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Transformation of siphon responses during conditioning of *Aplysia* suggests a model of primitive stimulus–response association

(associative learning/classical conditioning/neural model/pseudoconditioning)

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ABSTRACT **A semi-intact preparation was used to study the effects of classical conditioning on the type of siphon response elicited by a conditioned stimulus to the mantle of *Aplysia*. Five pairings of the conditioned stimulus with an unconditioned stimulus to nerves from the tail transformed the constricting alpha response of the siphon into a conditioned flaring response resembling the unconditioned response to stimulation of the tail nerves. Although some pseudoconditioning occurred, an associative component was indicated by the significantly greater incidence of flaring responses after paired training than after unpaired presentations of the conditioned and unconditioned stimulus or the unconditioned stimulus alone. Previously described cellular plasticity in the underlying neural circuits suggests a testable model based on cell-wide rather than synapse-specific mechanisms, which can account for specific conditioned responses. In this model, effective stimulus–response associations are produced by a concatenation of stimulus-specific facilitation of sensory neurons (a mechanism for alpha conditioning) and response-specific facilitation of motor neurons (a mechanism for pseudoconditioning).**

Activity-dependent neuromodulation of sensory neuron synapses has been proposed as a mechanism (ref. 1; see also ref. 2) underlying conditioning of siphon withdrawal in *Aplysia* (3–5). This mechanism provides a cogent explanation for the selective enhancement of a conditioned stimulus (CS) after the CS is paired with a strong unconditioned stimulus (US). A selective change in CS effectiveness could, in turn, account for quantitative changes in siphon withdrawal. Quantitative, pairing-specific modulation of preexisting behavioral responses to a CS represents perhaps the simplest form of classical conditioning—alpha (or type A) conditioning (6–8).

Until recently, it was assumed that there is only one type of siphon response and, therefore (since siphon withdrawal is elicited by the CS prior to conditioning) that pairing-specific changes in siphon responses only involve alpha conditioning (e.g., see ref. 8). However, we have found that the siphon displays qualitatively different responses, which are used to direct defensive secretions toward different sites of noxious stimulation (9). Two of these, constriction (elicited by anterior stimuli) and flaring (elicited by posterior stimuli), involve opposite movements and can easily be distinguished by using a photocell in a semi-intact preparation (ref. 10; see Fig. 1). Interestingly, an unsignaled US to the tail or head can pseudocondition siphon responses tested with midbody stimuli—i.e., change the preexisting (alpha) siphon response into a response resembling the unconditioned response (UR) of the siphon to the US (10). These observations raised the possibility that procedures used ostensibly for alpha conditioning of siphon withdrawal (3–5) might actually produce a qualitatively new conditioned response (CR). Earlier we found evidence for transformed CRs in the intact animal, using parapodial stimulation as a CS (11). Here I describe conditioning of a new siphon response to a mantle CS in a semi-intact preparation. Based on these results and current knowledge of sensory and motor facilitation in the siphon response circuit, I then suggest a model of primitive stimulus–response (S-R) association.

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MATERIALS AND METHODS

Aplysia californica (100–300 g) were supplied by Alacrity Marine Biological Services (Redondo Beach, CA) and kept in artificial seawater at 19°C–20°C. Experiments were conducted at 20°C–22°C between January and July 1988. A semi-intact preparation (Fig. 1*A*) allowed the use of a photocell for quantitative measurement of siphon responses (10). Constricting responses increased photocell exposure to a light beam, giving positive readings directly related to the degree of constriction (Fig. 1*B*). Conversely, flaring responses decreased photocell exposure, giving negative readings. Photocell readings were digitized and analyzed with an automated program (SPIKE; Hilal Associates, Englewood, NJ). A "net siphon response" was obtained by measuring the area under the curve of the change in photocell reading during the 20-sec period following each CS. The resulting values were normalized to the mean of the three pretest values. A major difference from the methods of Erickson and Walters (10) was that test stimuli were delivered to the mantle skin rather than to pedal nerves innervating the midbody region. This permitted the use of a CS and a protocol similar to that used by Carew and coworkers (4, 5). A differential procedure (5) was not used because of difficulty in obtaining similiar siphon responses from moderately separated test sites on the mantle and siphon. Indeed, there is a clear response gradient across the mantle/siphon, with stimulation of the anterior and right sides normally causing constriction and the posterior and left sides causing flaring responses (unpublished data).

