study. Subject loss was not related to training group condition.

16. This measure is similar to the suppression ratio commonly used as an index of conditioning in conditioned emotional response procedures [Z. Annau and L. J. Kamin, *J. Comp. Physiol. Psychol.* **54**, 438 (1961)]. During testing, cutoff scores of 180 minutes were used.
17. R. E. Kirk, *Experimental Design: Procedures for the Behavioral Sciences* (Brooks/Cole, Belmont, Calif., 1968), pp. 276–283. The "pairings" factor and "orientation" factor were between subjects; "trials" was within subjects. Total number of subjects in each group across the three replications were paired/caudal (N = 22), paired/cephalad (N = 24), random/caudal (N = 12), and random/cephalad (N = 12).
18. We thank J. Harrigan and I. Lederhendler for their helpful advice, T. Crow for discussions of this work, and A. Gelperin and J. Rudy for comments on an earlier version of this report. Supported in part by a research grant from the Marine Biological Laboratory "Steps Toward Independence" program to J.F.

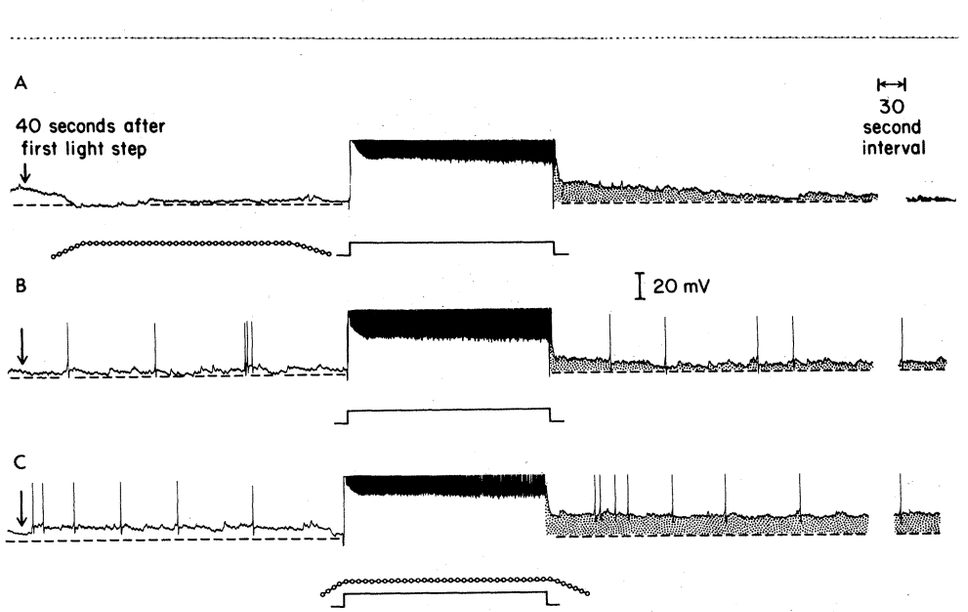
11 July 1980

Membrane Depolarization Accumulates During Acquisition of an Associative Behavioral Change

Abstract. *Long-lasting electrical changes of identified* Hermissenda *neurons, the type B photoreceptors, can account for concomitant associative behavioral changes. Depolarization of the type B cells after paired light and rotation accumulates (as monitored with intracellular electrodes) with repetition. This accumulation was specific to stimulus pairing (versus light alone or explicitly unpaired stimuli) and to the orientation of the nervous system with respect to the center of rotation; it provides a neural step in the acquisition of associative behavioral changes for gastropod mollusks and possibly other species.*

Type B photoreceptors of the nudibranch mollusk *Hermissenda crassicornis* undergo long-lasting depolarization (LLD) after a light step (*1*) of moderate ($\geq 10^3$ to 10^5 erg cm^{-2} sec^{-1}) intensity (Fig. 1 and Table 1). This LLD is a nonsynaptic process originating in the type B cell body. It arises at least in part from a light-induced, voltage-dependent Ca^{2+} conductance (*2*). The LLD and other nonsynaptic electrical characteristics of the type B cell show long-lasting changes (*3*) after exposure of intact *Hermissenda* to 3 days of light paired with rotation (compared with randomized and explicitly unpaired control tests). This paired-stimulus regimen produced short- (*4*) and long-term (*5*) behavioral changes, the latter having defining features of associative learning (*6*). In this experiment, the same sensory stimuli that produced the behavioral and neural (*5*, *7*) changes were presented to the isolated circumesophageal nervous system with intact eyes and statocysts through the use of an apparatus for continuous intracellular recording (*8*, *9*).

