Learning: A Model System for Physiological Studies

The laws of learning and the design of behavioral criteria must be aimed at testing cellular hypotheses.

George J. Mpitsos, Stephen D. Collins, Andrew D. McClellan

Just as Sherrington used behavioral criteria to deduce the mechansims of spinal reflexes (*1*), Pavlov thought of learned behaviors as "factual points of departure" for understanding the elementary properties of the brain (*2*). These properties, however, still remain obscure, owing in large measure to the complexity of the vertebrate brain and also to the lack of an experimental system that reliably shows learning and can be applied widely in studies that combine behavioral and cellular approaches (*3*). In recent years invertebrate animals have been used advantageously to investigate brain and behavior problems (*4*). Among these animals, the gastropod mollusks (the snails and slugs) have relatively simple and reliably elicited behavioral repertoires (*5*). Their nervous systems are easily accessible and contain large neuron somata that can be visually reidentified in successive dissections and penetrated with one or more recording microelectrodes. These technical assets have been important in the successful analysis of many behaviors, from fixed-action feeding (*6, 7*) and swimming (*8*) responses to more complex phenomena such as behavioral "choice" (*6, 9*) and sensitization (*10, 11*), the last being a possible evolutionary precursor of associate learning (*12, 13*). Although the literature over most of the past 75 years contains little evidence of the gastropods being capable of behavioral plasticity beyond the level of sensitization (*5, 12*), recent studies seem more successful and encourage the view that the gastropods might provide the means to explore the neuronal basis of associative learning (*14–17*).

People seeking to study learning in such primitive animals are faced with two problems. First, the definition of learning itself remains unsettled and has been historically in a state of controversy (*18–20*). Second, since the phenomenology and theories of learning have been derived from behavioral studies on higher animals, often in terms that are relatable to human experience, there is the difficulty of selecting from the behaviors of lower animals the appropriate response units to study. In this article we shall reconsider the definition of learning as it might be usefully applied to lower animals. Then, with control experiments, we shall apply this definition to an instance of trained food-aversion behavior in the marine gastropod *Pleurobranchaea californica*. These experiments were designed specifically for neurophysiological applications (*14*). Our purpose here will be to evaluate the food-aversion behavior as a viable instance of associative learning. Then, using the learned behaviors and the information gained from the control experiments as "factual points of departure," we shall discuss how the *Pleurobranchaea* preparation may be extended as a "model" system for studies on the cellular level.

The Definition of Learning

In the most global sense, learning may be defined learning as a behavioral change that results from experience (*21*). In a more restricted sense, learning may also be given operational definitions such as Pavlovian, avoidance, and operant conditioning which focus specifically on the associative aspects of learning (*13, 20, 22, 23*). Although such operational definitions may be encompassed by the more global definition, it is not certain whether they may be applied to all cases of apparent associations, as, for example, to the acquisition of symbolism in language (*13*). While not wishing to diminish the value of the global approach, we shall restrict the present discussion to the operational definitions because they provide a systematic way to study and compare associative phenomena in different animals and different behaviors.

As an illustration of the use of these operational definitions, let us consider the food-aversion behavior of *Pleurobranchaea* (*14*). These animals are voracious carnivores capable of ingesting amounts of food equivalent to 10 to 20 percent of their body weight in a single feeding, and will readily repeat this performance every 3 to 5 days (*9*). However, by means of an aversive procedure in which experimental animals received strong electrical shocks contingent on their responses to food, hungry *Pleurobranchaea* were rapidly trained to cease feeding and to withdraw from a natural food substance (*14*). It is useful to express these stimuli and responses in the terminology of Pavlovian conditioning which is thought to be descriptive of fundamental units of behavior and applicable to other conditioning paradigms (*20*). In Pavlovian conditioning an animal comes to respond to an initially ineffective stimulus much as it does to an innately effective one only after the animal has experienced the two stimuli in close temporal association (*24*). In Pavlov's terms, the initially ineffective stimulus is the conditioned stimulus (CS) and the response it elicits after training is the conditioned response (CR); the innately effective one is the unconditioned stimulus (UCS) and the response it elicits is the unconditioned response (UCR). Thus, the food substance applied to *Pleurobranchaea* is the CS, the withdrawal and suppressed feeding responses elicited by food after training comprise two components of the CR; the electrical shocks are the UCS and the withdrawal responses they elicit are the UCR. Evidence for learning is usually assessed on the basis of behavior differences between the experimental animals, which are given the CS and UCS closely "paired," and control animals, which are given the CS and UCS by some other procedure. In our previous experiments the control animals were given the two stimuli alternately and separated by the maximum amount of time between trials. This is the "explicitly unpaired" control and traditionally has been used to support the theory that the essential feature of associational conditioning in the experimental procedure is the close tem-

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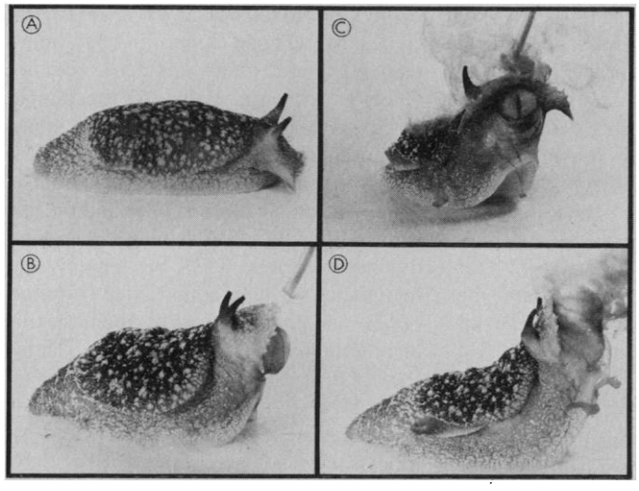
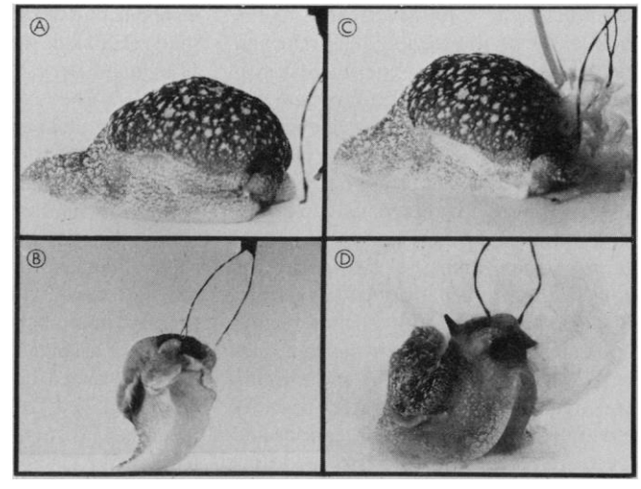
poral pairing or contiguity between the CS and UCS (*18, 20*).

An opposing theory states that the CS-UCS contingencies, rather than CS-UCS pairing, are the essential features of conditioning (*18*). The only "proper" control, therefore, is a procedure by which the CS and UCS are presented in a "truly random," noncontingent fashion (*18*). All the traditional controls (novel CS, CS alone, UCS alone, backward and discriminitive conditioning, and the explicitly unpaired procedure) used alone or together are all insufficient because they either present nonassociative factors not found in the experimental procedure or introduce CS-UCS contingencies of their own. Traditional theory in turn does not accept that the explicitly unpaired procedure involves "true" conditioning. Accordingly, responses resembling the CR which are obtained from animals after they have been given the explicitly unpaired procedure are usually ascribed in traditional theory to nonassociative phenomena such as sensitization or pseudoconditioning (*13, 20, 23, 25*). Nonetheless, there is evidence to support the view that even pseudoconditioning procedures in which the UCS is presented alone or unpaired with the CS involve associational conditioning (*22, 23, 26*), much as Pavlov originally contended for the acquisition of responses to contextual cues (*27*).

