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Classical Conditioning of *Hermissenda*: Origin of a New Response

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Training of the marine snail *Hermissenda crassicornis* with paired light and rotation was previously shown to result in acquisition and retention of a behavioral change with many features characteristic of vertebrate associative learning. Here, this behavioral change is demonstrated to be classical, Pavlovian-like conditioning. A new response to light is formed (the CR) that is pairing-specific and resembles the unconditioned response (UCR) to rotation. The conditioned and unconditioned responses are relatively rapid, occurring within seconds of the onset of light or rotation stimuli, and correspond to pairing-specific reductions in speed during the same time period. Since the CR is independent of the presentation of rotation, and it is also expressed by the same effector system (the foot) responsible for the UCR, light stimulation has assumed some of the functional character of rotation.

The development of invertebrate models of associative learning and memory depends on a compromise between nervous systems accessible to state-of-the-art analyses and learning behavior with features identified for vertebrate species. Some of the promising invertebrate models include instrumental leg-position learning in locust (Forman, 1984; Horridge, 1962; Tosney and Hoyle, 1977), odor aversion learning in Limax (Sahley et al., 1981a, b), chemosensory aversion learning in *Pleurobranchaea* (Mpitsos and Davis, 1973; Mpitsos et al., 1978), and gill- and siphon-withdrawal in *Aplysia* (Carew et al., 1981, 1983; Lukowiak and Sahley, 1981).

For *Hermissenda,* associative learning has been measured as reduction in positive phototaxis (Alkon, 1974). Pairing-specific increased latencies to enter the illuminated portion of a light-intensity gradient have been measured on days following experience with paired light and rotation stimuli (Crow and Alkon, 1978; Farley and Alkon, 1982; Harrigan and Alkon, 1985). Whereas during baseline tests before training, naive animals enter the light within several minutes, after training with paired light and rotation, these same animals may not enter for up to 2 hr. Individuals trained with random occurrences of the same number of light and rotation stimuli do not change significantly relative to their pretraining baselines. Aside from this pairing specificity, *Hermissenda* associative learning satisfies a host of criteria traditionally applied to vertebrate conditioning: stimulus specificity (Crow and Offenbach, 1983; Farley and Alkon, 1982), long-term retention (Crow and Alkon, 1978; Harrigan and Alkon, 1985), extinction (Richards et al., 1984), savings (Crow and Alkon, 1978), and contingency sensitivity (Farley, in press). Particularly important, nonassociative behavioral modifications could not be produced by repeated presentations of the conditioned stimulus (CS) or of the unconditioned stimulus (UCS) (Crow and Alkon, 1978). There is no persistent suppression of phototaxis that might be interpreted as sensitization, habituation, or pseudoconditioning (Farley and Alkon, 1985).

We know that at least some of the behavioral changes contributing to the overall reduction in positive phototaxis occur shortly after the test light is presented. One manifestation of this is the increased latency to initiate locomotion toward the light source (Crow and Offenbach, 1983; Farley and Alkon, 1982). Even though the time to initiate locomotion (measured as a latency to reach a criterion distance) is on the order of several minutes, it is clear from other observations (Barnes and Lederhendler, 1981; Lederhendler and Alkon, 1984; Lederhendler et al., 1983; I. Lederhendler and D. L. Alkon, unpublished observations) that some changes happen immediately on presentation of the light. Individuals do move and locomote in very dim light during the time prior to reaching the criterion distance by which the start of locomotion has been measured (Crow and Offenbach, 1983; Farley and Alkon, 1982). This means that even in the least illuminated regions of the gradient, individuals experience changing levels of illumination. Thus, the increased latencies to "initiate" locomotion may be the consequence of a fundamental change in the responses of associatively trained *Hermissenda* to contrasting levels of light intensity (Lederhendler and Alkon, 1984). Furthermore, in at least one identified visual pathway (Goh and Alkon, 1982, 1984), training-dependent reductions in motor output within the first 5 sec of light stimulation were directly correlated with the increased latency to enter the light (Goh et al., 1985; Lederhendler et al., 1982). In associative learning with *Hermissenda,* light has been used as the CS. In the present study, we have defined one response that occurs within 6 sec of light onset: foot-lengthening.

