show that the distribution of some human satellite sequences is chromosome specific (*18*). However, the method we have used is simpler and can be used to screen much larger numbers of individuals and chromosomes.

Several lines of investigation are suggested by the results of this study. First, restriction enzymes might produce chromosome banding and permit identification of all of the individual chromosomes in the genomes of such diverse organisms as amphibia, fish, and plants lacking easily banded chromosomes. Second, the distribution of subclasses of satellite DNA could be studied in species other than the human. Mouse satellite DNA is more nearly homogeneous than human satellite DNA, but certain restriction enzyme sites are present in only a fraction of the repeating units (*19*). Such variant sequences could either be concentrated on one or a small number of chromosomes (similar to the human situation) or be present on every chromosome. Information of this kind is important for understanding the evolution of repetitive DNA's. Third, the finding that regions containing amplified rRNA genes in human and rat chromosomes can be revealed by specific cutting with the enzyme Msp I suggests that cutting with one or more restriction enzymes might reveal regions containing other amplified genes; for example, the well-known homogeneously staining regions (HSR's) and double minutes (DM's) seen in many cancers and in drug-resistant cell lines. Finally, the effects on chromosomes exposed to these enzymes before fixation remain to be explored.

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30 August 1982; revised 29 November 1982

Differential Classical Conditioning of a Defensive Withdrawal Reflex in *Aplysia californica*

Abstract. *The defensive siphon and gill withdrawal reflex of* Aplysia *is a simple reflex mediated by a well-defined neural circuit. This reflex exhibits classical conditioning when a weak tactile stimulus to the siphon is used as a conditioned stimulus and a strong shock to the tail is used as an unconditioned stimulus. The siphon withdrawal component of this reflex can be differentially conditioned when stimuli applied to two different sites on the mantle skin (the mantle shelf and the siphon) are used as discriminative stimuli. The differential conditioning can be acquired in a single trial, is retained for more than 24 hours, and increases in strength with increased trials. Differential conditioning can also be produced within the field of innervation of a single cluster of sensory neurons (the LE cluster) since two separate sites on the siphon skin can serve as discriminative stimuli. The finding that two independent afferent inputs that activate a common set of interneurons and motor neurons can be differentially conditioned restricts the possible cellular loci involved in the associative learning.*

In classical conditioning, an animal learns to associate two stimuli by the specific temporal relationship between them. Conditioning is thus thought to represent a prototypical example of the learning of causal relationships by animals and humans. Although classical conditioning is well understood behaviorally (*1*), the cellular mechanisms that underlie conditioning are still unknown, in part because of the difficulty of cellular studies in vertebrates, where conditioning has predominantly been examined. A potentially important advance in the analysis of the cellular mechanisms of associative learning was therefore achieved when it was demonstrated that a variety of complex behaviors in higher invertebrates show classical conditioning (*2*), including cognitive features of learning once thought to be exclusively mammalian (*3, 4*). Classical conditioning was recently demonstrated in a simple reflex, the gill and siphon withdrawal reflex in *Aplysia* (*5*), which is mediated by a well-delineated neural circuit (*6*). We now report that the siphon withdrawal component of this reflex is capable of differential conditioning with discriminative stimuli applied to two different sites on the mantle skin—the mantle shelf and the siphon. The demonstration that differential conditioning can occur in an elementary withdrawal reflex mediated by a small number of cells indicates that this more advanced form of associative learning is not an exclusive feature of behaviors having complex neural circuitry. Moreover, the finding that two independent afferent inputs, each of which activates a common set of motor neurons and some common interneurons (*6*), can be differentially conditioned restricts the possible cellular loci for the associative changes. Finally, differential conditioning allows each animal to serve as its own control and thereby provides a useful behavioral tool for analyzing classical conditioning on a cellular level (*7*).