Such controversies over the fundamental nature of conditioning may not be solvable without further knowledge about the nervous system itself. This is because the control procedures may be interpreted in terms of potentially different neural mechanisms. For example, experiments in which the explicitly unpaired, backward, or random control procedures are used could indicate that the neural mechanisms of learning involve, respectively, processes for detecting the occurrence and temporal proximity of environmental events, their temporal order or temporal patterns, or all of these. Without our knowing how the nervous system functions under a variety of imposed control situations, it may be impossible to determine which controls point to the most fundamental process (if there is one) or which ones point to processes requiring several higher orders of neural integration. By placing behavior theory in the context of possible cellular mechanisms, it seems to us that there may not be a single fundamental mechanism in Pavlovian conditioning. This is to say that there may not be a single universally applicable definition of learning even within a given conditioning paradigm.

Given the present level of knowledge, therefore, it may be best to consider the utility of any control as the amount and kind of information it gives about the stimulus complexity to which the experimental animals may be attuned rather





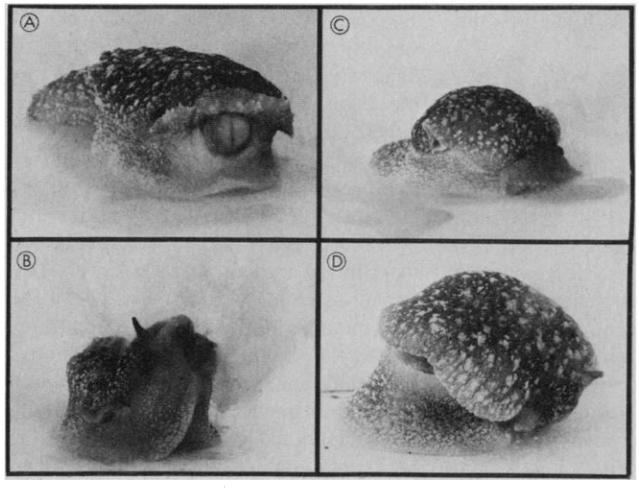


Fig. 1 (top left). Feeding behavior of untrained animals. (A) Natural, unrestrained posture of *Pleurobranchaea*. The animal (about 7.8 cm in length) is locomoting forward from left to right in the photograph. Structures pointing upward on either side of the head are the rhinophores; the oral veil, resembling a cowcatcher, is below and in front of the rhinophores; the mouth area lies below the oral veil. (B) The proboscis extension response (the proboscis is shown protruding below the oral veil). The food is being applied through a short piece of tubing; the slight cloudiness at the tip of the tube is due to a dye mixed with the squid homogenate to aid visibility for photography. (C) A more advanced extension response showing parted lips of the proboscis in a small bite response. (D) Fully developed bite-strike response in which the animal completely extends its proboscis and strikes the food with a biting action of the lips and mouth. Fig. 2 (top right). (A) Withdrawal to electrical shocks applied with hand-held electrodes (far right); notice retraction of the tentacles, rhinophores, and head. (B) Electrical shocks often evoke swimming escape responses. (C) Withdrawal during conditioning when food and shock are applied together. (D) Approach-avoidance behavior during conditioning; the animal is postured ambivalently between a withdrawal response and a feeding response: the rhinophores and oral veil are only partially withdrawn, the head and foot have begun to swing away, and the body is flexed dorsally in a partial initiation of a swimming escape response. Shocks were delivered at 70 volts, 10-msec pulses at a rate of 15 per second. Fig. 3 (bottom left). Conditioned aversive response to food (CS) alone. The animal was first conditioned with electrical shocks (UCS) contingent upon its response to food (see Fig. 4); same animal as in Figs. 1 and 2. (A and B) Approach-avoidance behavior; compare with Fig. 2D. (C) Withdrawal response similar to response observed during conditioning in Fig. 2C. (D) Full withdrawal response similar to unconditioned response shown in Fig. 2A.

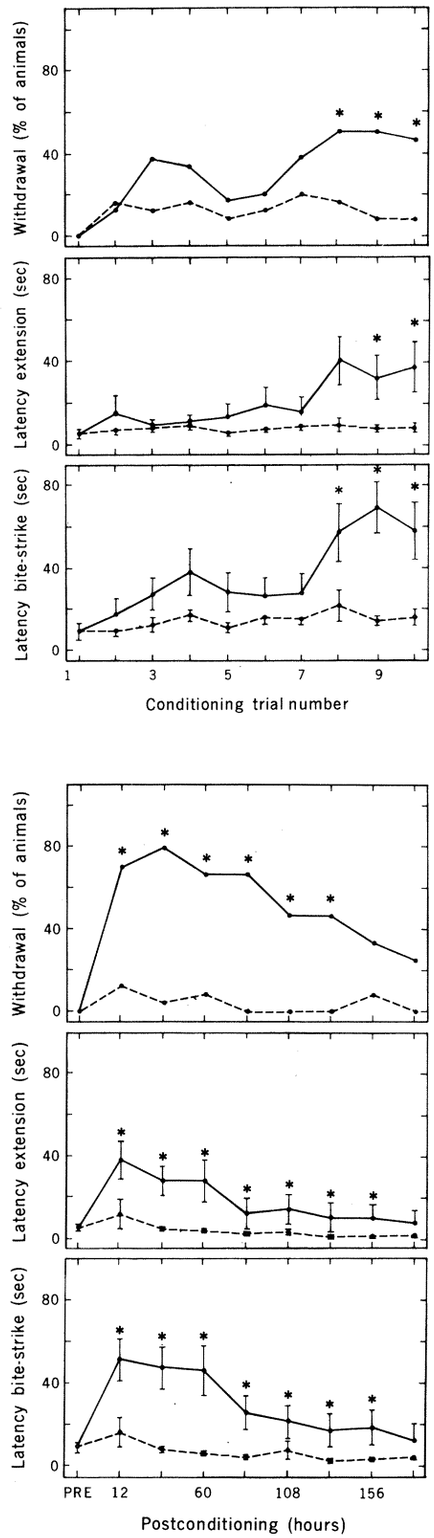
than to seek the "required" or "proper" control for "incontrovertible demonstrations of true conditioning" (*28*). This use of the controls is attractive for application to invertebrate animals and for considerations of the possible phylogenetic evolution of the mechanisms of learning, because it allows the investigator to determine the complexity of information an animal can learn instead of imposing on the animal a preestablished definition. What is most important for our purposes is that this use of the controls keeps the amount and kind of information gained from behavioral studies in close parallel correspondence with the complexity of mechanisms to look for in the nervous system.

Learning in a "Simple" Animal

Rapid behavior modification. To be suitable for neurophysiological applications, learned behaviors should involve an obvious switch in motor responses, be measured in parameters that can be directly applied to neural activities such as latencies and thresholds, and occur rapidly. The criterion of rapid learning seems particularly necessary because it may accentuate the quantity of cellular or biochemical changes that might occur and provides the opportunity to observe these changes within the space of time imposed by the life-span of experimentally manipulated nervous systems. Our studies on food-aversion learning in *Pleurobranchaea* have been designed specifically to meet these criteria (*14*).

Figure 1 illustrates the aggressive feeding behavior of *Pleurobranchaea*. This behavior can be rapidly suppressed for long periods of time by giving, in only a few trials, strong electric shock together with food (Fig. 2). During training, the behavior of the animals changes progressively from full feeding (Fig. 1) to approach-avoidance (Fig. 3A) and then to an obvious avoidance withdrawal response (Fig. 3, A to D). This food-aversion behavior consists of a dramatic motor switch which is obvious without statistical statements; that is, since our intent is to carry the investigation to the cellular level, the behavioral phenomenon in question ought to be demonstrable in each animal examined.