Furthermore, in this paper we show that in tubes, in the absence of light, the response to rotation (the UCR) is a reduction in the length of the foot. Alkon (1974) originally noted that *Hermissenda* placed in open beakers containing seawater responded to turbulence by increasing contact with the substrate through a "clinging" response. One feature of this clinging appeared to be a configurational change of the foot (the single organ of locomotion) as it increased its contact with the substrate. We present evidence that after training with paired light and rotation stimuli, a new response to light occurs that resembles the UCR—a decrease in length of the foot.

These findings suggest that both of the essential characteristics of classical conditioning as a model of associative learning may be satisfied here (Gormezano, 1984; Gormezano and Moore, 1969). The CR (light-elicited foot-shortening) is independent of the presentation of the UCS. And, in addition, the CR is elicited by the same effector system (the foot) that elicits the UCR. The light therefore appears to have taken on some functional character of the rotation.

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Materials and Methods

General methods

Adult *Hermissenda crassicornis* 2–4 cm in length were provided by M. Morris (Sea Life Supply, Sand City, CA) and D. Anderson (Charleston, OR) and maintained individually at 13°C in a recirculating aquarium. The aquarium was filled with 50 gal of fresh filtered (100 μM) sea water. Animals were maintained in this system for 4–5 d before an experiment began. They were fed 20–40 mg of tunicate viscera daily. The animals were maintained on a diurnal light cycle of 12 hr light: 12 hr dark. Testing always began after at least 2 hr of light and individuals were always returned to the aquarium before the light went off. The intensity of light from a modified 40 W fluorescent tube that reached the animals in the aquarium was approximately 5×10^{-1} μW cm^{-2}. All light measurements were made with or calibrated to a radiometer (Yellow Springs Instrument Co., Yellow Springs, OH; Model 65A).

Photography

The principle that guided the design of the photographic techniques described here was to obtain clear images of the foot under conditions of varying illumination (e.g., before and after stimulation with light) without needing to adjust camera settings. This was accomplished by using film sensitive to red light and filtering the strobes and subilluminating lights with red filters. We used a motor-driven Nikon FM2 35 mm camera with a 55 mm Micro-Nikkor lens, fitted with a Schott RG-630 and Tiffen polarizing and dichroic filters. Four Vivitar 283 flash units with VP-1 Varipower modules and two Tensor high-intensity subilluminating lights, all fitted with Schott RG-665 and Tiffen polarizing and dichroic filters, were used to illuminate the subject. Some of the experiments used three 6.6 W fluorescent tubes filtered with a Kodak No. 2 safelight and polarizing filters for subillumination.

The camera and flash units were mounted on a modified motorized x–y plotter to manually track the moving animal. The shutter release was triggered manually or by a photoelectric cell linked to a delay circuit. We used Kodak Recording 2475 film throughout. Time was monitored with a digital chronometer mounted in the camera field of view.

This system provided clear images of *Hermissenda* in the "dark," during rotation, as well as in the light, without the need to adjust camera settings. Intracellular recordings from Type B photoreceptors, dark-adapted for 10 min and stimulated with the filtered flash units, showed no generator potential or increased discharge frequency for at least 60 sec after the flash.

Response to rotation (UCS)

Animals were placed in a 1.4 cm diameter Plexiglas tube filled with filtered seawater. The tube was mounted on a motorized turntable 25 cm in diameter 15 cm below the camera. The animals were restrained to the end of the tube and dark-adapted to the red subilluminating light for 7 min. At this time, the restraint was removed and the position of the tube was adjusted so that the foot of the animal was toward the camera. The animals began to locomote in the dark, and several baseline photographs were obtained at the rate of one per second. Rotation, at 97 rpm, was started and photographs were obtained during the next 10 sec.

