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3. Animals were injected with isotonic $MgCl_2$ (50 percent of body weight), and the central nervous system was surgically exposed (*2*). In studies of siphon and ink motor neurons, the mantle organs were isolated from the body except for a connection through the pleuroabdominal connectives between the abdominal ganglion and the rest of the central nervous system. After surgery, the $MgCl_2$ was cleared by perfusing artificial seawater through the aorta for at least 1 hour.
4. Parameters of this stimulus were the same as in the behavioral studies (*1*).
5. These data do not rule out the possibility that the CS might induce changes in premotor neurons in the locomotor system of paired animals.
6. We recorded from ink motor neurons L14A or L14B [T. J. Carew and E. R. Kandel, *J. Neurophysiol.* **40**, 692 (1977)] and a novel class of siphon motor neurons that are uniquely sensitive to tail stimulation. Sometimes both classes of cells were examined simultaneously. Fifteen ganglia were examined, seven paired and eight unpaired. Siphon motor neurons were hyperpolarized to permit measurement of the complex excitatory postsynaptic potential (EPSP).
7. Weak electrical shocks (approximately 1 mA) were applied through thin wires implanted in the tail. Each train was approximately 2 seconds long, with 2-msec pulses (6 Hz).
8. The EPSP's were traced, cut out, and weighed to obtain a measure of EPSP area (a combined measure of amplitude and duration). The weights of EPSP tracings were then compared.
9. Preliminary results suggest that *Aplysia* receiving unpaired training may learn a form of conditioned inhibition—the CS becomes a "safety signal" rather than a "danger signal"—and thus the CS may inhibit defensive responses in these animals (E. T. Walters, T. J. Carew, E. R. Kandel, in preparation).
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11. Using electrical stimulation of the posterior pedal nerve (which innervates the tail) to produce a complex EPSP in the siphon and ink motor neurons, we found that 35 to 50 percent of the EPSP remained with no change in latency when the threshold of interposed interneurons was elevated by bathing the central nervous system in a solution containing high concentrations of Ca^{2+} and Mg^{2+} ions.
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11 August 1980; revised 21 November 1980

Associative Learning in *Aplysia*: Evidence for Conditioned Fear in an Invertebrate

Abstract. *Aversive classical conditioning of* Aplysia californica, *a gastropod mollusk suited for neurobiological study, produces a learned reaction to the chemosensory conditioned stimulus that is expressed as a marked facilitation of four defensive responses: two graded reflexes (head and siphon withdrawal), an all-or-none fixed act (inking), and a complex fixed action pattern (escape locomotion). In addition, the conditioned stimulus produces a concomitant depression of at least one appetitive response, feeding. These extensive and selective actions of the conditioned stimulus in* Aplysia *resemble the actions of conditioned fear stimuli in higher mammals and suggest that the functional equivalent of fear occurs in invertebrates and thus may be an adaptive mechanism that is widespread in the animal kingdom.*

It has recently proven possible to analyze the cellular mechanisms of nonassociative forms of learning in several simple invertebrate preparations (*1*). The apparent generality of these mechanisms (*2*) has in turn encouraged the development of invertebrate models for the study of mechanisms of associative learning. Four independent studies have now shown that aversive associative learning occurs in gastropod mollusks, animals that offer many advantages for cellular neurobiological studies (*3, 4*). However, an analysis of the underlying neuronal mechanisms and an assessment of the generality of these mechanisms will require an understanding of what the animals actually learn during aversive associative conditioning. In particular, one needs to know how the consequences of aversive conditioning are organized in these animals and the extent to which these effects resemble those seen in mammals after aversive conditioning.

Recently we found (*4*) that *Aplysia californica* can rapidly acquire a temporally specific aversive reaction to a chemosensory conditioned stimulus (CS) paired with a noxious unconditioned stimulus (US). This learned reaction was expressed as a facilitation of escape locomotion triggered in the presence of the CS. Because the CS did not elicit obvious conditioned responses after training, we proposed that the animals might have learned to associate the CS not with specific motor responses but instead with a central defensive state that modulates escape locomotion. To test this interpretation, we have examined the effect of the CS on four defensive responses: two graded reflex acts (head and siphon withdrawal), an all-or-none fixed act (inking), and a complex fixed action pattern (escape locomotion). In addition we have examined the effects of the CS on an appetitive behavior, feeding. We have found that aversive conditioning in *Aplysia* produces a conditioned internal state which appears functionally equivalent to conditioned fear in mammals.