The primary purpose of all such experimental conditioning procedures is to generate the desired behavioral changes. The following control experiments provide what we believe is some of the most crucial behavioral information for guiding investigations on the cellular mecha-



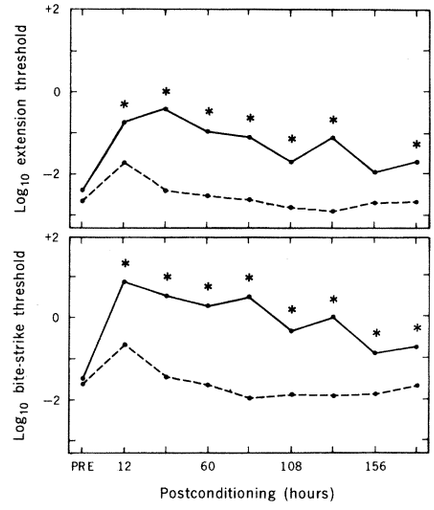


Fig. 4 (top left). Aversive conditioning compared to explicitly unpaired control procedure; pooled data of four experiments. Experimental animals (solid curve; $N = 25$) were given ten conditioning trials with an intertrial period of 1 hour. In each trial a food CS, consisting of a standard mixture of homogenized squid (*14*), was presented at a constant rate (0.2 ml per second) over the oral veil as shown in Fig. 1. Electrical shocks were applied concurrently with the CS, as shown in Fig. 2, for 60 seconds contingent on the animals' response to food: shocks were given immediately after the animals exhibited bite responses, but shocks were withheld if a withdrawal response was sustained for 180 seconds; if animals did not bite but appeared to behave indifferently to food, or only with extended proboscis, the 60 seconds of shock was given after 180 seconds [see (*14*)]. Control animals (dashed curve; $N = 24$) were individually matched with experimental animals and exposed to the same amounts and durations of food and shock; however, the two stimuli were separated by intertrial periods of one-half hour. Here, as in all following graphs, asterisks indicate statistically significant differences between experimental and control animals at $P < .01$; χ^2 test for parametric withdrawal data, Mann-Whitney U test for nonparametric latency data (*48*). Vertical bars indicate standard errors, but where the variance of data is small, error bars have been excluded. Figures 4 through 9 illustrate two cycles of conditioning and reconditioning of the same four groups of animals [see (*14*)]. Fig. 5 (bottom left). Withdrawal behavior and latencies of feeding responses for the same animals as in Fig. 4. Responses were measured beginning 12 hours after the end of the conditioning session and repeated every 24 hours thereafter. Preconditioning response values to standard food stimulus are shown at *PRE* on abscissa. Latencies for proboscis extension and bite-strike responses were measured and recorded if they occurred, otherwise a value of 180 seconds was assigned in order to avoid infinite latencies in the computation of means (*48*). Fig. 6 (top right). Thresholds of proboscis extension and bite-strike responses. Thresholds were measured by applying over the oral veil serial tenfold dilutions of standard food stimulus, beginning with the least concentrated (10^{-5}) and progressing toward the most concentrated solution (10^{0}). If a response was not obtained with even the most concentrated solution, we made the conservative estimate that the response would have been obtained with the next tenfold greater concentration (10^{+1}) were it available; this avoided the use of infinite threshold values in the computation of means. Similarly, if neither the proboscis extension nor bite-strike response were obtained, their thresholds were assigned the values of 10^{+1} and 10^{+2}, respectively, since the proboscis response has been found consistently to have a tenfold lower threshold than the bite-strike response.

nisms that may underlie these changes.

The explicitly unpaired control. We describe here the behavior of animals taken from four experiments involving two cycles of conditioning and extinction. All of the procedures have been reported elsewhere and critically discussed (*14, 28, 29*). Briefly, in ten trials spaced 1 hour apart, experimental animals were given food and then electrical shocks contingent upon their response to food. Control animals were given as much stimulation and handling, but food and shock were presented alternately and separated by one-half hour (Fig. 4). During conditioning we noted whether the animals fed or withdrew when they were given food, and determined the latencies of the two major components of the feeding response, namely, the extension of the proboscis and bite-strike responses (see Fig. 1); before and after conditioning we obtained all of this information from each animal as well as the thresholds of the feeding components (Figs. 5 and 6).

The differences between the experimental and control animals in these experiments are as quantitatively robust as the behavioral differences shown in Figs. 1 to 3 are qualitatively obvious. Even with so few conditioning trials, significant differences between experimental and control animals appeared late in the conditioning session and remained for more than 8 days after conditioning (Figs. 5 and 6). Although the behavior of the control animals changed somewhat during conditioning, it quickly returned to normal shortly after the conditioning. To demonstrate a savings of learning, we reconditioned the same groups of animals on day 9 after the first conditioning (Fig. 7). The experimental-control differences were further exaggerated during and after the reconditioning (Figs. 7 to 9).

The effectiveness of the aversive-conditioning procedures on the experimental animals is clearly illustrated by the withdrawal and latency data. After the first conditioning session 80 percent of the animals withdrew when presented food, and after the second conditioning session 100 percent of the animals withdrew; similarly, the latency of the feeding components increased by several orders of magnitude. The means of the threshold data are more difficult to interpret, especially since the positive threshold values are actually a conservative representation of infinite feeding thresholds (*14*). To better appreciate these data, we have reinterpreted in Fig. 10 the bite-strike threshold observations reported previously (*14*). This report included the

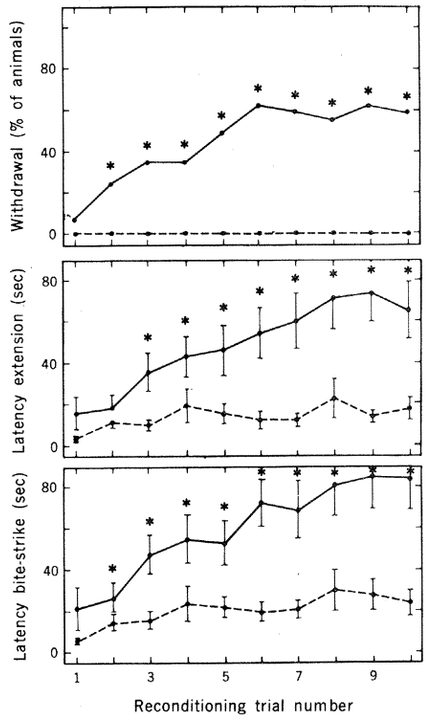


Fig. 7. Reconditioning of the same animals as shown in Figs. 4 to 6. Training was begun 24 hours after the last postconditioning test session. Procedures were as described in Fig. 4. Experimental (solid curves) and control (dashed curve) animals remained in their respective groups from the first conditioning session.

four experiments described here and a fifth in which the animals were not reconditioned. Figure 10 shows that the experimental animals may be divided into two groups. One group, which includes more than 50 percent of the animals, did not exhibit bite responses on 7 of the 8 days after conditioning when they were tested for feeding thresholds; that is, they had infinitely high feeding thresholds. Even the second group, which exhibited some feeding behavior, was significantly different from the controls. We have now conducted many aversive-conditioning experiments in our laboratory and have obtained similar results. Learning in *Pleurobranchaea* may involve relatively simple neural components. However, the task of finding these components and analyzing them may still be a difficult and time-consuming task, even in such a "simple" animal. If this analysis is to proceed rapidly, the reproducibility of the procedures and the capability to produce obvious changes in many animals are requisites for extending the experimental utility of the food-aversion learning in *Pleurobranchaea* in our own and other laboratories.