Response to light (CS)

The techniques for obtaining pictures of the response to light were the same as those described in the previous section. A fiberoptic bundle, mounted 10 cm above the center of the tube, and fitted with an optical diffuser was fed from a single 150 W tungsten halogen source. This directed illumination along the length of the tube to form a horizontal gradient 2.3×10^{3} μW cm^{-2} at the center and 0.2×10^{2} μW cm^{-2} at the periphery. Animals were dark-adapted to the dim red subillumination for at least 6 min while restrained to the end of the tube. The restraint was removed and the position of the tube adjusted so that the foot of the animal was toward the camera. After the animal had begun locomotion, several photographs were taken to provide baseline measurements in the dark. When the subject reached a preset position 20 cm from the center of the turntable, the light was turned on. The intensity of illumination reaching the animal at this point was 1.3×10^{2} μW cm^{-2}. One picture was always obtained simultaneously with the onset of the light to provide a calibration point for stimulus duration. Two to four pictures were obtained during the next 6 sec of light stimulation.

Training

After baseline responses were obtained, usually over a 2 d period, individuals were randomly assigned to each of the treatment groups: Paired, Random, or Naive. For the Paired group, training consisted of 50 trials a day for 3 d in which 30 sec of diffuse white light (1.3×10^{2} μW cm^{-2} in the periphery) was presented at random intervals (0.5–4 min), together with 30 sec of rotation (97 rpm). Maximum rotation was estimated to begin about 500 msec after the onset of light. The Random group was presented with the same total number of light and rotation stimuli but on separate random programs such that no consistent association between them occurred. In fact, the program that determined random stimulus presentations contained 25–33% of trials in which the light and rotation stimuli coincided by chance alone. The presentation of light and rotation was controlled by an automated timing circuit (Tyndale and Crow, 1979). The Naive group was retested after 3 or 4 d of standard maintenance. Paired and Random animals were tested 16–28 hr after the conclusion of training.

Measurement and statistics

After the end of training and testing, the negatives were mounted as slides. The slides were coded to hide identification of the treatment groups until after measurements were obtained. Slides were projected onto a Photooptical Digitizer (L-W International, Woodland Hills, CA, model 110) for direct scaled measurements of the foot. Length measurements for each slide were scaled to a standard 2 cm calibration mark etched onto the test tube. Each measurement was repeated seven times and the mean ($\pm$SD) of these measurements recorded. If the SD was greater than $\pm$0.005 cm the measurement was repeated and the set of values with the smaller SD was used.

The greatest length of the foot in the dark before light stimulation was used as the standard referent value. During rotation, the lengths after 1, 3, 6, and 9 sec of stimulation were subtracted from the prerotation baseline referent to provide a difference score. Isochronal points could be obtained from individual to individual because the turntable speed acted as a precise timer. Pictures of the response to light were obtained manually so that the time intervals between pictures varied $\pm$1 sec. To estimate the lengths in response to light, therefore, we used the mean of the smallest and largest values obtained during the first 6 sec of light stimulation.

We also chose to monitor displacement in response to light adjusted for varying time intervals. This measure (speed) was obtained during comparable intervals before and after training for each individual within 6 sec before and after light onset. We hoped the measure would help assess the relationship between foot shortening and other measures of phototaxis (particularly latency measures) that have been used previously. The findings of previous studies (Crow and Alkon, 1978; Crow and Offenbach, 1983; Farley and Alkon, 1982) showed that, after training, Paired animals locomote more slowly than either the Random or Naive animals. We wanted to see if this finding held during the initial seconds of light stimulation. It should be noted that this is a direct measurement of changes in speed in response to light, not a latency to initiate locomotion, because the animals were already locomoting in the dark.

Several criteria were applied to the photographic images to determine their acceptability for inclusion in the sample. Two of these were technical, and one monitored the well-being of test animals. Individuals were judged as "not thriving" if their dark baseline lengths after training were significantly shorter ($p < 0.05$; t test) than before training. Nine individuals were excluded on this basis. This selection occurred before identification of their treatment group. The two technical selection criteria dealt with the positions and configurations of the animals, which we could not control once the photography began. To assure valid measurements, the entire image of the foot had to be within the central two-thirds of the long axis of the tube. In addition, the entire length of the foot had to be in contact with the substrate. This was determined directly from the photographs as portions of the perimeter that were not in focus. These technical difficulties resulted in numerous individuals whose responses we could not measure.