In a previous study of classical conditioning of the siphon and gill withdrawal reflex, Carew, Walters, and Kandel (*5*) used a light tactile stimulus to the siphon, which produces a weak siphon and gill withdrawal, as the conditioned stimulus (CS) and a strong electric shock to

the tail, which produces a strong withdrawal reflex, as the unconditioned stimulus (US). Specific temporal pairing of the CS and US significantly enhanced subsequent siphon and gill withdrawal in response to the CS. To examine whether the siphon withdrawal component of this reflex could be differentially conditioned, we used two CS's. One CS (CS+) was paired with the US, the other (CS−) was specifically unpaired. Conditioning was assessed in each animal by comparing the responses to the CS+ and CS− after training. The specific protocol we have used is illustrated in Fig. 1, A and B (*8*). We applied two weak CS's: one, a light tactile stimulus to the siphon, the other a weak electrical shock to the mantle shelf (*9*), and measured the duration of siphon withdrawal to each CS using standard blind procedures (*10*). The experiments consisted of three phases: (i) a pretest, (ii) training, and (iii) one or more retention tests after training. In the pretest, the duration of siphon withdrawal was measured for each animal, first in response to the siphon CS and then to the mantle CS. For training, animals were matched on the basis of their pretest scores and assigned to one of two groups (Fig. 1B): the siphon-paired group ($N = 12$) received the siphon CS specifically paired with the US (tail shock) and the mantle CS specifically unpaired; the mantle-paired group ($N = 12$) received the mantle CS paired and the siphon CS unpaired. For paired presentations of stimuli, the US was delivered 0.5 second after CS+ onset; for unpaired presentations, the CS− was delivered 2.5 minutes after the US. The intertrial interval was 5 minutes (*11*).

If *Aplysia* is capable of differential conditioning, the siphon-paired group should show greater responses to the siphon CS than to the mantle CS, and the mantle-paired group should show the opposite. This prediction was confirmed (Fig. 1C). Thirty minutes after training, animals receiving 15 siphon-paired training trials showed significantly longer siphon-withdrawal responses to the siphon CS than to the mantle CS (mean times of 43 and 27.4 seconds, respectively; $P < .05$) (*12*). In contrast, animals receiving mantle-paired training showed significantly longer responses to the mantle CS than to the siphon CS (means, 49.4 and 10.2 seconds, respectively; $P < .01$). As before (*5*), paired presentations of the siphon CS with the US during training produced significantly greater responses than unpaired presentations ($P < .005$, comparing responses to the siphon CS in siphon-paired and mantle-paired animals). The same result held for paired and unpaired presentations of the mantle CS ($P < .005$, comparing responses to the mantle CS in mantle-paired and siphon-paired animals). Thus, each CS pathway is competent to undergo classical conditioning. Moreover, the degree of conditioning in response to each CS is comparable. A between-group comparison of the mean differences between test and pretest scores for each pathway revealed that paired responses from both the siphon and the mantle increased by a significantly greater amount than respective unpaired responses ($P < .005$ in each case). Furthermore, there was no significant difference in the magnitude of this difference between the two pathways (mean difference: siphon, 30.2 seconds and mantle, 22.5 seconds; $t_{22} = .84$). Thus, under our training conditions, not only is each CS pathway competent to produce classical conditioning, but the two pathways are also of approximately equivalent efficacy.

Since each animal provides its own (unpaired) control, data from the paired and unpaired pathways can be pooled. The results from all CS+ pathways can be combined (siphon scores from siphon-paired and mantle scores from mantle-paired animals), and compared to those from the CS− pathway (mantle scores from siphon-paired and siphon scores from mantle-paired) and to their own pretraining scores (Fig. 1D). This comparison illustrates that the unpaired (CS−) pathway exhibits significant sensitization (*13*) (means of 10.8 and 18.9 seconds, respectively, for CS− pretest and test scores; $P < .01$) and that the paired pathway exhibits significant differential conditioning. The latter conclusion is supported by two comparisons. First, the CS+ pathway elicits significantly greater responses than the CS− pathway (means, 46.7 and 18.9 seconds, respectively, $P < .005$); and second, the difference between the CS+ response and its pretest score is significantly greater than the difference between the CS− response and its pretest score (means, 34.8 and 8.1 seconds, respectively, $P < .005$). The latter comparison also shows that the associative effect (classical conditioning) exhibited by the CS+ pathway is significantly greater than the nonassociative effect (sensitization) exhibited by the CS− pathway. Together these results show that even a