One hundred and seventeen *A. californica* were studied. Animals were trained according to procedures previously described (*4*). Paired animals received the CS (shrimp extract for 90 seconds) 1 minute before the onset of the US (electric shock to the head for 30 seconds) (*5*). Unpaired controls received the same CS 90 minutes after the US. In the experiments shown in Fig. 1, paired (N = 17) and unpaired (N = 16) animals received nine trials, three trials per day, with an intertrial interval of 3 hours. During training, all animals responded to the US with maximal withdrawal of the head; secretion of ink, opaline, and mucus; siphon withdrawal; turning away from the shock, and (after a delay of several minutes) escape locomotion. Animals were then tested 1 and 2 days after the last day of training (*6*). In each test session, the CS was delivered to the head and left in the chamber for the entire test period (6 minutes). In all tests, the observer was unaware of the identities of the animals.

We first examined head withdrawal, the only overt response elicited by the CS after conditioning. We had not previously seen significant differences between paired and unpaired animals in the incidence of CS-evoked head withdrawals after conditioning (*4*), and we had observed that the CS produced weak withdrawals in untrained animals. To see if conditioning might produce a difference in the intensity of head withdrawal to the CS, we rated the magnitude of each withdrawal on a three-point scale: strong, weak, or no withdrawal. Significantly more paired animals withdrew strongly from the CS than unpaired animals did (Fig. 1A) (Fisher exact probability test, $P < .01$). Similar differences were noted in separate experiments (*7*) (N = 12 per group) in which the amplitude of head withdrawal [in a restrained preparation (*8*)] was measured with a tension transducer. Thus, although an unconditioned head withdrawal response to the CS can be seen in both untrained and unpaired animals, aversive conditioning significantly facilitates the amplitude of this withdrawal in animals receiving paired training.

Other defensive responses do not seem to be directly elicited by the CS after training, but the effects of conditioning can be seen on these responses when test stimuli are used to trigger them

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in the presence of the CS. We tested the effect of the CS on siphon withdrawal by applying weak shock to the tail (*9*). The duration of withdrawal was measured from the offset of tail shock until the siphon reappeared above the parapodia. The duration of siphon withdrawal was significantly greater in paired than in unpaired animals (Fig. 1B) [$U(17, 16) = 38$, $P < .005$]. Similar significant differences have also been obtained in three separate experiments (combined $N = 30$ per group).

To examine the effect of the CS on defensive inking behavior we applied a series of progressively stronger shocks to the tail (*10*). Inking sensitivity was measured as the reciprocal of the threshold (in milliamperes) for ink release. The median sensitivity for ink release was significantly greater in paired animals than in unpaired animals (Fig. 1C) [$U(12, 12) = 15$, $P < .005$]. Similar results were found in other experiments (paired $N = 15$, unpaired $N = 12$) in which the test shocks were applied to the side of the animal rather than to the tail (*11*).

The results indicate that the CS facilitates two graded defensive reflexes and an all-or-none defensive act. We showed previously that aversive conditioning enhances escape locomotion, a complex fixed action pattern (*4*). Confirming these earlier findings, in this experiment the amount of escape locomotion elicited by weak tail shock (*9*) in the presence of the CS was significantly greater in paired animals than in unpaired animals (Fig. 1D, [$t(31) = 3.81$, $P < .005$].

In addition to these defensive responses, we also examined the effect of the CS on an appetitive response, feeding. To maintain a high level of feeding motivation, all animals were deprived of food for 3 days. The test stimulus was a narrow strip of seaweed, which was gently brought into contact with the chemosensitive rhinophores, a procedure that causes the animal to lift its head and orient toward the food (*12*). When the animal lifted its head, the experimenter transferred the seaweed to the lips, evoking biting responses. The latencies from contacting the rhinophore with the seaweed to lifting the head and to the first bite were recorded, as were the interbite intervals for ten bites. In order to display depressive effects clearly, we have analyzed the data in terms of the speed (reciprocal of latency) to the first response. After training, the CS significantly depressed the speed to lift [$U(17, 16) = 83$, $P < .05$] and the speed to the first bite [$U(17, 16) = 84$, $P < .05$] (Fig. 1E) in paired, compared with unpaired, animals. No significant differences were found in biting frequency after the initial bite.