The CS was a 0.5-sec train (10 Hz; 4–6 mA) delivered through an electrode implanted (2) in the region between the center of the mantle shelf and the anterior half of the ink gland. Stimulation of this region in 115 untrained preparations in several studies repeatedly caused alpha responses of pure constriction in 81%, mixed constriction and flaring in 14%, and pure flaring in 5% of the preparations. Preparations (n = 9) showing pure or mixed flaring responses to the CS during baseline were excluded from the present study. Experiments began at least 60 min after washout of isotonic $MgCl_2$ solution used for anesthesia during dissection. A CS was delivered every 30 min and, after the third pretest, the type of training

Abbreviations: CS, conditioned stimulus; CR, conditioned response; US, unconditioned stimulus; UR, unconditioned response; S-R, stimulus–response; S_{CS}, sensory neurons representing the CS; M_{AR}, motor neurons mediating the constricting alpha response; M_{UR}, motor neurons mediating the flaring UR.

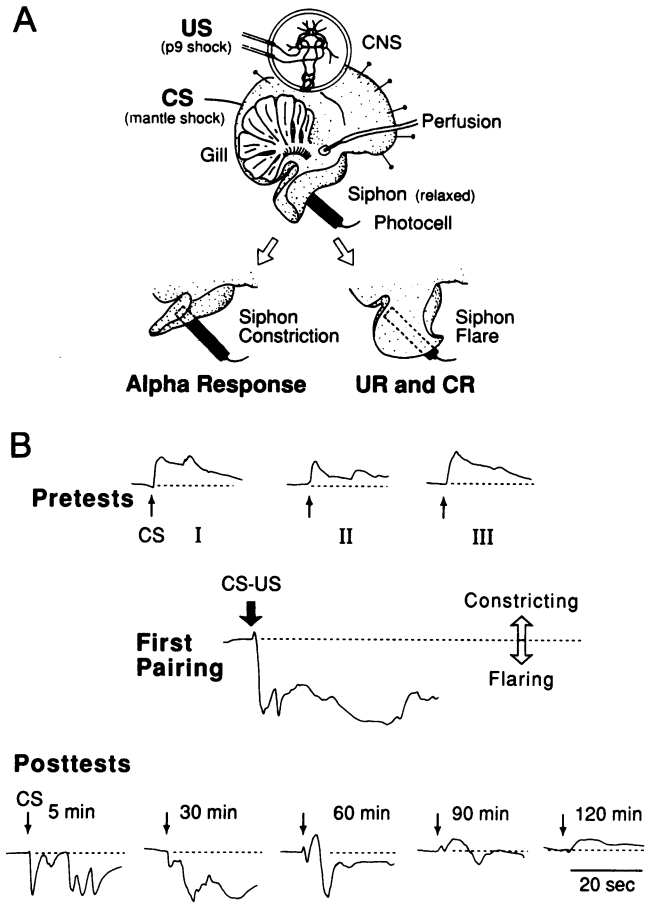

FIG. 1. (*A*) Semi-intact mantle organ and central nervous system (CNS) preparation (ventral side). The CS is delivered to the dorsal surface of the mantle (see text). The US is delivered to p9 nerves. Arrows indicate responses involved in conditioning. (*B*) Siphon responses monitored during conditioning. The 60- and 90-min posttests show mixed responses, with the former yielding a net flaring response (negative area) and the latter yielding a small net constriction (positive area) during the 20-sec test period.

