tested at interneuron synapses) or cell-wide activity-dependent facilitation of specialized interneurons.

These several hypotheses should be testable at the cellular level. Such tests may provide further insights into general psychological issues such as what determines the conditioned response and what the animal learns to associate in conditioning. The psychological issues may in turn suggest cellular experiments that reveal specific properties of neurons in the circuit for the withdrawal reflex that underlie the psychological phenomena.

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