These results show that aversive conditioning in *Aplysia* produces a learned reaction to the CS that is characterized by three features. (i) Conditioning has widespread effects, significantly influencing at least five separate behavioral responses. (ii) The effects of conditioning are selective: Whereas defensive responses are enhanced, at least one appetitive response is depressed. (iii) The expression of the conditioning depends largely upon modulatory mechanisms; the effects of training are seen most clearly in the alteration by the CS of the magnitude of responses elicited by test stimuli. In each of these features, aversive learning in *Aplysia* resembles that in mammals. Taken together, our observations indicate that *Aplysia*, like vertebrates, can learn to associate the CS with

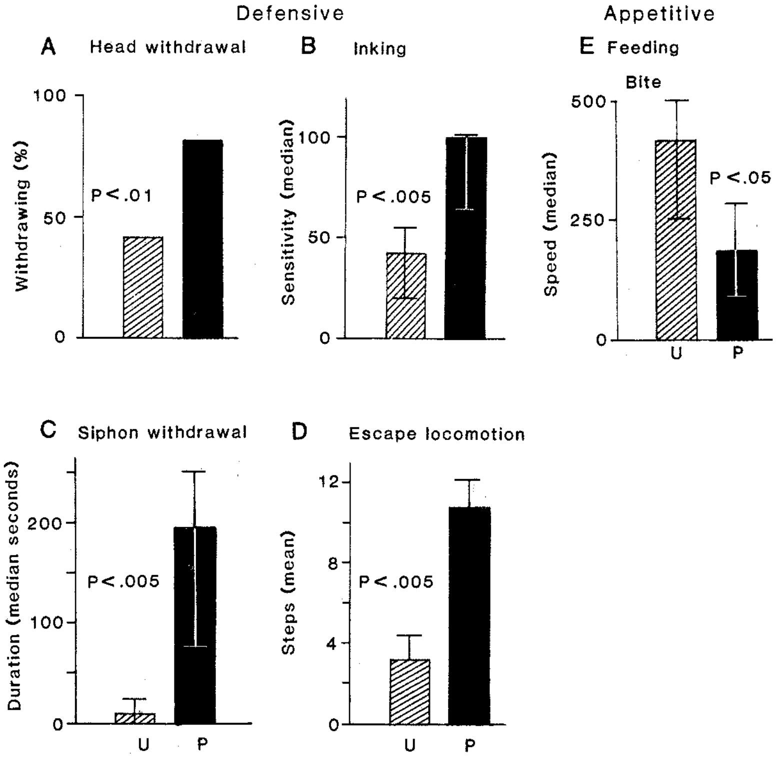

Fig. 1. Effects of the CS on various responses after aversive conditioning. In each part, *U* refers to unpaired animals ($N = 16$) and *P* to paired animals ($N = 17$). These data are from multiple tests of the same animals after nine training trials. All test stimuli were applied after delivery of the CS. (A to D) Defensive responses. (A) The percentage of animals in each group exhibiting strong withdrawals in response to CS delivery in the first test (*14*). (B) The median duration of siphon withdrawal in response to weak tail stimulation. (C) Median sensitivity of ink release in response to a series of progressively stronger shocks to the tail. Sensitivity is expressed as the reciprocal of the threshold (in milliamperes) $\times 10^4$. (D) The mean number of steps (+ standard error) in response to weak tail stimulation. (E) Appetitive response (feeding). Median speed to take the first bite after stimulation of the head with seaweed. Speed is defined as the reciprocal of the latency to bite and is expressed as the reciprocal of the latency (in seconds) $\times 10^4$. Medians are used for measurements subject to a ceiling and are shown ± interquartile ranges.

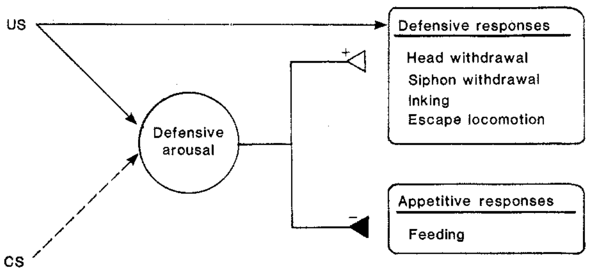

Fig. 2. A model of conditioned fear in *Aplysia*. The noxious US, in addition to eliciting an ensemble of motor responses, produces defensive arousal, a central state that selectively facilitates (open triangle) defensive responses and inhibits (filled triangle) appetitive responses. An initially neutral chemosensory CS, when paired with the US, is proposed to become capable of eliciting this same defensive state. The mechanisms by which this state is expressed are not yet known and might involve peripheral competition between responses or interactions among motor systems as well as the central modulatory actions suggested.

a defensive arousal state, which organizes the animal's behavior for defensive action. This central arousal state [which also appears to be elicited transiently by the US (*13*)] is expressed as a priming of defensive behaviors and a concomitant depression of behaviors incompatible with defense, such as feeding. A model summarizing these results and incorporating previous findings on the effects of the US (*13*) is shown in Fig. 2.