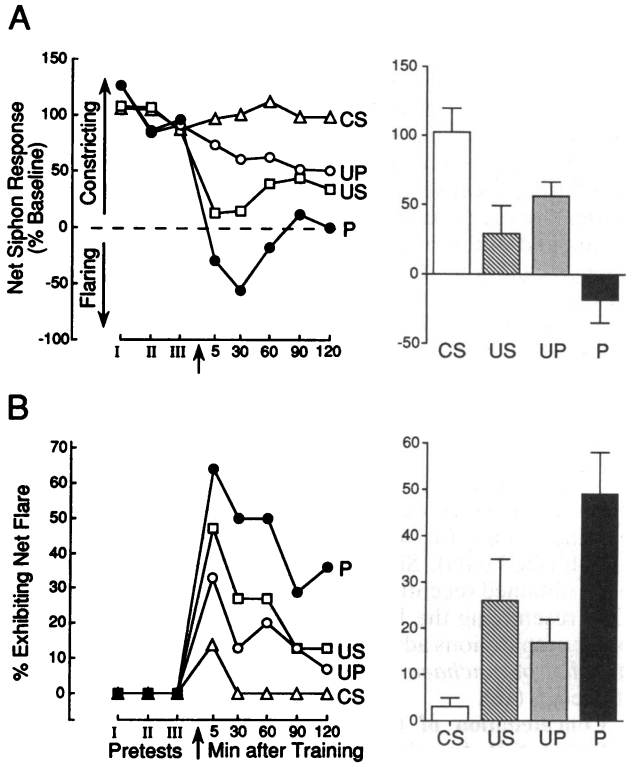

FIG. 2. Effects of training with paired (P; $n = 14$), unpaired (UP; $n = 15$), US alone (US; $n = 14$), and CS alone (CS; $n = 14$) protocols. (*Left*) Mean responses on each trial. (*Right*) Means of all five posttests. (*A*) Response topography indexed by net siphon responses. (*B*) Percentage of each group exhibiting CRs (net flaring responses).

was decided by coin flips. The US was a 1-sec train (25 Hz; 1–2 mA) through suction electrodes on both p9 nerves (which innervate the tail). This stimulus usually caused inking and a strong flaring UR (Fig. 1*B*) like that produced by noxious cutaneous stimulation of the tail (9, 10). Twelve preparations were excluded because p9 stimulation failed to produce flaring (possibly because of nerve damage during dissection). The paired group received the 1-sec US immediately after offset of the 0.5-sec CS on five training trials at 5-min intervals. The unpaired group received the CS either 2.5 min before or 2.5 min after each US. Other control groups were given five presentations of the US alone or the CS alone at 5-min intervals.

RESULTS

After paired training, the response of the siphon to the CS often changed from pure constriction to a predominantly flaring response—i.e., into a response qualitatively similar to the UR (Fig. 1*B*). The mean of the net siphon responses of the paired group ($n = 14$) was negative (flaring) on the 5-, 30-, and 60-min tests (Fig. 2*A*). Individual preparations in the unpaired ($n = 15$) and US alone ($n = 14$) groups also showed some flaring responses after training, but the positive means for these groups indicated that constriction continued to predominate (Fig. 2*A*). In the CS alone group 2 of 14 animals showed very weak flaring responses (near the noise level) on the 5-min posttest; all other responses were constricting. The near absence of flaring in the CS alone group indicates that flaring posttest responses in the other groups were an effect of the US. The mean of the five posttest responses for each animal (Fig. 2*A Right*) was used to compare net siphon responses in each group. A one-way analysis of variance showed an overall effect of training ($F_{3,53} = 6.58$; $P<0.001$). Multiple comparisons using the Student–Newman–Keuls test showed that the paired group was significantly different from each of the control groups ($P<0.05$), which were not significantly different from each other. This indicates a temporally specific association between the CS and a CR resembling the UR in the paired group.

Effects on response selection are perhaps shown most clearly as changes in the incidence of flaring CRs following training. This is plotted as the percentage of each group displaying net flaring responses on each test trial (Fig. 2*B Left*) and as the means of these percentages for all posttests (Fig. 2*B Right*). During the pretests, there were no net flaring responses in any of the animals accepted for the study. After training, the paired group displayed a significant increase in flaring response incidence compared to the pretest, but so did the US alone and unpaired groups (Wilcoxon test, $P<0.05$ in each case). All three groups exposed to the US also showed more flaring responses than did the CS alone group (Mann–Whitney U test; $P<0.05$ in each case). Differences of the US alone and unpaired groups from the CS alone group indicate that the US can pseudocondition siphon responses elicited by mantle stimulation, as it can with test responses to other stimuli (10). However, an additional associative effect was demonstrated by the significantly greater incidence of CRs in the paired group than in the unpaired and US alone groups (Mann–Whitney U test; $P<0.05$ in each case).

DISCUSSION

These results demonstrate that procedures similar to those used previously to condition siphon withdrawal in *Aplysia* (3–5) can change the type of siphon response elicited by a CS delivered to the mantle after only five training trials. The siphon response changes from a constricting alpha response to a flaring CR resembling the UR. Because constriction and flaring are incompatible responses involving opposite movements and different muscles (ref. 9; unpublished data), this change cannot be due to a simple quantitative enhancement of the alpha response. The transformed CRs may, in part, reflect a contribution of pseudoconditioning since the US alone and unpaired groups also showed increases in flaring responses. However, the fact that paired training produced significantly more CRs than did the other training procedures shows that acquisition of a CR resembling the UR is enhanced by pairing of the CS and US. This pairing-specific transformation is unlikely to be an artifact of the semi-intact preparation, since we have also found evidence in the freely moving animal for new CRs resembling the UR (ref. 11; unpublished data). Similar results in the intact animal have been obtained recently by Hawkins *et al.* (12). Acquisition of CRs resembling the UR has also been described in several other preparations advantageous for cellular analysis, including *Pleurobranchaea* (13), *Hermissenda* (14), *Limax* (15), and the leech (16).