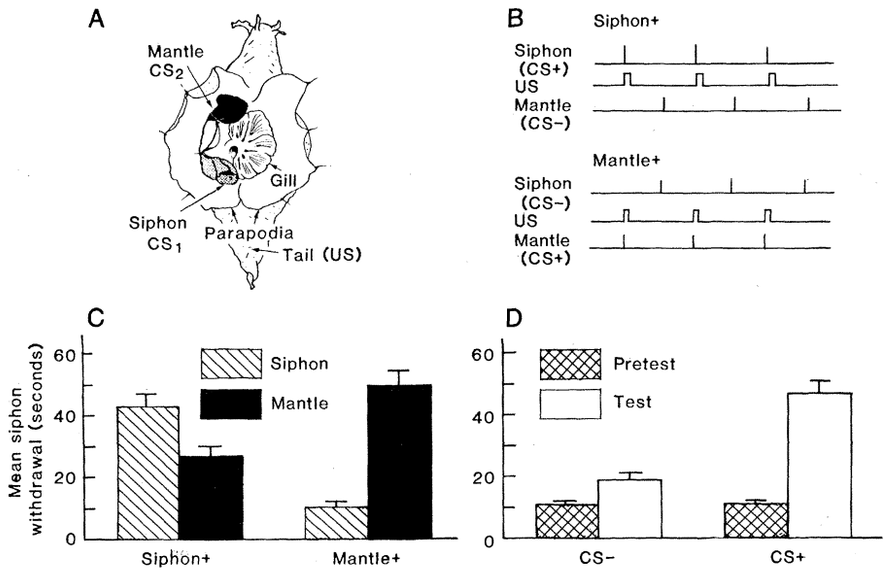

Fig. 1. (A) Dorsal view of *Aplysia* illustrating the two sites used to deliver conditioned stimuli: the siphon and the mantle shelf. The unconditioned stimulus (*US*) was an electric shock delivered to the tail. For illustrative purposes, the parapodia are shown intact and retracted. However, the behavioral studies were all carried out in freely moving animals whose parapodia were surgically removed (*8*). (B) Paradigm for differential conditioning: one group (*Siphon+*) received the siphon CS (*CS+*) paired with the US and the mantle CS (*CS−*) specifically unpaired with the US; the other group (*Mantle+*) received the mantle stimulus as CS+ and the siphon stimulus as CS−. The intertrial interval was 5 minutes (*11*). (C) Results of an experiment using the paradigm of (B). Testing was carried out 30 minutes after 15 training trials. The *Siphon+* group ($N = 12$) showed significantly greater responses ($P < .05$) to the siphon CS than to the mantle CS, whereas the *Mantle+* group ($N = 12$) showed significantly greater responses ($P < .01$) to the mantle CS than to the siphon CS. Data in this and all other figures are expressed as means ± standard error of the mean. (D) Pooled data from (C). Tests scores from the unpaired (CS−) and paired (CS+) pathways are compared to their respective pretest scores (see text for details of pooling). The CS+ test scores are significantly greater than the CS− test scores ($P < .005$), demonstrating that differential conditioning has occurred.

simple behavior, the siphon withdrawal reflex of *Aplysia*, can be differentially conditioned.

We next determined the number of trials necessary to produce differential conditioning. We exposed different groups of animals to 1, 5, or 15 trials and tested them twice: once within 1 hour after training and again 1 day after training. In each experiment, half the animals received siphon-paired training and the other half received mantle-paired training (Fig. 1B). We found significant differential conditioning after a single training trial, both when tested immediately and 24 hours later ($P < .005$ in each case). The differential effect (the difference between the response to the CS+ and the CS−) increased with more training trials [the mean at 24 hours was 4.8 seconds for one trial ($N = 21$), 9.8 seconds for five trials ($N = 23$), and 45.3 seconds for 15 trials ($N = 24$)] (Fig. 2A). A comparison of test and pretest scores for the CS− pathway illustrates that sensitization also increases with more training trials, since it does not appear after one training trial (*14*), but is evident after five trials and is more developed after 15 trials (Fig. 2A). Also, as in previous experiments (*4*), after 5 and 15 trials, both sensitization and differential conditioning were more developed 24 hours after training than within 1 hour after training (*15*). Thus our data show that the magnitude of both sensitization and differential conditioning is affected by two variables: (i) the number of trials used in training and (ii) the time between training and testing. Preliminary experiments indicate that there is significant retention of differential conditioning 3 days after a single training trial.