The classically conditioned central state in *Aplysia* resembles states in mammals that have been defined operationally as conditioned fear (*14*). Indeed, the conditioned state in *Aplysia* satisfies functional definitions of fear as a general, preparatory defensive state elicited by stimuli signaling imminent danger (*15*). That such a state can be conditioned in an invertebrate indicates that the capability to anticipate danger and prepare for flexible defensive action is of considerable adaptive value and may be widespread in the animal kingdom. In addition, the discovery of a fearlike state in a simple invertebrate whose nervous system is amenable to neurobiological analysis may permit the use of cellular approaches to explore aspects of aversive learning not accessible to study with behavioral techniques alone. In the accompanying paper, we describe experiments in *Aplysia* in which we begin to examine aversive associative learning on the cellular level (*8*).

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5. The US was a 30-second train of 450-mA (peak-to-peak) a-c (60-Hz) current pulses applied through Ag/AgCl electrodes spanning the head. Each pulse in the train was 1.5 seconds long; interpulse interval was 3 seconds.
6. Tests were given in the following sequence: (i) head withdrawal and feeding, 18 hours after training; (ii) siphon withdrawal and escape locomotion, 22 hours after training; and (iii) inking, 42 hours after training.
7. Animals were tested in the semi-intact preparation used for neuronal studies (*8*). Head withdrawal in paired animals was significantly greater than that in unpaired animals [$t(21) = 3.63$, $P < .005$].
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9. A 15-second train of 45-mA a-c (60-Hz) current pulses applied through bipolar electrodes spanning the tail. Pulse duration was 1.5 seconds and interpulse interval, 3 seconds.
10. A series of 1.5-second shocks (a-c, 60 Hz) were applied at 15-second intervals through Ag/AgCl electrodes spanning the tail in the sequence 50, 100, 150, 200, 250, 300, 350, and 700 mA.
11. Test shocks were applied at 60-second intervals in the sequence 20, 40, 60, 80, and 100 mA through thin capillary electrodes (filled with seawater) held in contact with the side body wall. Again, the median inking sensitivity was greater in paired animals than in unpaired animals [$U(15, 12) = 35$ ($P < .01$].
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11 August 1980; revised 21 November 1980

Suckling Infant Rats Learn a Preference for a Novel Olfactory Stimulus Paired with Milk Delivery

Abstract. *When presented a novel olfactory stimulus while suckling a passive dam, 11- to 14-day-old rat pups acquire a conditioned preference for that stimulus. The magnitude of the conditioned preference is greater if the pups received milk while suckling than if they did not. The results indicate that infants are capable of learning while suckling and that milk delivery plays a role in this associative process.*

Freud believed infants capable of learning that milk comes from the mother and thought that this was one of the more important reasons the infant sought to maintain proximity with her (*1*). This position has received some support over the years (*2*), but many have criticized it because there has been no clear demonstration that the infant associates the reinforcing properties of the milk it receives while suckling with any stimulus in the environment, including the mother (*3*). I now report what is to my knowledge the first demonstration in the suckling mammalian infant of learning based on milk reward and the transfer of that learning to a situation removed from the suckling environment.

Rat pups are capable of learning a remarkable variety of responses (*4*) but the only attempt to demonstrate that infant rats can learn while suckling the mother was that of Martin and Alberts (*5*). They found that pups suckling a passive dam were unable to learn an illness-mediated aversion to flavored milk unless they were of weanling age, even though much younger pups learn this same aversion if they received the flavored milk when they were not suckling the mother. However, suckling, even in the absence of milk, is rewarding in its own right, so that any experimentally arranged negative contingency might be overshadowed by the inherently positive value of suckling itself. Thus, a question of more immediate importance becomes whether an infant rat might learn a preference for stimuli present in the environment when it suckles and whether mother's milk plays any role in this learning.

The study consisted of two phases. In

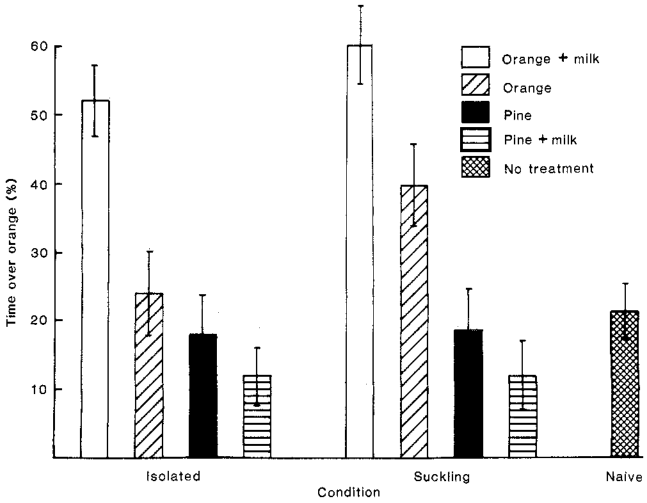

Fig. 1. Mean percentage of time spent in the presence of orange-scented shavings by pups conditioned while isolated or suckling, and by naïve pups.