Experiments with a random control procedure. The above results satisfy traditional theory in that they show that the food-aversion behavior of *Pleurobranchaea* is attributable to association of contiguous or paired stimuli. By the opposing contingency theory, however, it is expected that we should obtain fewer withdrawals and stronger feeding responses from the control animals than from the experimentals. The explicitly unpaired control procedure contains the contingency that the appearance of the food stimulus signals a "safe" period when the shocks will not appear (*18*). When there is close temporal pairing of food and shock, the experimental animals are exposed to a contingency in which food signals an "unsafe" period. There is some evidence in the above conditioning-reconditioning observations that might be used to support the contingency argument. For example, up to 20 percent of the control animals withdrew from food during the first conditioning session (Fig. 4), whereas none of the control animals withdrew from food during the second session (Fig. 7). Similarly, for the control animals, there are noticeable differences between the "pre-" and 12-hour postconditioning observations associated with the first conditioning (Figs. 5 and 6), but these differences are only minimal in the second session (Figs. 8 and 9). Thus, there is evidence not only to support the statement that there is a savings of learning in the experimental animals, but also to support the statement that there is a savings of learning in the animals that were given the explicitly unpaired control procedures. If the contingency theory is correct, the differences obtained in the first experiments (Figs. 4 to 7) might not have been as great with a noncontingency control.

To compare the effects of the aversive procedure that we applied to the experimental animals with a noncontingency control, we conducted studies in which the control animals were given electrical shocks at random intervals with respect to the time they were given the food stimuli. The pooled results of three replicate experiments are shown in Figs. 11 to 13. During training we obtained only minimal differences (Fig. 11), but this was due to the lack of avoidance responses in the experimental animals rather than to increased avoidance responses in the control animals. Nonetheless, we obtained strong, statistically significant differences between the experimental and control animals beginning with the 12-hour postconditioning tests and lasting over the entire 8-day observation period (Figs. 12 and 13). Altogether the effect of the random procedure on

the control animals is essentially the same as that produced by the explicitly unpaired control (compare Figs. 4 to 7 with Figs. 11 to 13). Although it is difficult to completely exclude CS-UCS contingencies that might arise from the successive presentations of the stimuli in the control procedure, these data suggest that the major factor in establishing the food-aversion response is the close temporal pairing between the CS and UCS.

The increase in the food-aversion behavior of the experimental animals that occurs between the conditioning and postconditioning tests has been observed in all our studies, and it is possible that the food-aversion behavior may be primarily established within the 12 hours after conditioning (*14*). This could prove of considerable advantage since it would allow us to observe and manipulate a functionally changing nervous system during a time when the processes of the nervous system do not have to be disrupted by conditioning trials.

Forward versus backward conditioning. Pavlovian conditioning typically involves forward pairing of stimuli such that the CS or its onset precedes the UCS, but in backward conditioning the UCS precedes the CS. Here, too, there is controversy about whether responses obtained with the backward procedure that resemble the CR are associational or nonassociational (*13, 22, 30*). The information gained with the backward procedure, however, can be useful for neurophysiological interpretation of behavior, since the temporal order of events, just like latencies and thresholds, can be directly correlated with the functional properties of the nervous system. In order to focus explicitly on the temporal order of the stimuli, we conditioned experimental and control animals with only one trial and on only 1 day. In this way we avoided any complications of CS-UCS contingencies that might occur between trials in a multitrial training session as well as the contingencies that might occur between training days.

Experimental animals were given a typical Pavlovian conditioning procedure in which we first applied the standard food stimulus alone over the oral veil (Fig. 1) and then presented food and shock together (Figs. 2 and 14). We used two forward "control" groups in which the food and shock stimuli were separated by 1 and 10 minutes (+1 and +10 groups) and two backward control groups in which the stimuli were similarly separated by 1 and 10 minutes but in reverse order (−1 and −10 groups). To test for the effects of each of the stimuli, we also used a control group that was given only the food stimulus and another group that was given only shocks.

The results of four experiments (N = 135) are summarized in Fig. 14. The procedure in which we paired food and shock consistently produced the greatest amount of food-aversion behavior, though by no means was one-trial training as effective as ten trials. The most important comparisons to be made are between the experimental and backward conditioning groups. The experimental group was significantly different from both control groups, though on fewer of the test days when compared to the −1 group than to the −10 group. In the 1 minute following shock the −1 animals were still showing pronounced effects of the electrical stimulation and avoided the food when it was presented to them. By contrast, the −10 animals seemed to have fully recovered in the 10 minutes following shock and exhibited strong feeding behavior when the food stimulus was given to them. Some association between the aftereffects of the electrical stimulation and food may have occurred in the −1 group. This conclusion is supported by the finding (Fig. 14) that the −1 group showed stronger food-aversion behavior than the −10 group despite the fact that both groups of animals were given equal amounts of stimulation. An especially interesting though surprising finding is that the +1 procedure had essentially no effect on the animals. At present we can only speculate that strong electrical stimulation presented alone somehow disrupts the consolidation of an experience that just precedes it. Nonetheless, when one compares the behavior of the experimental and +10 animals with all other groups, the major conclusion to be drawn from these findings is that forward pairing of food and shocks is more effective than backward, food alone, and shock alone procedures.

Conditioned stimulus specificity. Although we have attempted to minimize

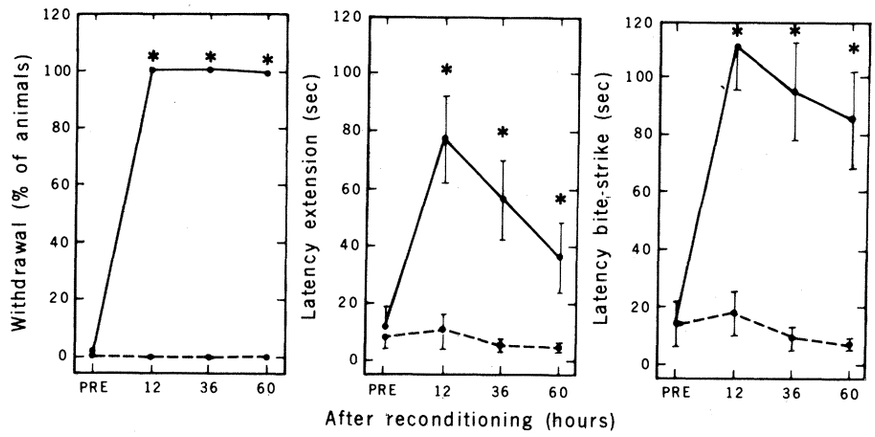
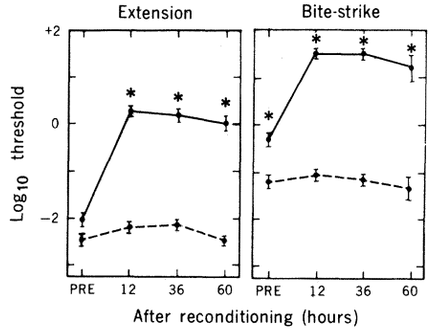


Fig. 8. Withdrawal behavior and latencies of feeding after reconditioning. Tests were begun 12 hours after the last reconditioning trial shown in Fig. 7 and repeated every 24 hours. For measurement procedure see Fig. 5. Preconditioning values (*PRE*) were measured on the first trial of the reconditioning session.