When the circumesophageal nervous system was rotated in the caudal orientation (with the statocysts' caudal poles oriented away from the center of rotation) during the light step, the LLD was increased in amplitude and prolonged (Fig. 1 and Table 1). Accumulation of this depolarization, measured instantaneously at 20 and 60 seconds after the light step, was apparent when these paired sensory stimuli were repeated 90 seconds after the first paired stimulus presentation. Cumulative depolarization after two stimulus pairs was greater than that after two lights alone (at 90-second intervals) or that after light and rotation in an explicitly unpaired sequence (Fig. 1 and Table 1). The same number of explicitly unpaired stimuli were presented over the same total time period as for the paired stimulus regimen. For the caudal orientation, with repeated stimulus pair presentations the cumulative depolarization progressively increased (Fig. 2A) and persisted for many minutes after the stimulus pairs (Fig. 2B). Cumulative depolarization after stimulus pairing did



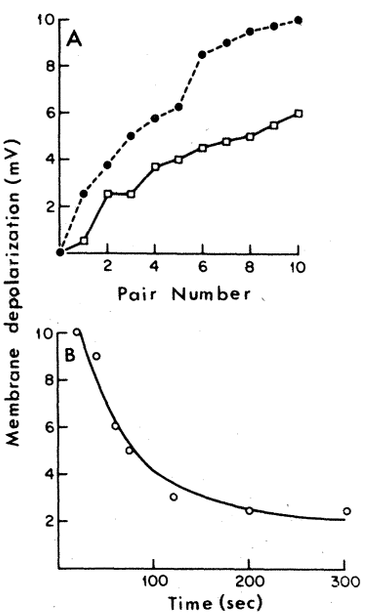


Fig. 1 (left). Intracellular voltage recordings of *Hermissenda* neurons during and after light and rotation stimuli. (A) Responses of a type B photoreceptor to the second of two succeeding 30-second light steps (with a 90-second interval intervening). The cell's initial resting potential, preceding the first of the two light steps in (A), (B), and (C), is indicated by the dashed lines. Depolarization above the resting level after the second of the two light steps is indicated by shaded areas. (A) Light steps (~ 10^4 erg cm^{-2} sec^{-1}) alternating with rotation (caudal orientation) generating ~ 1.0 g. The end of the rotation stimulus preceded each light step by 10 seconds. (B) Light steps alone. (C) Light steps paired with rotation. By 60 seconds after the first and second light steps, paired stimuli cause the greatest depolarization and unpaired stimuli the least. The minimal depolarization was in part attributable to the hyperpolarizing effect of rotation. Depolarization after the second presentation of paired stimuli was greater than that after the first. Fig. 2 (right). (A) Increase of type B membrane depolarization with repetition of the stimulus pairs. Membrane potential was measured instantaneously 20 seconds (filled circles) and 60 seconds (open squares) after successive presentations of light steps paired with rotation. (B) Decrease of type B membrane depolarization after repeated presentation of stimulus pairs as described in (A).

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Table 1. Mean [± standard deviation (S.D.)] depolarization (in millivolts) of type B cells 60 seconds after light and rotation.