Between-group differences in changes in length were assessed with a one-way analysis of variance (Winer, 1971). *A posteriori* t tests were used to compare means directly. Non-parametric comparisons were based on Siegel (1956).

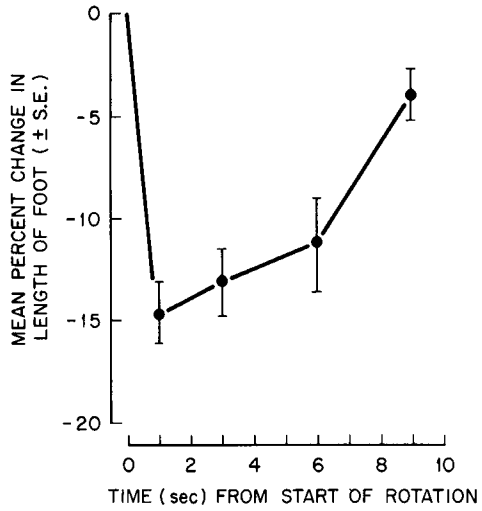

Figure 1. Unconditioned response of the foot of *Hermissenda* elicited by rotation (97 rpm) in the dark. Time intervals monitored 1, 3, 6, and 9 sec after the start of rotation were compared to those just prior to the start of rotation.

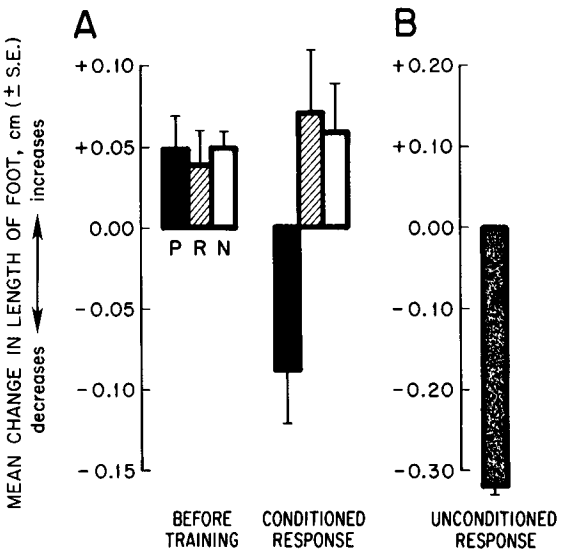

Figure 2. Classical conditioning of a new response to light in *Hermissenda*. *A*, Before training, foot length increased in response to light onset. Random (▨) and Naive (□) groups continue to show light-elicited lengthening, but Paired (■) animals became conditioned to shorten the length of the foot in response to a light stimulus. *B*, Unconditioned response after 6 sec of rotation. The CR is about 28% of the UCR. Note the difference in scale between *A* and *B*.

Results

Figure 1 shows the change in foot length as a function of the duration of rotation in the dark. The response itself is illustrated photographically in the bottom panel of Figure 3. The length of the foot decreased in all 20 animals tested. Mean length (±SD) in the dark just prior to rotation was 2.91 ± 0.49 cm. Average increases in foot length were 14.8% at 1 sec into rotation (n = 20), 13.1% after 3 sec (n = 20), 11.1% after 6 sec (n = 4), 3.7% after 9 sec (n = 4). The sample size diminished rapidly after 6 sec as a result of a combination of configurational and positional changes with continued rotation. Usually, this meant the axis of the foot shifted too far to one side of the tube. But the results indicate that foot shortening is greatest within 6 sec after the start of rotation and subsequently appears to diminish.

The three groups of animals whose responses to light were tested did not differ in baseline lengths of the foot (maximum length in the dark). Mean lengths (cm ± SD) were as follows: *Before training*—Paired, 2.78 ± 0.32; Random, 3.14 ± 0.69; Naive, 2.85 ± 0.32. *After training*—Paired, 3.05 ± 0.35; Random, 3.27 ± 0.67; Naive, 3.06 ± 0.44.

Table 1 shows that before training 19 out of the sample of 25 individuals responded to the onset of light by lengthening the foot. The binomial probability associated with such a distribution occurring by chance is $p = 0.007$. This small but quite reliable lengthening is illustrated photographically in the upper-left panel of Figure 3.

