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10 April 1978; revised 6 July 1978

Retention of an Associative Behavioral Change in *Hermissenda*

Abstract. *The nudibranch mollusk* Hermissenda crassicornis *is normally attracted to a test light. Three days of training consisting of 50 trials per day of light paired with a rotational stimulus led to a significant increase, lasting for days, in the animal's response latency to enter a test light. The group that received light associated with rotation was significantly different from groups subjected to nonassociative control procedures. Modifications of well-known sensory networks may be related to a behavioral change that shares several operational features with associative learning.*

Invertebrates have been selected for study in investigations of the physiology of learned behavior because their simpler nervous systems are more amenable to a cellular analysis (*1–4*). Some of the characteristics of learned behavior have been examined at the behavioral and cellular levels for nonassociative behavioral modifications such as habituation and sensitization (*2*). For gastropod mollusks, behavioral changes that may be dependent on the temporal association of two sensory stimuli have only recently been explored (*3*, *4*). Methodological questions have been raised concerning behavioral changes reported to be examples of aversive conditioning for the mollusk *Pleurobranchaea californica* (*3*). In addition, for this preparation an analysis has not been made of the neuronal interactions within the relevant sensory pathways.

Stimulation of two sensory systems in the nudibranch mollusk *Hermissenda crassicornis* with natural stimuli has resulted in a short-term change in both the intact preparation and the isolated nervous system (*4*, *5*). We now report a long-term behavioral change that shares several operational features with associative learning. This behavioral change lasts for several days, persists during repeated testing, is reversible, and is dependent on the temporal association of two sensory stimuli. An examination of the cellular mechanisms underlying this behavioral change in *Hermissenda* may be useful, therefore, in understanding cellular mechanisms of associative learning.

The visual and statocyst pathways of *Hermissenda* consist of a relatively small number of cells whose synaptic relations and cellular organization have been examined in detail with intracellular recording and histological techniques (*6*). Stimulation of these pathways with light and rotation in subjects trained and tested en masse has resulted in a short-term change in the animals' normal attraction to a light stimulus (*4*) and in correlated neural changes (*5*).

To determine whether this behavioral change is long-term and dependent on the association of light and rotation we examined changes in response latencies of individual animals to enter a test light following paired, unpaired, and random presentations of light and rotation.

Animals (N = 115) weighing 0.2 to 1.2 g were maintained separately in plastic mesh containers in a fresh flow-through seawater system (1 liter/min at 15°C). The animals (*7*) were fed small pieces of squid daily and placed on a cycle of 6½ hours of light in 24 hours. Training and testing were carried out during the daily light cycles. After at least 3 days of this light schedule, the animals' baseline response to enter a light spot was measured. The data collection system and training procedures were completely automated. The animals were transferred from the plastic containers to glass tubes 228 mm long filled with seawater (Fig. 1). A plug inserted through an opening in the tubes confined the animal to one end of the tube. The tubes were then inserted into ten holders on a modified turntable (Fig. 1) enclosed in an incubator at 15°C. A light spot (*8*) was projected onto the center of the turntable, illuminating a circular area 10.16 cm in diameter with ten photocells on its circumference. Response latencies were recorded when a *Hermissenda*, entering the light spot, interrupted the light between the source and a photocell, and thus triggered an event marker on a ten-channel recorder.

After the baseline measurements, the animals were randomly assigned to treatment and control groups. Training during the acquisition phase consisted of a total of 150 trials (50 trials per day). A trial for the treatment group (N = 22) consisted of 30 seconds of light (*8*) paired with 30 seconds of rotation (*9*) with a variable intertrial interval (range, 1 to 3 minutes). Five groups were run to control for ef-

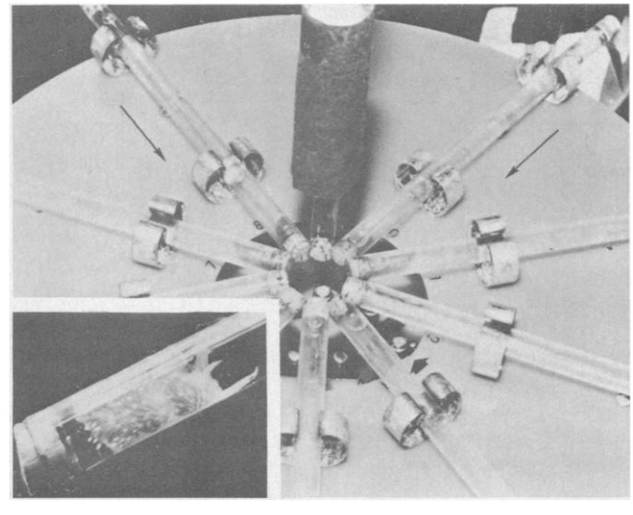

Fig. 1. Training and testing apparatus. The response latencies to enter a light spot projected onto the center of the turntable by an overhead illuminator were recorded automatically when the *Hermissenda* moved toward the light source (direction of arrows) and interrupted the light between illuminator and photocells (arrowhead). (Inset) *Hermissenda* were subjected to different behavioral treatments consisting of light and rotation while confined to the end of glass tubes filled with seawater.