To further study the relationship between sensitization and differential conditioning, we carried out experiments in which no CS− stimulation was delivered to the control pathway during training, so that this pathway served as a US-alone control (*16*). We found that the pathway receiving the US-alone exhibited sensitization 24 hours after five training trials ($N = 24$, $P < .005$, comparing test and pretest scores, having means of 15.2 and 11.7 seconds, respectively). Moreover, the paired pathway exhibited differential conditioning ($P < .005$, comparing CS+ and US-alone scores, whose means were 24.5 and 15.2 seconds, respectively). Finally, the difference between the CS+ pathway and its pretest score was greater than the difference between the US-alone pathway and its pretest score ($P < .005$), indicating once again that the associative effect of conditioning was significantly greater than the nonassociative effect of sensitization.

Our results indicate that two separate afferent pathways can mediate differential conditioning. The two afferent inputs are carried into the abdominal ganglion by two independent clusters of identified mechanosensory neurons: the LE cluster from the siphon and the RE cluster from the mantle (*17*). The fact that differential conditioning can be established by the activation of two populations of sensory neurons that have similar mechanoreceptive properties led us to try to differentially condition subpopulations of sensory neurons within a single cluster. We stimulated two separate sites on the siphon skin (one dorsal, the other ventral) as CS+ and CS− inputs (Fig. $2B_1$) using fine silver implanted electrodes (*18*). The site that received CS+ training elicited significantly greater response than the site that received CS− training ($N = 14$; means, 21.9 and 13.4 seconds, respectively; $P < .005$) (Fig. $2B_2$). These results suggest the hypothesis that the differential associative effect may be produced by neuronal changes within subpopulations of a single homogeneous cluster of sensory neurons. In addition to helping to pinpoint possible cellular loci of the associative changes, this finding is experimentally advantageous since it allows cellular studies (*7*) to be focused on a single cluster of neurons, the LE sensory cluster, which has been extensively investigated in studies of nonassociative learning (habituation and sensitization) (*19*).

Although we have achieved reasonably good stimulus control over the behavior and understand several features of the differential conditioning, much remains to be studied. For example, we have begun to examine the effects of varying the temporal relationships between the CS and US. Preliminary studies indicate that the interstimulus interval function of the conditioning is quite steep and that backward conditioning does not occur (*20*). It will also be interesting to explore the time course of retention of the learning, and to examine extinction and reacquisition of the conditioned response. Since two independent conditioned stimuli can mediate conditioning, it also now becomes possible to examine higher-order features of learning that are interesting from the perspective of cognitive psychology. These include contingency effects and second-order conditioning, as well as blocking, sensory preconditioning, and conditioned inhibition, which can be studied with compound conditioned stimuli (*21*). Differential conditioning and several higher-order learning phenomena have been demonstrated in other mollusks by using complex appetitive behaviors (*3*).