No between-group differences were found before training in the changes in foot length in response to light (Fig. 2). After training with either paired or random presentations of light and rotation stimuli, significant between-group differences were found in the changes of foot length in response to light ($F(2,22) = 5.61$, $p < 0.05$). *A posteriori* comparisons of the means indicated that Paired animals were significantly different from both the Random ($t(14) = 2.98, p < 0.01$) and Naive ($t(15) = 2.95, p < 0.01$) groups. The latter two groups were not significantly different from each other. Examination of Table 1 shows that all the paired animals showed a reduction in foot length in response to light stimulation after training. And, in fact, only the Paired group showed a significant change to a shortening response after training compared to before training (McNemar Test of the significance of changes; $\chi^2 = 7.00$, $p < 0.01$).

Changes in speed in the dark and within 6 sec of presentation of the light were assessed as a consequence of training. No significant between-group differences were found for speed in the dark before training (Paired, 2.03 ± 0.30 mm/sec; Random, 1.90 ± 0.44 mm/sec; Naive, 1.89 ± 0.30 mm/sec). Similarly, speeds in the light before training did not differ significantly ($F(2,20) = 2.57$) between the groups (Paired, 2.30 ± 0.75 mm/sec; Random, 1.67 ± 0.56 mm/sec; Naive, 2.29 ± 0.47 mm/sec). However, since the Random group did appear to be slower, *post hoc* paired comparisons were applied, and they supported the original conclusion of no differences. In any case, an intrinsically lower Random group would have a conservative influence on the directional hypothesis that the Paired animals become slower than Random or Naive animals. In fact, as Figure 4 illustrates, the mean relative change in speed in the light, but not in darkness, was significantly different between the treatment groups ($F(2,20) = 4.21$, $p < 0.05$). *A posteriori* paired comparisons of the means showed that the Paired animals were significantly slower than either the Random ($t(12) = 2.18$, $p < 0.05$) or Naive ($t(14) = 2.45$, $p < 0.05$) animals.

These results indicate that after training, Paired animals exhibit a new response to light—shortening rather than lengthening

Table 1. Frequency of occurrence of increased or decreased lengths of the foot of *Hermissenda* to light onset

	Before Training		After Training	
Treatment	Number increased	Number decreased	Number increased	Number decreased
Paired	7	1	0	8
Random	5	3	5	3[a]
Naive	7	2	6	3[b]
Total	19	6	11	14

[a] These individuals were different from those that decreased in length before training.

[b] One of these individuals decreased in length of the foot before training.