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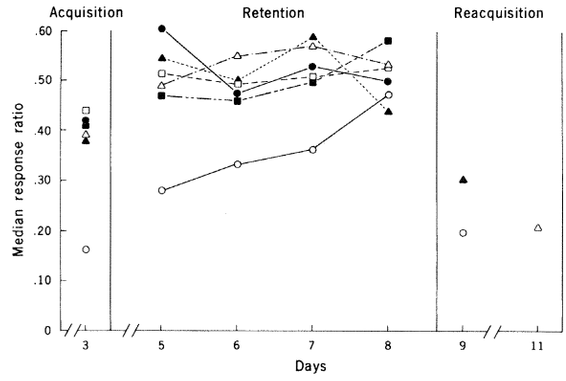

Fig. 2. Median response ratios for acquisition, retention, and reacquisition of a long-term behavioral change in response to a light stimulus in *Hermissenda* [random rotation (●), random light (□), unpaired light and rotation (△), random light and rotation (▲), nothing (■), and paired light and rotation (○)]. The response ratio [in the form of 1 − A/(A + B)] compared the latency during the test (A) with the baseline response latency (B). Group data consists of two independent replications for all control groups and three independent replications for the experimental group.

fects of nonassociative factors (*10*): rotation programmed on a random schedule (N = 19), random light and rotation (N = 17), light programmed randomly (N = 20), unpaired light and rotation (N = 19), and no light or rotation (N = 18) (*11*). The stimulus presentations, stimulus duration, interstimulus intervals, intertrial intervals, and number of training trials were automatically controlled with solid-state programming equipment. A detailed description of the circuits will be available elsewhere (*12*).

At the end of acquisition (day 3) the response latencies to enter the light were measured for all groups (Fig. 2). Two days after the last acquisition trial the animals were tested for retention of the response on each of four consecutive days (days 5 through 8), and response latencies to enter the light spot were measured. One day after the last retention test, some animals received additional training. This reacquisition phase (days 9 through 11) consisted of 50 trials of light paired with rotation for the previous experimental group and previous random control group, while the previous unpaired control group received 150 trials of light paired with rotation (50 trials per day) over three consecutive days (days 9 through 11).

The response to enter the light at the end of acquisition, during retention, and during reacquisition was assessed by means of ratios that compared the latency during the test to the baseline response latency (*13*). A ratio of .50 indicated that the baseline and treatment latencies were identical, while a ratio of greater than .50 indicated a decrease in the response latency to enter the light during testing and less than .50 indicated an increase in response latency to enter the light during testing (Fig. 2).

There were no significant differences between groups before the start of acquisition. In contrast to the baseline latencies there were significant differences between the groups on day 3 of acquisition (Kruskal-Wallis one-way analysis of variance, $P < .001$). Dunn's multiple comparison procedure revealed that the treatment group that received paired light and rotation was significantly different from all of the respective control groups ($P < .001$), and none of the control groups were significantly different from each other (*14*). Although the unpaired and random control groups did not differ from each other or the remaining control groups, their scores were significantly different from their pretest scores (Wilcoxon matched-pairs signed-ranks test, $P < .01$ and $P < .05$, respectively). This could be accounted for on the basis of chance pairings between stimuli during random presentations, although all control group latencies during the acquisition phase were significantly longer than the baseline latencies. Significantly longer latencies were not observed for the control groups over the retention days. There is evidence that random stimulus schedules may yield some positive behavioral results (*15*). The significant differences between the control groups and the paired group suggest that the random control group provided a conservative baseline against which associative factors could be evaluated.

Differences between groups during the retention phase (days 5 through 8) were evaluated by pooling response ratios over days 5 and 6 and days 7 and 8. The overall difference between groups for days 5 and 6 was significant (Kruskal-Wallis test, $P < .001$). Dunn's multiple comparisons revealed that the experimental group was significantly different from all control groups ($P < .005$), and the control groups did not differ from each other. An identical analysis of the pooled data from retention days 7 and 8 did not reveal significant differences. This finding can be attributed to the data from retention day 8. A separate analysis of overall differences between groups on retention day 7 was significant (Kruskal-Wallis test, $P < .05$), but no significant overall differences were obtained on day 8. The results again demonstrate that paired light and rotation lead to a significant increase in response latencies, retained for several days, to enter the test light. This behavioral change lasts for several days, and the response latencies gradually return to pretest values (day 8).

One day after the last retention day (day 9), some animals received additional training (reacquisition) to determine whether previous control animals could exhibit the associative behavioral change. Animals from the second replication of the paired group (N = 10) and random control group (N = 8) received 50 trials of light paired with rotation and were tested immediately after this training session. Animals from the previously unpaired control group (N = 9) now received 3 days of paired training, consisting of a total of 150 trials of light paired with rotation as in original acquisition.