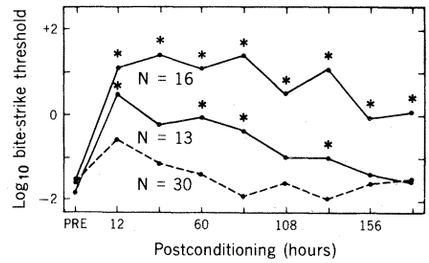
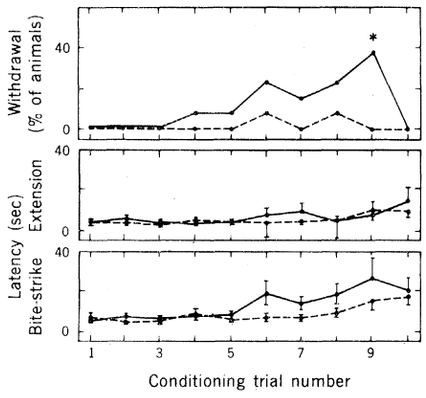


Fig. 9 (left). Proboscis extension and bite-strike response thresholds; same animals as in Figs. 7 and 8. Measurements were obtained as described in Fig. 6. Values prior to reconditioning (*PRE*) were obtained just before the beginning of the reconditioning session shown in Fig. 7. Fig. 10 (right). A reanalysis of data from (*14*, figure 2F) on bite thresholds. The experimental animals fell into two categories: one (upper curve, N = 16) whose feeding was completely suppressed during nearly all the tests (positive threshold values), and a second whose feeding behavior was statistically different from that of the control animals but only about 50 percent of the time. Measurements were obtained as described in Figs. 4 and 7.



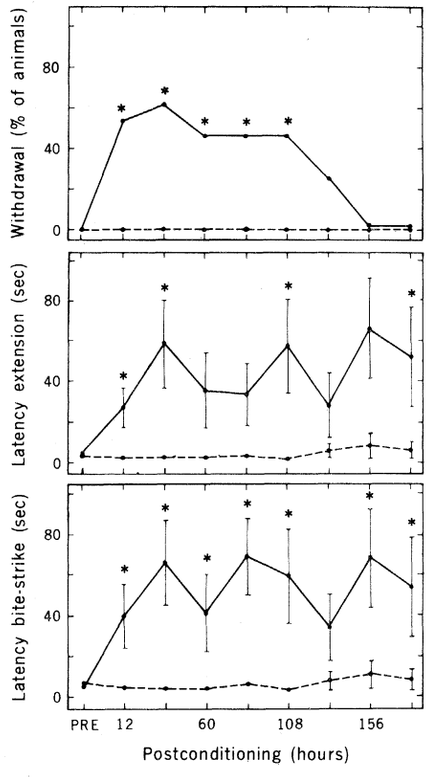
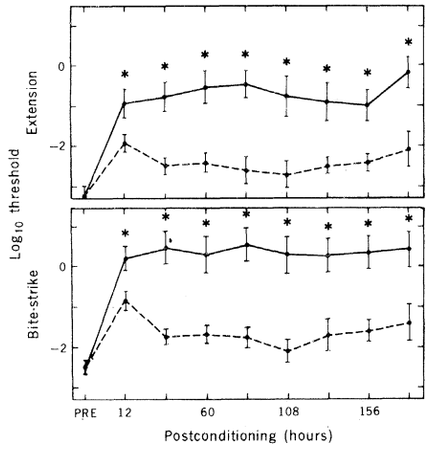


Fig. 11 (left). Aversive conditoning compared to a random control procedure; pooled data of three experiments. Experimental animals (N = 24; solid curves) were given ten conditioning trials (1 hour between trials) as described for experimental animals in Fig. 4. Control animals (N = 25; dashed curves) were again matched with experimental animals and given the same amounts of shock and food, but shocks were given after a random time interval following the food presentation. Random time intervals were selected by a "card in the hat" routine and were restricted to discrete intervals of multiples of 1 minute (for example, 1 minute, 2 minutes, . . . minutes). We used schedules that generated uniformly random intertrial intervals. Measurements were obtained as described in Figs. 4 and 7. Fig. 12 (right). Withdrawal responses and latency of feeding in the same animals as in Fig. 11. Tests were begun 12 hours after the last conditioning trial and repeated every 24 hours thereafter. For the measurement procedure see Fig. 5. Preconditioning values (*PRE*) were determined on the first trial of the conditioning session.



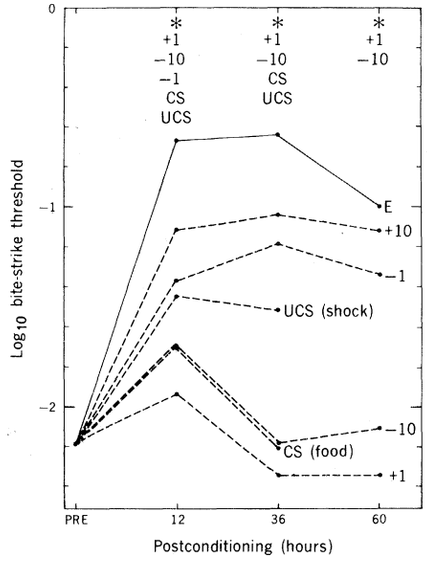


Fig. 13 (left). Proboscis extension and bite-strike thresholds: same animals as in Fig. 11. Measurements were obtained as described in Figs. 6 and 9. Fig. 14 (right). One-trial classical aversive conditioning; pooled data from four experiments. Experimental animals (solid curve, E; N = 36) were given 75 seconds of food stimulation, the last 60 seconds of which overlapped with electrical shock. Control groups (dashed curves) were given as much of each stimulus but in nonoverlapping forward and backward combinations: two forward groups received food and then shock separated by 1 and 10 minutes, respectively (+1, N = 12, and +10, N = 12); similarly two backward groups received shock and then food separated by 1 and 10 minutes, respectively (−1, N = 33, and −10, N = 12). Shown also is a control group that was given food alone (CS, N = 15) and another group that was given shock alone (UCS, N = 15). The food stimulus (3 ml of the standard food concentration) was initially applied over the oral veil as shown in Fig. 1 at a rate of 0.2 ml/sec; after 15 seconds an additional 17 ml was rapidly dispersed throughout the 250 ml of seawater in which the animals were trained. For shock procedure see Fig. 2. All animals (mean size 125 g) received equal handling. Groups under asterisks indicate statistically significant differences between experimental and control animals (P < .05, Mann-Whitney U test); total animals, N = 135. Error bars have been excluded for clarity of presentation.

tactile cues in our procedures, some tactile stimulation must undoubtedly accompany the presentation of the food and shock stimuli. Therefore, part or all of the aversive responses might have been conditioned to tactile cues rather than to the food stimulus. Tactile stimulation of the oral veil normally produces withdrawal responses similar to the ones obtained with the food stimulus after aversive conditioning (*15*). It is possible to measure the amplitude and duration of these withdrawal responses by means of a device affectionately called the Twanger (*31*) which delivers a constant but fairly innocuous tactile stimulus to the oral veil.

The rationale behind the experiments described here is that if aversive conditioning involves tactile cues, we should see a change in the amplitude or duration of the withdrawal response after conditioning. Table 1 shows, however, that the various parameters of tactile withdrawal responses obtained from experimental animals are the same as those obtained from control animals, and that the measurements obtained from all animals after conditioning are essentially the same as those obtained before conditioning. The effects of conditioning might not be detectable if the Twanger stimulus produced near-maximal withdrawal responses. This possibility seems unlikely since the animals are capable of withdrawal movements equivalent to at least one-third of their body length, and these are considerably longer than the withdrawal movements obtained with the Twanger stimulus. We conclude, therefore, that tactile cues have a minor role, if any, in the CS and that the proper CS is the food stimulus. The findings described here also support previous conclusions (*14*) that the food-aversion behavior is not caused by sensitization, since sensitization would probably augment withdrawal responses to other stimuli as well as to food.