Concatenation of Cell-Wide Plasticity May Produce an Effective S-R Association. Current cellular models of S-R association are dominated by "Hebb synapses" (17, 18), and a contribution of this form of synaptic plasticity to siphon response conditioning in *Aplysia* is an interesting possibility. However, the present results and earlier results in the intact animal (11) can also be explained parsimoniously by a quite different mechanism that takes into account known neural plasticity in *Aplysia* and evidence that plastic synapses in the siphon response circuit lack Hebbian properties (19). In this model of primitive S-R association (Fig. 3), mantle sensory neurons representing the CS (S_{CS}) initially have stronger connections to motor neurons mediating the constricting alpha response (M_{AR}) than to motor neurons mediating the flaring UR (M_{UR}). Thus, prior to conditioning (Fig. 3*A*), the CS elicits a constricting alpha response. Since constriction and flaring are mutually incompatible, the alpha response blocks simultaneous expression of the UR even though M_{UR} is also excited. The mechanism of mutual inhibition among siphon responses is not yet known.

During conditioning (Fig. 3*B*), the noxious US causes intense activation of M_{UR} and a strong flaring UR, which prevents simultaneous expression of the alpha response. The US also activates a modulatory system that causes general sensory facilitation. Activity-dependent amplification of facilitation in S_{CS} produces pairing-specific enhancement (1, 2) of the CS (i.e., the paired CS shows a larger increase in effectiveness than do unpaired stimuli). Because both sensory activity and US-evoked neuromodulatory effects appear to spread to most of the sensory neuron (increasing synaptic release and general excitability), the specificity of this mechanism is encoded at the level of the whole cell, rather than the individual synapse (cf. refs. 1, 2, and 20–23, but see ref. 24). Consequently, all responses to the CS are enhanced similarly by this mechanism: it provides stimulus specificity but not response specificity. This argument suggests that activity-dependent sensory facilitation cannot account for all features of conditioning in *Aplysia* (see also ref. 25 and 26).

The US produces another facilitatory effect that is response specific rather than stimulus specific. Stimulation of the tail or p9 nerves increases (facilitates) the tonic firing rate of siphon motor neurons (M_{UR}), which mediate the flaring UR, with little or no alteration of motor neurons (e.g., M_{AR}) producing other siphon responses (ref. 27; unpublished observations). The selectivity mechanism is unknown. Two possibilities are (*i*) selective distribution of US-evoked facilitatory modulation to motor neurons mediating the UR (Fig. 3*B*), or (*ii*) activity-dependent modulation (1, 2) of siphon motor neurons activated by the US. The selective and persistent enhancement of motor neuron firing rate produces neuromuscular facilitation (27, 28). This response-specific facilitation of the output of M_{UR} increases the probability that previously subthreshold stimuli will evoke the UR. Response-specific facilitation may also enhance motor neuron excitability, as occurs in ink and opaline motor neurons (29, 30). Both mechanisms (increased output and increased input