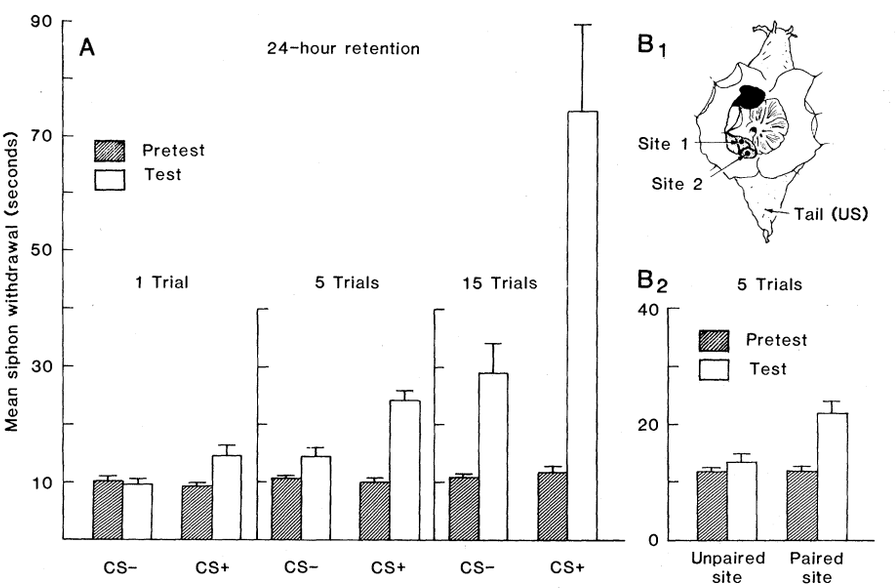

FIG. 2. (A) The effect of the number of training trials on differential conditioning. Different groups of animals were given 1, 5, or 15 trials and tested twice, once within 1 hour after training (not shown) and again 24 hours later. Data are pooled as in Fig. 1D. A significant differential effect was present after one trial ($P < .005$), and the differential effect progressively increased with more trials ($P < .005$ in each case). Sensitization (comparing pretest and test CS− scores) was not evident after one trial (*14*), but did appear after five trials and was larger after 15 trials. (B) Differential conditioning of two sites on the siphon skin. (B_1) Dorsal view illustrating the approximate location of the two implanted (CS) electrodes in the siphon (*18*) and the (US) electrodes in the tail. (B_2) Pooled test scores from paired and unpaired sites compared to their respective pretest scores. Significant differential conditioning ($P < .005$) was exhibited 15 to 30 minutes after five training trails.

It will therefore be of theoretical interest to determine whether these advanced features of learning can be demonstrated in a simple behavior mediated by a neural circuit containing a small number of nerve cells.