Selective versus whole-animal stimulation. In all the preceding experiments, shocks were selectively applied to the oral veil of animals as shown in Fig. 2. We originally used this method because it best produced the behaviors we wanted to condition. In addition, since the oral veil and other appropriate sensory structures can be left intact in an otherwise isolated but functioning nervous system, the procedures for presenting food and electrical stimulation to the oral veil can be directly applied to the conditioning of nervous systems in vitro. A disadvantage of using this method, however, is that the shocks cannot be applied "blind" since the person who applies the shocks automatically gains the informa-

Table 1. Differences between experimental and control animals in response to food stimulation but not to tactile stimulation. Experimental animals ($N = 5$) were given "paired" food and shock, control animals ($N = 5$) were given the food and shock "explicitly unpaired" (see Fig. 4). Preconditioning values are shown at Pre; postconditioning values are shown at hours 12, 36, and 60. Bite-strike threshold measurements as in Fig. 5. Response to tactile stimulation was measured as described elsewhere (*31, 47*).

Time of test (hour)	Food stimulation		Tactile stimulation		
	Log_{10} bite-strike threshold*	Animals withdrawing (%)	Withdrawal amplitude (cm)*	Withdrawal time (sec)*	Return time (sec)*
		Experimental animals			
Pre	−2.80 ± 0.37	0	1.22 ± 0.28	0.74 ± 0.15	10.00 ± 1.02
12	+1.60 ± 0.40†	100†	0.80 ± 0.17	0.76 ± 0.09	11.80 ± 4.70
36	+0.80 ± 0.58†	80†	0.86 ± 0.15	1.10 ± 0.10	15.80 ± 6.13
60	+1.00 ± 0.45†	100†	0.76 ± 0.14	1.10 ± 0.36	18.92 ± 4.86
		Control animals			
Pre	−2.40 ± 0.24	0	1.14 ± 0.19	0.70 ± 0.11	8.34 ± 1.81
12	−0.60 ± 0.24	20	2.38 ± 1.66	1.30 ± 0.35	8.92 ± 2.58
36	−0.80 ± 0.20	20	0.70 ± 0.19	0.82 ± 0.09	9.22 ± 2.19
60	−1.20 ± 0.37	0	0.88 ± 0.17	0.74 ± 0.07	11.04 ± 5.04

*Data show means ± standard error. †Statistical differences at $P < .05$ (χ^2 test for percentage withdrawal; Mann-Whitney U test for other data).

tion of whether an animal is an experimental or control subject by the presence or absence of food in the conditioning tray [for a critical discussion see (*28, 29*)]. Nonselective whole-animal stimulation, though less attractive for neurophysiological applications, is the closest approximation to the selective shock procedures that provides the means for presenting the shocks blind.

In the following experiment, the procedure was as described above except that whole animals were stimulated electrically by an automated procedure (see Fig. 15). During training, feeding was suppressed on each pairing of food and shock, but on each successive trial the animals again exhibited strong feeding responses. Consequently, few differences in the performance of the experimental and control animals were obtained during conditioning. Similarly, we did not obtain food-elicited withdrawal responses during or after conditioning. However, statistically significant experimental-control differences were obtained in the measurements of thresholds and latencies of the proboscis and bite responses; and these were extinguished within 3 to 4 days. The magnitudes of the latency changes were not impressive, being only 300 to 400 percent of the preconditioning values. The latency changes obtained with selective electrical stimulation were 50 to 100 times greater than the preconditioning values and lasted longer than those obtained with whole-animal stimulation.

The two methods of electrical stimulation thus clearly produced different responses in the animals. Selective electrical stimulation of the oral veil provided a discrete stimulus from which the animals could withdraw or attempt to escape. Whole-animal stimulation did not effectively stimulate the oral veil and was less effective in suppressing the feeding response than was selective stimulation, despite the fact that we used sizable electrical currents (see Fig. 15). When used in conjunction with the food stimulus, selective stimulation produced strong conditioning of both the suppression of feeding behavior and withdrawal responses of the oral veil. The method of whole-animal stimulation produced conditioning of only the suppression of feeding behavior, and this suppression was less effective than obtained with selective stimulation. In each case, however, the conditioned response elicited by the food was the same as the unconditioned response elicited by the particular type of electrical shocks used in the conditioning, as is required by the definition of learning (*24*); that is, the CR resembled the UCR.

Conclusions and Outlook

Although studies on learning in gastropod mollusks were recorded before the turn of the century (*5*), the first indication of the capability for associative learning in these animals was obtained only recently when it was demonstrated that *Aplysia* could be trained to come to rest at unaccustomed positions in their aquariums (*32*). Aside from the two previous studies on *Pleurobranchaea* (*14, 15*), three other studies provide evidence for associative learning; two studies on the reversal of negative geotaxis in snails (*17*) and one study on avoidance of food in the common garden slug *Limax* (*16*). The results of food-aversion learning in *Pleurobranchaea* provide what we believe is extensive evidence for associative learning in an invertebrate animal.

The ability of *Pleurobranchaea* to undergo rapid associational behavioral changes may in part be due to its being a carnivore which must be able to adjust opportunistically to a labile food source. However, the paucity of demonstrations of associative learning in other gastropods may be due to the difficulty of assessing the kinds of stimuli that can be meaningfully related to the natural behavior of these animals. It was suggested (*14*) that the behavioral hierarchy (*9*) might be used as one means of selecting candidate stimuli for the CS and UCS. The behavioral hierarchy and its related list of stimuli provide some indication of what an animal will do when it is confronted simultaneously by several different stimuli. Given this set of an animal's interrelated stimulus preferences, it was proposed that stimuli at the lower end of the hierarchy could be used most often as the CS; stimuli at the top of the hierarchy would act most effectively as the UCS; and stimuli in between could be used either as the CS or UCS depending on the relative strength of the particular stimuli with which they were paired (*14*). While higher animals seem to have developed the capacity for broad stimulus generalizations and can make associations between many different stimuli, lower animals may be restricted in the range of associations that they can make between the various stimulus pairs derived from the behavioral hierarchy. The lead we are following in *Pleurobranchaea* is that before conditioning the CS has a small but finite probability of eliciting the same response as the UCS (*14, 15*); that is, *Pleurobranchaea* seems to learn most readily those tasks that it has some innate capacity to achieve before the conditioning. This suggests to us that learning in this animal might somehow occur in preexisting connections of the nervous system (for example, by some functional or structural change) rather than by a completely de novo process.

To bridge the gap between behavior and neurophysiology, a conceptual framework is needed which states associative learning in the context of a poten-

tially identifiable nerve network that can be supported or refuted by appropriate experimentation. Since learning is defined behaviorally, the putative neuronal connections and mechanisms one might propose must be consistent with the behavioral observations. Therefore, as a first approximation in formulating a testable hypothesis in studying food-aversion learning, we shall begin with the results of various control experiments that provide some indication of what the experimental animals may be associating. The similarity of the results obtained with the explicitly unpaired and random control procedures, taken together with the observation that one-trial conditioning produces significant behavior changes, suggests that the essential feature of food-aversion learning in *Pleurobranchaea* is the temporal pairing between the food and shock stimuli. Food-shock contingencies that might occur between trials may be involved but it seems that, with only ten conditioning trials, this involvement is minimal. The controls also suggest that the order of the stimulus pairing is more effectively weighted in the forward direction with the onset of the CS preceding the UCS. The relatively weak effects produced by all the control procedures compared to those of stimulus pairing indicate that pseudoconditioning and sensitization have minor roles in establishing the food-aversion behavior. Although electrical shock is a relatively nonspecific form of noxious stimulation, the results of the control procedures show that it can be treated as a discrete stimulus with respect to the temporal and causal effects of the food stimulation.