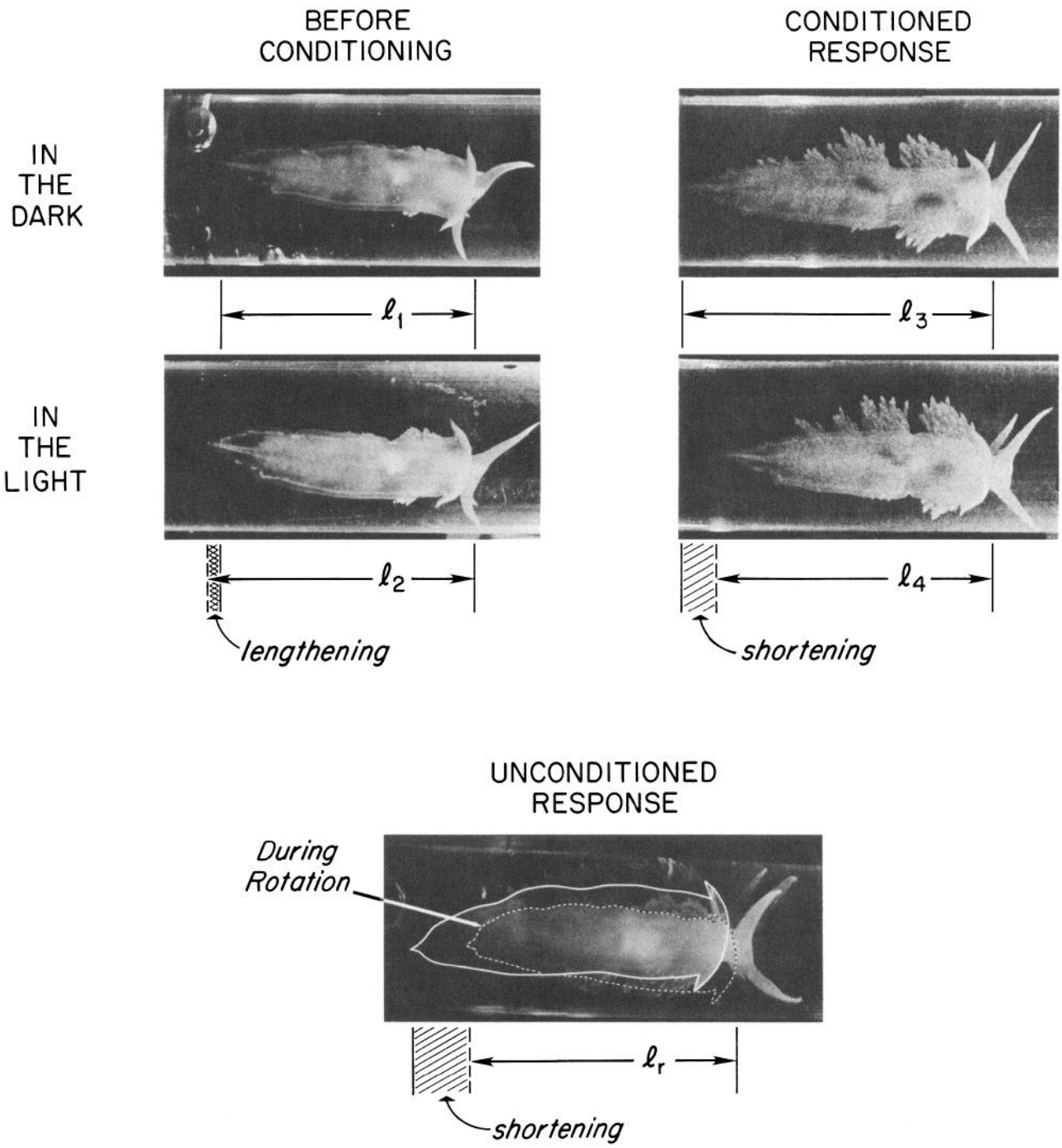

Figure 3. Photographic representation of classically conditioned responses of *Hermissenda* foot. *Bottom panel,* An overlay of two photographs taken in the dark; *solid white line,* the outline of the foot 1 sec before rotation; *dashed line,* outline of the foot after 3 sec of rotation at 97 rpm. *Upper panels,* Comparisons of lengths in the light to lengths in the dark before and after conditioning with paired light and rotation stimuli. ℓ_1, Length in the dark before training. ℓ_2, Length in the light before training ($\ell_2 > \ell_1$ = lengthening). ℓ_3, Length in the dark during retention of learned behavior. ℓ_4, Length in the light during retention ($\ell_3 > \ell_4$ = shortening). ℓ_r, Length during rotation was always smaller than before rotation began.

the foot. This response happens within the first 6 sec of light stimulation. During this same 6 sec period, these same animals, as a group, exhibit a significantly reduced speed in the light.

Discussion

In *Hermissenda* the experience of pairings of light and rotation stimuli has the effect of forming a new response to light (the CR)—foot-shortening—that resembles the response to rotation (the UCR). However, since locomotion in *Hermissenda,* as in gastropods generally, depends on muscular, secretory, and ciliary processes, foot-shortening need not occur exclusively through muscular contractions. Increased adhesion of the mucus or reduced ciliary beating might produce similar effects.