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8. *Aplysia californica* (80 to 300 g) were used. The parapodia of all animals were surgically removed to permit complete visualization and direct access to the mantle shelf and siphon. All animals were then housed in individual pans in a 200-gallon aquarium for approximately 1 week before an experiment was begun. For details of housing and preparation of animals, see (*5*).
9. The tactile stimulus to the siphon was applied with a nylon bristle (*5*); the electrical stimulus to the mantle was a very weak electric shock (60 Hz a-c) with an intensity of approximately 15 mA for 0.5 second delivered in seawater with bipolar capillary electrodes separated by approximately 5 mm. The electrode was in series with a 10-kilohm resistor to provide relatively constant current. This level of shock was chosen because it produced siphon withdrawals comparable to those elicited by the tactile stimulus to the siphon. The US was delivered with spanning electrodes across the tail (*5*) and consisted of a 1.0-second pulse of 60-Hz a-c current at an intensity of 75 mA in seawater.
10. Siphon withdrawal was measured with a stopwatch from stimulus onset until the siphon stopped contracting and began to relax. All responses were measured (blind) by an observer who did not know the experimental history of the animals (*5*).
11. Both CS's and the US were delivered by hand, with the timing of the two stimuli guided by electronically controlled signals. Thus the interstimulus interval is approximate, although it was usually accurate to within 0.25 second. Moreover, the intertrial interval in group studies was also somewhat variable (between 5 and 6 minutes on average). In other experiments in which both the interstimulus and the intertrial intervals were precisely controlled (*18*), comparable results were obtained (Fig. $2B_2$).
12. For all experiments, the results of two independent replications were pooled. Data were analyzed for each animal by first computing a difference score for each pathway (each test score minus the pretest score for that pathway) and then determining the difference between CS+ and CS− scores. Thus each animal provided a single score that reflected the difference in that animal's response to the paired and unpaired conditioned stimuli. Since multiple statistical comparisons were not made, within-group and between-group statistical comparisons were made by means of *t*-tests for correlated means or independent means respectively. All probability values are two-tailed.
13. These results confirm earlier findings (*5*) of significant sensitization 30 minutes after 15 trials of US-alone training [see figure 6B in (*5*)]. In addition to sensitization (a generalized nonassociative increase in responsiveness), increased responsiveness in the CS− pathway may also be due to stimulus generalization. On the other hand the CS− pathway may underestimate sensitization because of habituation. Additional behavioral experiments are in progress to distinguish among these possibilities.
14. Sensitization can readily be observed after one trial in other experimental contexts such as when stronger unconditioned stimuli are used or when sensitization is superimposed on a previously habituated response (dishabituation) [T. J. Carew, V. F. Castellucci, E. R. Kandel, *Int. J. Neurosci.* **2**, 79 (1971); *Science* **205**, 417 (1979)].
15. An example of this can be seen by comparing the 15-trial data in Fig. 1D (where testing was done 30 minutes after training) with those in Fig. 2A (the same animals as in Fig. 1D 24 hours later); both sensitization and differential conditioning significantly increased. Similar though less dramatic results were also seen after five trials; no sensitization was evident 15 minutes after training, while modest sensitization was evident 24 hours later (Fig. 2A), and the differential effect also showed a slight increase.
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18. Electrodes were implanted in the siphon skin as described (*5*). Overlap in the receptive fields of the two sites (*17*) was minimized by implanting one electrode on the dorsal surface of the siphon (near the caudal margin of the shell) and the other in the base of the siphon (near the anus). For each site, current was passed between the implanted electrode and a ground electrode in the tank. Electrical stimulation consisted of a 0.5-second pulse of 60-Hz a-c current stepped down from a variable transformer and dropped across a 10-kilohm resistor in series with the electrode to produce relatively constant current. Currents used ranged between 5 and 10 mA. The site at the base of the siphon usually required slightly less current to produce a withdrawal comparable to that produced from the other site. Stimulus intensities were first adjusted to produce siphon withdrawal pretest scores from each site comparable to those in previous experiments (Figs. 1D and 2A) and then one site was randomly chosen to be CS+ or CS−. The intensity of the US [60-Hz a-c current for 1 second, delivered by a second pair of electrodes implanted in the tail (*5*)] was adjusted before training so that it produced a pronounced withdrawal of the whole animal. Typical current range for the US was 20 to 30 mA. Five trials were delivered (intertrial interval, 5 minutes), and testing was carried out 15 to 30 minutes after training. Timing of stimuli was controlled by a programmable timing device (*5*).
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20. A CS-US interval of 0.5 second produced optimal learning; an interval of 1.0 second produced significant but reduced learning; and intervals of 2.0, 5.0, and 10.0 seconds as well as backward intervals (US-CS pairings) of 0.0 and 0.5 second (US offset to CS onset) produced no learning (T. J. Carew, R. D. Hawkins, E. R. Kandel, in preparation).
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22. Supported by NIH career awards 5KO2 M0081 (to T.J.C.) and 5KO5 MH18558 (to E.R.K.), by NIH grants NS 12744 and GM 23540, and a grant from the McKnight Foundation. We thank L. Bursztajn for technical assistance, J. H. Schwartz for comments on an earlier draft of this manuscript, H. Ayers for manuscript preparation, and L. Katz and K. Hilten for the illustrations.

7 October 1982

A Cellular Mechanism of Classical Conditioning in *Aplysia*: Activity-Dependent Amplification of Presynaptic Facilitation

Abstract. *A training procedure analogous to differential classical conditioning produces differential facilitation of excitatory postsynaptic potentials (EPSP's) in the neuronal circuit for the siphon withdrawal reflex in* Aplysia. *Thus, tail shock (the unconditioned stimulus) produces greater facilitation of the monosynaptic EPSP from a siphon sensory neuron to a siphon motor neuron if the shock is preceded by spike activity in the sensory neuron than if the shock and spike activity occur in a specifically unpaired pattern or if the shock occurs alone. Further experiments indicate that this activity-dependent amplification of facilitation is presynaptic in origin and involves a differential increase in spike duration and thus in Ca^{2+} influx in paired versus unpaired sensory neurons. The results of these cellular experiments are quantitatively similar to the results of behavioral experiments with the same protocol and parameters, suggesting that activity-dependent amplification of presynaptic facilitation may make a significant contribution to classical conditioning of the withdrawal reflex.*

One of the major goals of neuroscience has been to specify the cellular mechanisms of associative learning. Although cellular correlates and analogs of associative learning have been observed in several vertebrate species (*1, 2*), it has been difficult to study these events with intracellular techniques, and it has been impossible to establish a causal relationship between the cellular events and behavior. In the last decade, neural correlates of associative learning have been