Food-aversion learning in *Pleurobranchaea* might therefore be described as the result of appropriately timed (contiguous or paired) activity in two converging inputs to the nervous system, one associated with food stimulation and the other with noxious stimulation. The statement of convergence seems appropriate from the definition of learning itself, since, if conditioning is to be successful, the effects of the UCS must somehow be communicated with the effects produced by the CS. In the simplest anatomical configuration, this convergence may be represented in terms of two input neurons synapsing on the same third or follower neuron. Such a representation of the neuronal components that might underlie learning in *Pleurobranchaea* is similar to a model proposed by Eccles (*33*) and to an earlier physiological postulate proposed by Hebb (*34*) as the neural basis of learning in higher animals and which has recently been applied in mathematical models of learning in the cerebellum (*35*). Hebb's postulate states that "When an axon of cell A is near enough to excite cell B and repeatedly and persistently *takes part in firing it [B]*, some growth or metabolic change takes place in one or both cells such that A's efficiency *as one of the cells firing B* is increased." The italics are ours and indicate our interpretation of Hebb's postulate that cell A is equivalent to the input of the CS and that its activity is paired with activity in another cell which represents the input of the UCS.

As noted in Hebb's postulate, the alterations in the converging inputs may occur presynaptically between the inputs themselves, postsynaptically through the third cell, or at both loci. In a simple three-neuron model such as we propose,

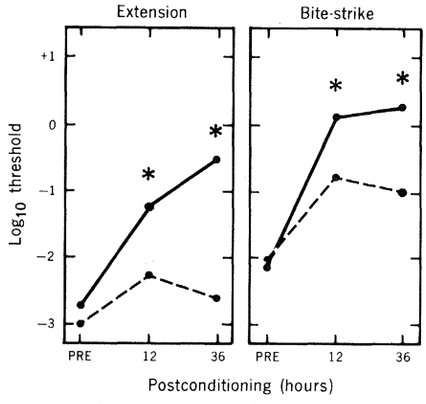


Fig. 15. Effects of electrical stimulation (constant current) on whole animals in aversive conditioning. Experimental animals ($N = 8$) were given the standard food-shock contingency procedure described in Fig. 4; controls ($N = 8$) were given the random procedure described in Fig. 7. The following procedural modification was made to protect the experimenter from the constant current electrical pulses. The food stimulus was applied over the oral veil (0.2 ml/sec) for a maximum period of 90 seconds or 18 ml; as always, food application and resultant observations were conducted by a "blind" observer. If the animal exhibited a bite response within 90 seconds, whatever remained of the food stimulus was quickly dispersed in the conditioning tray and another person independently decided whether or not to activate the electrical stimulator; this decision depended on whether the animal belonged to an experimental or control group. If the animal made no response or only extended its proboscis, the decision to shock was made after 90 seconds. Experimental animals could avoid shocks by remaining in a withdrawal position for 90 seconds. All of these decisions and all shock procedures were withheld from the person who applied the food. Experimental and control animals were individually matched and received equal stimulation and handling. The shocks (200 ma, 10-msec biphasic pulses, 15 pulses/sec) were supplied by a constant current stimulator (*49*) via platinum electrodes placed at each end of the rectangular conditioning trays. These trays were filled with 250 ml of seawater and had dimensions (21 by 11 by 7 cm) which kept the animals oriented head-to-tail with respect to the electrodes.

the experimental-control differences obtained with the paired and unpaired procedures might be less readily handled by presynaptic interactions, as we now know them, than by postsynaptic ones. If collateral axons of the UCS input presynaptically contacted the CS input, similar changes in the efficiency of the CS would result whether the UCS were presented alone, paired, or unpaired with respect to the CS. The only long-term behavioral modification that involves the activation of two afferent pathways and has been studied in detail on the neuronal level is the gill withdrawal response of *Aplysia* (*10*). Repeated tactile stimulation of the siphon produces habituation of gill withdrawal, but a few presentations of a noxious stimulus to the head of the animal causes dishabituation of the gill response. Since temporal pairing of the two stimuli is not required, the dishabituation was attributed to sensitization rather than to associative learning. The sensitization apparently is produced by a serotonergic interneuron which is activated by tactile stimulation from the head and has a presynaptic input on the sensory neurons from the siphon (*11*). With an attractive cellular mechanism of long-term modification already in hand, it is tempting to speculate that the capacity for associative learning might have evolved in the gastropods by including such known presynaptic mechanisms within a more complex neural network or by the addition of cellular interactions between the presynaptic inputs that are not seen in the gill withdrawal response of *Aplysia*. The criteria for associative learning could be met, for example, if the activation of the sensitizing neurons required temporal pairing between the two presynaptic inputs (*36*).

Alternatively, if the communication between the converging CS and UCS pathways were mediated postsynaptically through their common follower neuron, the experimental-control differences obtained in associational conditioning could be accounted for without increasing the complexity of the model nerve network. In this way, one or any number of CS inputs that converged on the same follower neuron could be reinforced as long as their activities were appropriately timed with the activity in the input of the UCS. In such a three-cell model, specificity for the stimulus to be conditioned would be a consequence of the particular occurrence of the environmental events. Specificity in the above presynaptic models would have to be set by the wiring of the nervous system since each pair of conditioned and unconditioned stimuli would require its own sensitizing neuron. But while the

greater simplicity of the postsynaptic three-cell model is attractive, we have no cellular mechanism with which to explain reinforcement, that is, how changes in the efficiency or structure of the CS input occur when there is contingent CS-UCS stimulation.

In a synthesis of information taken from studies on binocularity in neonatal mammals and on regenerating nerve-muscle systems, Stent (*37*) has proposed a cellular model for Hebb's postulate of learning that in fact places the locus of interaction between two converging neurons postsynaptically on their common follower neuron. The central feature of Stent's proposal is that the two inputs compete for occupancy of the postsynaptic sites and that this competition is mediated by action potentials in the follower neuron. Apparently it is the reversal of polarity of the postsynaptic membrane during the action potentials that would weaken the efficiency or completely remove nonactive synaptic sites. Thus, synapses which are coactivated with action potentials in the follower neuron and which do not reverse polarity would be spared. The neonatal animal, however, may be likened to an animal that has already been conditioned. The proposed mechanism shows how activity in both eyes allows binocularly driven cortical neurons to remain binocular, and how by blinding one eye a previously binocular neuron becomes monocular. But to satisfy the conditions for associative learning, one would have to show how renewed and appropriately paired activity in both eyes would cause monocular cortical neurons to become binocular again. By Stent's proposal we would also predict that frequently repeated presentations of the CS by itself might sustain the capacity of the CS to initiate the CR (much as continued vision in one eye sustains the input synapses on the target cortical neuron), but such treatments lead instead to the extinction of the CR. Although Stent's synthesis of disparate lines of behavioral, physiological, and cellular information represents the type of approach that is needed to give directon to physiological investigations, it does not address the necessary criterion for associative learning in Hebb's postulate, which is that pairing of activity in two inputs produces an increase (or decrease, depending on the kind of conditioning) in A's efficiency of firing B.