The description of classically conditioned features of associative learning in *Hermissenda* became possible with the specification of an objective measure of the UCR. There may be other UCRs to rotation, or to different intensities of rotation stimuli, that we have not yet examined. These other measures

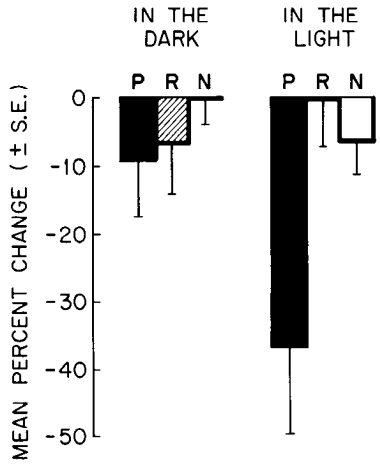

Figure 4. Percentage change in speed in the light and in the dark during retention, as compared to before conditioning. Speeds were calculated from photographic measurements of displacement 6 sec before and 6 sec after light onset. The reduction in speed in the Paired animals (*P*) was significantly greater than in the Random (*R*) or Naive (*N*) treatments in response to light stimulation, but not to dark.

may or may not show classical conditioning. However, the accumulated evidence to date (Alkon, 1984; Alkon et al., 1982, 1985; Crow and Alkon, 1980; Farley et al., 1983; Goh et al., 1985; Lederhendler and Alkon, 1984; West et al., 1982) indicates that primary and intrinsic conductance changes in the medial Type B photoreceptor, which persist after training, initiate a cascade of effects within different identifiable neuronal pathways that modify the expression of at least several different visually dependent behaviors. Specific conditioning-induced output changes in the photoreceptors need not be expressed in all visual behaviors. Rather, different components of the photoreceptor output can influence various behaviors, depending on which neural pathways are excited and the circumstances of the behavioral observations.

It is important to emphasize that the ionic conductance changes have been related to *Hermissenda* visual behavior after dark adaptation and for an identified Type B photoreceptor—the medial B cell. Crow's (1985b) report of reduced depolarization and impulse activity is related to different stimulus conditions (after 5 min of light adaptation) and for unidentified Type B photoreceptors. It is significant, therefore, that in the present study we have demonstrated behavioral changes within seconds of the onset of light stimulation.

Conditioned foot-shortening may mark the beginning of a series of learned modifications of visual responses. This series includes a delay in the time to start locomotion at the dim end of the gradient (Crow and Offenbach, 1983; Farley and Alkon, 1982), reduced speed of locomotion while moving toward the center of the gradient (Farley and Alkon, 1982), reduced response to the contrast differences that make up the gradient (Lederhendler and Alkon, 1984; and unpublished observations), increased latencies to enter the brightest part of the gradient (Crow and Alkon, 1978; Crow and Offenbach, 1983; Farley and Alkon, 1982; Goh et al., 1985; Harrigan and Alkon, 1985), and, finally, reduced preference for the more intense areas of illumination (Crow, 1985a; Lederhendler et al., 1980). Modifications that may be primary have been identified in two of these behaviors: they occur early and they may greatly influence the other response measures. These modifications are conditioned foot-shortening, as reported in the present study, and the reduced ability to detect contrast differences in the gradient (Lederhendler and Alkon, 1984; and unpublished observations). Both are especially noteworthy because they match closely the time course of cellular correlates.