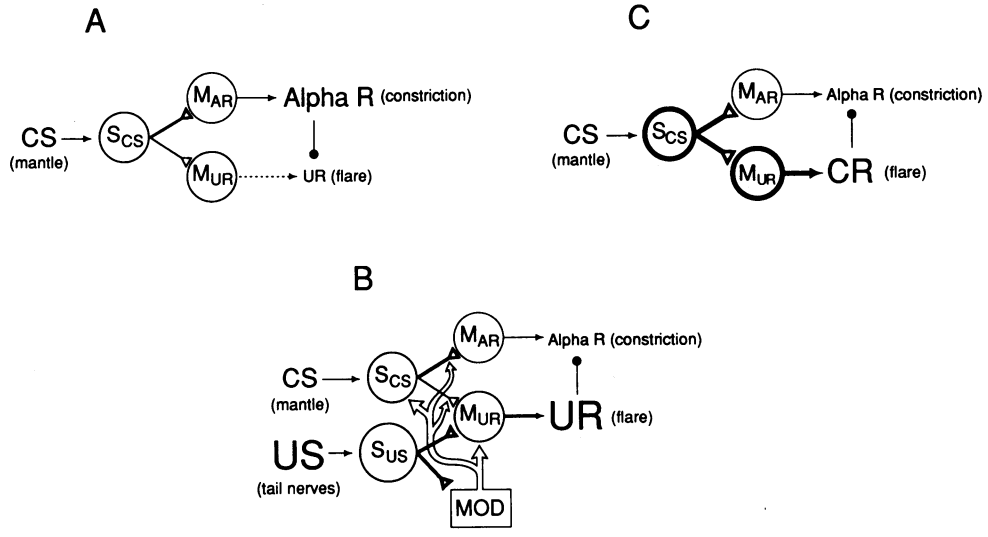

FIG. 3. Concatenation model of S-R association. S, sensory neuron; M, motor neuron, MOD, modulatory interneurons. (*A*) Before conditioning, the connection from S_{CS} to M_{AR} is stronger than the connection to M_{UR}. The CS evokes the alpha response, which inhibits (●) incompatible responses such as the UR. (*B*) During conditioning, S_{CS} undergoes activity-dependent neuromodulation, and M_{UR} (but not M_{AR}) is also facilitated. Neuromodulation of S_{US} (22) is not important for this model and is not shown. (*C*) After conditioning, concatenation of CS-specific sensory facilitation and UR-specific motor facilitation results in preferential facilitation of signals from S_{CS} to M_{UR}. Facilitated signaling strength (input sensitivity and output) is indicated by thickened lines. With sufficient pre- and postsynaptic facilitation, S_{CS} excites M_{UR} more than M_{AR}, and the CS evokes a CR resembling the UR (which inhibits the alpha response).

sensitivity of M_{UR}) will selectively enhance the flaring response mediated by M_{UR}, and this enhancement will be generalized across all stimuli that excite M_{UR}, thus accounting for the observed pseudoconditioning (10).

I propose that effective S-R associations can result from a concatenation of stimulus-specific sensory facilitation and response-specific motor facilitation. Activity-dependent enhancement of S_{CS} signaling during training causes the CS to show the largest increase in effectiveness in eliciting the UR. Selective facilitation of M_{UR} during training causes the UR to show the largest increase in responsiveness to the CS. Thus, the connection between the CS and UR is preferentially strengthened by independent pre- and postsynaptic facilitation that is cell specific rather than synapse specific. Simulations of this monosynaptic model and of more complex networks have confirmed that cell-wide facilitation of sensory and motor neurons can lead to a newly effective S-R link (31). S-R associations produced by this mechanism are particularly clear when the alpha response and the UR are mutually incompatible motor actions, such as flaring and constriction of the siphon (Fig. 1).

Like many others (e.g., refs. 6, and 32–34), this model builds a basic form of learning from elementary units of neural plasticity that are shared in different combinations with other forms of learning. In this case, a S-R association is formed by concatenating putative mechanisms of alpha conditioning and pseudoconditioning (11). The actual contribution of the concatenation mechanism and of other potential mechansims, such as Hebb synapses and branch-specific neuromodulation of S_{CS} synapses to M_{UR} (10, 24, 27), can now be explored intracellularly in the same semi-intact preparation (Fig. 1*A*) used to obtain the behavioral results.

Potential Scope of Cell-Wide Associative Mechanisms. Changes in cell excitability following behaviorally important events have been described in a wide range of animals, including invertebrates (20–23, 27, 29, 35–38) and vertebrates (39, 40). This suggests that cell-wide facilitation, which might contribute to associative effects, may be widespread. In simple S-R systems, synapse-specific associative mechanisms may be less developed (e.g., ref. 19), and associations may depend largely on cell-wide mechanisms. A limitation of a cell-wide mechanism, however, is that it cannot readily store multiple S-R associations (i.e., linking several CSs with different CRs) in the same network without losing CS and CR specificity. In contrast, synapse-specific associative mechanisms, which have been implicated in mammalian hippocampus (e.g., ref. 41), should allow a number of specific associations to be stored in the same network. Thus, if both occurred in a brain, it seems likely that the two types of associative mechanism would have complementary rather than identical functions. For example, concatenation of sensory and motor facilitation might ensure that effective responses to important CSs are acquired rapidly, albeit with partial CS and CR specificity. These crude associations might then be supplanted by more precise, synapse-specific associations after additional CS–US pairings. By enhancing activity along particular S-R pathways early in training, concatenation of cell-wide facilitation mechanisms might also guide and accelerate the formation of associations by more specific activity-dependent mechanisms, such as Hebb synapses. It will be interesting to look for both kinds of mechanism in circuits controlling defensive behavior of *Aplysia*.

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