The paired group was still significantly different from the random group on the reacquisition test (day 9) (Mann-Whitney U test, $P < .025$), even though the groups were not different on day 8 and each group received the same number of training trials on day 9. The unpaired group that had initially received unpaired stimuli in the acquisition phase, and now received paired light and rotation, was not significantly different from the originally paired group after the three days of additional training. The performance of the paired group on day 9 was not significantly different from their performance at the end of acquisition (day 3), but the random group was significantly different from their own baseline level (Wilcoxon test, $P < .01$). These results from the reacquisition phase suggest that the paired group exhibited behavior similar to savings (*16*). After 50 reacquisition trials these animals were not significantly different from their performance at the end of 150 training trials (acquisition day 3), although they were different from random controls which received 50 reacquisition trials of light paired with rotation. In addition, these data show that the behavior of control animals could be modified by the appropriate behavioral manipulations. The performance of the previously unpaired control group at the end of reacquisition (day 11), following 150 trials of light paired with rotation, was not significantly different from the paired group on reacquisition day 9.

This behavioral change to a light stimulus in *Hermissenda* was retained for several days following training, gradually returning to baseline levels after several days of testing for behavioral retention. The subsequent reacquisition suggests that acquisition was more rapid following original training. The significant differences between the paired group and controls at the end of acquisition and retention demonstrates that the behavioral change was dependent on the temporal association of light and rotation. Therefore, this change in behavior exhibited some of the defining characteristics of associative learning (*17*). An examination within well-defined neural networks of the cellular mechanisms underlying this associative behavioral change in *Hermissenda* may provide a basis for studying operationally similar processes in more complex neural systems.

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8. The intensity of the training and testing lights (7.2×10^3 ergs cm^{-2} sec^{-1} and 5.2×10^3 ergs cm^{-2} sec^{-1}, respectively) was measured with a radiometer (Yellow Springs 65A).
9. The subjects received a rotational stimulus during the acquisition phase equivalent to 2.24*g* at 93.6 revolutions per minute. The near maximum velocity of the turntable followed the onset of the light stimulus with a 250 msec delay, reaching maximum velocity in 5 seconds.
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11. The light and rotation were independently programmed to occur an equal number of times on a random schedule over the same time periods as the training session for the experimental group. The unpaired control group received stimuli programmed on a random schedule of interstimulus intervals with the restrictions that the stimuli could not overlap in time and could not occur more than once without being followed by the second stimulus.
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18. Supported by National Research Service Award 1 F32 NS05021-12 to T.J.C. We thank C. Tyndale for technical assistance.

9 January 1978; revised 10 May 1978

Modification of Attention in Honey Bees

Abstract. *Honey bees were trained in two consecutive two-dimensional (color-position) problems with one dimension (color or position) relevant and the other irrelevant in each problem. As in analogous experiments on dimensional transfer in rats and monkeys, performance in the second problem was more accurate when the relevant and irrelevant dimensions were the same as in the first problem than when they were interchanged. The results of further experiments suggest that the transfer is mediated by different modes of responding that develop in color and position problems rather than by some special process of dimensional selection, such as has been assumed to operate in vertebrates.*

Transfer experiments with rats and monkeys (*1*) suggest that discriminative training may alter attention not only to the stimuli encountered in training but also to novel stimuli varying in the same dimensions (*2*). The procedure is to train animals in consecutive two-dimensional problems with one relevant and one irrelevant dimension in each problem and with a different set of stimuli in each. For one group of subjects (the intradimensional group), the relevant and irrelevant dimensions remain the same from one problem to the next; for another (extradimensional) group, the relevant and irrelevant dimensions are interchanged. If attention to the relevant dimension tends to be increased by differentially reinforced experience with stimuli varying in that dimension and attention to the irrelevant dimension tends to be reduced by nondifferential reinforcement, intradimensional performance should be more accurate than extradimensional performance. Although such results have been obtained in experiments with rats and monkeys, the results for pigeons are inconclusive (*3*, *4*) and those for carp and goldfish entirely negative (*4*, *5*). It is particularly interesting, therefore, to find results like those for rats and monkeys in honey bees.

One of the dimensions used in our experiment was color. On each trial, two square targets of plastic, each 4.5 cm on a side, were presented, one yellow and the other orange or one green and the other blue. These pairs of colors provided equal discriminability and negligible generalization from pair to pair. The targets were laid on a square white background, 40 cm on a side, which was fixed to the top of a rectangular table in a small laboratory room just before a door opening to the outside.

The second dimension used was position. On each trial, the two targets were arranged either latitudinally (one to the right and one to the left of the entrance) or longitudinally (one to the front and one to the rear), 15 cm apart edge to edge. The spatial dimension was chosen on the basis of earlier research (*6*) and after pilot experiments had shown that the animals could readily learn to go to position (right rather than left or front rather than rear) independently of color in both spatial arrangements.

The 32 subjects were trained in each of two consecutive 20-trial problems, the first with one of the two pairs of colors in one of the two spatial arrangements and the second with the alternative pair of colors in the alternative spatial arrangement. Within each problem, each color appeared equally often in each of the two positions. For example, in the longitudinal yellow-orange problems, yellow was half the time at front and half the time at rear in quasi-random order (*7*). Half the subjects were trained first with yellow and orange targets and half with green and blue targets; half were trained first with the latitudinal arrangement of the

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