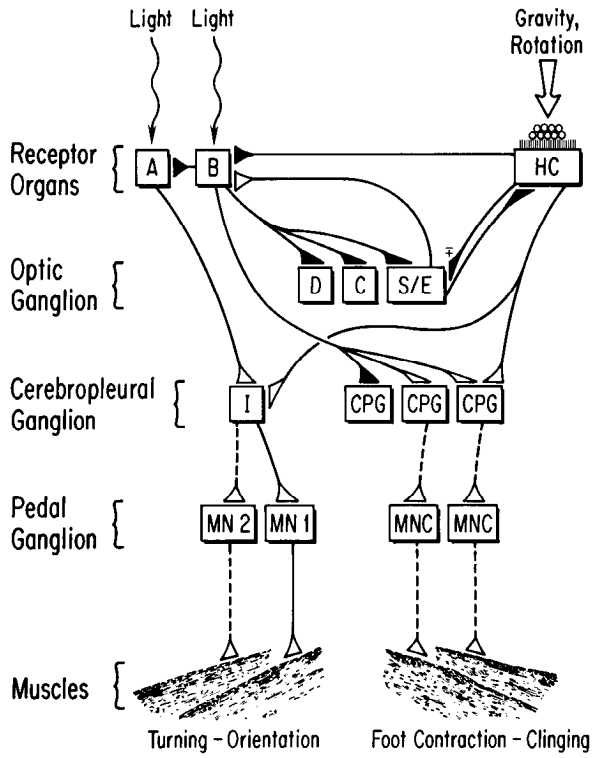

Figure 5. The flow of visual and graviceptive information and their learned interactions may be distributed through identified (*solid lines*) and predicted (*dashed lines*) neural pathways in such a way that different and even opposite behavioral responses will result. Convergence of information about the simultaneous occurrence of light and rotation is known to occur at the receptor level, the optic ganglion, and in interneurons found in the cerebropleural ganglion. Pairing-specific increases in the activity of Type B photoreceptors may lead to foot-contraction in an excitatory pathway (*open triangles*) through cpg interneurons (Akaike and Alkon, 1980). In a test situation requiring responses to contrast, reduced orientation capabilities may be produced via an inhibitory pathway (*solid triangles*) through the medial Type A photoreceptors (Goh et al., 1985; Lederhendler and Alkon, 1984).

For example, one neural pathway has been identified (Goh and Alkon, 1984) that can account for the disruption of *Hermissenda*'s ability to respond to contrast differences in the gradient (Lederhendler and Alkon, 1984), and the consequent increase in latencies to enter the light (Goh et al., 1985; Lederhendler et al., 1980). In this pathway, pairing-specific changes in the output of the network (putative motorneuron, MN1) were measured during the first 5 sec of light stimulation (Goh et al., 1985). Impulse activity in MN1 was found to be inversely related to firing frequency in the medial Type B photoreceptor. The inverse relationship occurs because the medial Type B cell inhibits the medial A photoreceptor, which, in turn, excites identifiable interneurons presynaptic to MN1. After training with paired light and rotation, enhanced excitability in the Type B photoreceptor was correlated with reduced excitation in MN1 in response to light. Reduced excitation in MN1 causes less turning of the animal toward the stimulated side (Goh and Alkon, 1984).

But the interneuronal relationships described for the turning pathway do not also account for the neural control of the behavioral changes described in the present study. However, consideration of the behavioral observations together may provide certain guidelines for studies of cellular correlates of classical conditioning of *Hermissenda*. In the present study, foot-lengthening in response to light stimulation is associated with forward movement, but not with the initiation of locomotion, since

locomotion was already in progress when the light was presented. Turning toward the light, as discussed above, is also associated with forward movement. Thus, we can distinguish an orientation system that involves approach behavior and turning movements. On the other hand, foot-shortening is an opposite response associated with clinging, stopping, and withdrawal behaviors which seem to disrupt locomotion.

The organization of behaviors mediated by visual and graviceptive input can be summarized into three functional systems: (1) An arousal system in which light and rotation initiate locomotion; we know this system is sensitive to light intensity, sensory adaptation, and diurnal factors (Barnes and Lederhendler, 1981; Crow, 1985a; Crow and Offenbach, 1983; Lederhendler et al., 1980). (2) An orientation system in which turning and forward (approach) locomotion would depend on a balance of inputs to the left or right side and, at the same time, minimal input to the foot-contraction pathway. (3) A system relating to foot-shortening, reduced forward locomotion, withdrawal, or stopping. This would be a high-threshold system responsive to intense rotation or turbulence. Thus, for example, in the presence of strong bilateral rotational stimuli, the foot-contraction pathway would be activated and override turning, forward movement, and lengthening.

Visual information may therefore be distributed through the nervous system to allow for expression of different behavioral responses. Figure 5 outlines identified neural connections that provide a working model to account for the two major response systems of interest in the present study: the orientation system and the foot-shortening system. The turning pathway is activated via the Type A photoreceptor and modulated by inhibitory input from the Type B cell. It is also excited directly by hair-cell input (Goh and Alkon, 1984). The foot-contraction pathway, on the other hand, is activated by excitatory input from the Type B photoreceptor. Interneurons of this type, which are also excited directly by the hair cells, were described by Akaike and Alkon (1980).

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