The concept of competition between converging neurons has considerable heuristic value in considerations of associative learning because it presupposes structural or at least functional mobility in the nervous system. Evidence for competition stems from studies on amphibian and fish neuromuscular regeneration (*38*), the effects of strabismus and visual deprivation in neonatal mammals (*39*), and nerve-nerve regeneration in bilaterally innervated neurons of amphibian cardiac ganglia (*40*). There is, however, little evidence in the behavioral literature that such competition might occur during learning. Insofar as the evidence for learning stems from conditioned changes in the efficiency of the CS, most studies have treated the UCS as an implicit variable, using it in ways to optimize its quality as a reinforcer (*23*). Also, since a common interpretation of Pavlovian theory is that the UCS and UCR should be innate "visceral" reflexes (*41*) attention has been drawn away from possible changes that might occur in the efficiency of the UCS before and after conditioning. Recent studies on rabbits, however, have shown that repeated presentations of a food UCS by itself produce significant changes in its efficiency to elicit the UCR (*42*). But to our knowledge a systematic evaluation of interrelated changes in the efficiencies of the CS and UCS's have not been reported for any experimental system. We have found that water-soluble UCS's, as opposed to electrical shocks, can be quantitatively applied by methods similar to those that we have used to measure the latency and threshold of feeding responses. By comparing the efficiencies of such UCS's with those of the CS before and after conditioning in control and experimental animals it may be possible to obtain behavioral evidence that is interpretable as neuronal competition (*43*).

The three-cell network and competition are obviously a minimal representation for a complex behavioral phenomenon. Missing are explicit statements for important elements such as motivation, drive, and feedback. As we have already indicated in the case of Stent's proposal, competition by itself does not sufficiently account for reinforcement. The three-cell model, however, is consistent with many of the theoretical and experimental aspects of associative learning; it is testable and suggests further behavioral and physiological experimentation. If competition is involved, its cellular processes may be linked with those that underlie reinforcement. With so little known for any experimental system, any physiological mechanism one might imagine is possible. Conditioned associations could be established by holographic, statistical, and other emergent properties that need not be discernible from the specific connectivity of the nervous system (*44*). The particular direction we have discussed here takes advantage of the special technical properties of our experimental animal.

Just as important as the statement of a model system to guide our studies of the nervous system is the technical transition that must be made from investigating behavior to designing experiments appropriate to the nervous system. We believe that it is not enough to look for any neural change. Since learning is defined behaviorally, it seems necessary to show in the functional properties of the nervous system the range of changes observed behaviorally. Even with model behaviors, considerably more may be happening in the animal than is anticipated by the experimenter. Changes may be observed in the nervous system that are experimental-control dependent but which may not be related directly to the mechanisms that generate the learned response; for example, the learned response may reflexively inhibit or excite nonlearned behaviors. It may be necessary for us to record the activity of the nervous system with permanently implanted electrodes while the animal is freely moving and being conditioned; to correlate the behavioral changes with the recorded neural activities; and then, leaving the implanted electrodes attached, excise the nervous system to show the same changes when the nervous system is prepared for single-cell analysis. Eventually, it may be possible to correlate all the various changes observed behaviorally with the properties of the residual nerve network. In this way the defining characteristics of the learned behaviors could be retained at each technical juncture in the analogous responses of the nervous system. By such a combined approach we might eventually be able to direct studies on the identified nerve network to the central feature of associative learning, namely, the cellular basis of reinforcement or of stimulus pairing. It is this feature that sets associative learning apart from other forms of behavior plasticity, and it is the elucidation of this feature that may add a new dimension to the understanding of the nervous system.

Assuming that we shall be able to account for associative learning in *Pleurobranchaea* by means of the properties of a simple nerve network, how applicable will these findings be to other animals? The notion that physiological mechanisms are phylogenetically conserved is widespread, and it has been stated that the mechanisms underlying learning in primitive animals will be found to be the same throughout the animal kingdom (*45*). Although there is evidence for the conservation of mechanisms, there is al-

so evidence for evolutionary analogies (convergence and parallelism) by which different mechanistic strategies have been taken to meet similar adaptive requirements (*46*). Thus, while the comparative approach may show that associative learning ubiquitously follows similar behavioral laws, it is entirely possible that there is considerable species diversity of the underlying cellular mechanisms. Much is known about the properties of single nerve cells and about the kinds of interactions that can take place between two cells. The interaction that can take place between three cells and the behavior that can result from such interaction are largely unknown. The point of our discussion has been that general properties of the nervous system, such as convergence between two neurons onto a third, provide an important transition in language from the widely applicable behavioral terms to those of the nervous system. It is this transition that may provide the necessary conceptual framework for asking experimental questions about the cellular interactions that underlie the behavioral phenomena. Perhaps the most we can expect initially of the comparative approach is some verification of this language, the information it can handle and, eventually, some understanding of the range of biological properties that might be expected of neural tissues.

Summary

For progress to occur in the understanding of the physiological basis of associative learning, two interrelated advances seem necessary, one technical and the other conceptual: a reliable experimental system and a union of theory with experimental findings in a testable statement of what to look for in the nervous system. Toward this goal we believe that a relatively simple animal like the gastropod *Pleurobranchaea* may provide model experimental material for studying learning in terms of the function and structure of visually reidentifiable nerve cells. We have reexamined the definition of learning and discussed some of the problems that have arisen from major controversies over the fundamental nature of learning. We conclude that there may be no single or proper definition of associative learning that can be applied ubiquitously. However, control procedures, which have been at the crux of these controversies, provide important information about the kinds of associations the experimental animals may be making. It is this information that offers the necessary direction for designing cellular studies. Using a broad spectrum of control criteria, we have shown that the food-aversion behavior of *Pleurobranchaea* is attributable to associative learning. The question foremost in our minds is: Can this associative learning be accounted for on the basis of a definable nerve network, one composed possibly of as few as three neurons? To answer this question, our experimental system was designed to have the following properties: rapid and obvious behavior changes so that the quantity of the underlying processes will be maximized at any given time; a conditioned motor switch, rather than the cessation or appearance of a response, so that there will be available continuously in the output of the nervous system some active indication of learning; the use of parameters to measure the stimulus efficiencies and motor responses in ways that can be interpreted directly in neuronal activities; and reproducibility, in order to have a ready supply of animals for neurophysiological studies and for the continued development of the behavioral foundation.

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43. For example, the use of a food CS, with KCl as the UCS rather than electrical shocks, indicates that there is an increase in the threshold of withdrawal responses to KCl once the withdrawal responses are conditioned to the food stimulus; the threshold of withdrawal responses to KCl in control animals that received the food and KCl randomly did not increase. If these findings are substantiated by further experiments with KCl and other water-soluble UCS, the interrelated change in the efficiencies of the food CS and the paired UCS represents the kind of information that may be interpreted as the behavioral manifestations of neuronal competition. This, of course, does not prove the competition hypothesis but provides important information for incorporating and testing in a cellular model.
44. K. H. Pribram, *Languages of the Brain: Experimental Paradoxes and Principles in Neuropsychology* (Prentice-Hall, Englewood Cliffs, N.J., 1971); in *Macromolecular and Behavior*, J. Gaito, Ed. (Academic Press, New York, 1966); E. R. John, *Science* **177**, 850 (1972); W. R. Adey, *Int. J. Neurosci.* **3**, 271 (1972).
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46. D. Kennedy, *J. Exp. Zool.* **194**, 35 (1975); K. Z. Lorenz, *Science* **185**, 229 (1974); G. J. Mpitsos, *J. Neurophysiol.* **36**, 371 (1973).
47. A detailed account of the effect of aversive conditioning on the behavioral hierarchy is being prepared by G. J. Mpitsos, S. D. Collins, W. J. Davis, and J. M. Pinneo; this includes a substantiation of present findings.
48. The χ^2 test was used for all withdrawal data; the Mann-Whitney U test was used for all latency and threshold data. Unless otherwise indicated, significance values are at $P < .01$, as labeled by asterisks.
49. A. D. McClellan, *Behav. Res. Methods Instrum.*, in press.
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