Training	Orientation			
	Caudal		Cephalic	
	N	$\bar{X}$ ± S.D.	N	$\bar{X}$ ± S.D.
Unpaired				
Trial 1	6	−0.417 ± 1.20	3	0.833 ± 1.04
Trial 2	6	−0.167 ± 1.169	3	1.33 ± 3.215
Paired				
Trial 1	6	3.58 ± 2.29	4	0.250 ± 0.289
Trial 2	6	6.833 ± 4.107	4	−1.0 ± 1.080
Light alone				
Trial 1	6	0.5 ± 0.632	4	0.125 ± 0.25
Trial 2	5	1.800 ± 0.570	4	0.625 ± 0.75

not occur (Table 1) when the circumesophageal nervous system was oriented with its cephalic pole away from the center of rotation (cephalic orientation).

To quantitatively compare the depolarization of the B photoreceptors for each combination of orientation and stimulus pattern conditions, the data in Table 1 were subjected to an analysis of variance (*10*). Photoreceptors receiving paired presentations of light and rotation with caudal orientation were more depolarized (~ 7 mV) than cells of all other conditions (*10*). Moreover, depolarization of B photoreceptors significantly accumulated with repeated presentation of light and rotation for caudal orientation (from 3.6 to 7.0 mV). For the cephalic orientation, unpaired training (trial 2) produced more depolarization than paired (trial 2), whereas the reverse was true for the caudal orientation. This impression was confirmed statistically through Scheffé multiple comparison tests of the interaction means ($\alpha = .05$); no other differences between pairs of means were significant. The triple interaction of orientation, stimulus pattern, and trials was also significant; the depolarization of the B photoreceptors accumulated with repeated paired presentations of light and rotation for the caudal orientation (Table 1). Scheffé comparisons of the triple interaction means indicate that depolarization was greater after two versus one pairing for the caudal orientation–paired group.

These observations are predicted by our previous knowledge of those neural system features (*2, 11–13*) responsible for rotation-induced enhancement of the LLD. Type B photoreceptors inhibit type A and other type B cells, optic ganglion cells, and caudal hair cells of the ipsilateral statocyst. Type B cells are inhibited by type A photoreceptors and ipsilateral caudal hair cells. Type B photoreceptors are excited by at least one ipsilateral optic ganglion cell (*14*). Caudal hair cells also receive synaptic inhibition (from the ipsilateral optic ganglion), which is blocked during a light step. The result of these interactions [compare with figure 1 in (*15*)] is prolonged synaptic excitation of the type B cell after a light step. By turning the circumesophageal nervous system 180° with respect to the center of rotation—from the caudal to the cephalic orientation—the type B photoreceptor does not receive this synaptic excitation after stimulus pairing. The synaptic excitation cannot then facilitate the light-induced depolarization (and vice versa) of the type B cell, as has already been described for the associative learning model (*2*). Thus, the synaptic organization of these sensory neural systems provides clear specificity of the stimuli necessary to produce cumulative depolarization.

The cumulative depolarization shown in this report to be specific to stimulus pairing is probably the beginning of a long-term neural change that occurs during acquisition of the associative behavioral change. If cumulative depolarization is causally related to acquisition of this associative behavior change retained by *Hermissenda*, we would predict that restricting the animal to the cephalic orientation would prevent the training effects and may even reverse them. Restricting the animal to the caudal orientation should not prevent the training effects and may even exaggerate them. These predictions were in fact confirmed by the behavioral experiments of the accompanying study (*15*).

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References and Notes

1. D. L. Alkon and Y. Grossman, *J. Neurophysiol.* **41**, 1328 (1978).
2. D. L. Alkon, *Science* **205**, 810 (1979).
3. T. J. Crow and D. L. Alkon, *ibid.* **209**, 412 (1980); *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39**, 596 (1980).
4. D. L. Alkon, *J. Gen. Physiol.* **64**, 70 (1974).
5. T. J. Crow and D. L. Alkon, *Science* **201**, 1239 (1978); *Soc. Neurosci. Abstr.* **3**, 174 (1977).
6. N. E. Miller, *Proc. Am. Philos. Soc.* **111**, 315 (1967); in *The Neurosciences: A Study Program*, G. C. Quarton, T. Melnechuk, F. O. Schmitt, Eds. (Rockefeller Univ. Press, New York, 1967), vol. 2, p. 643.
7. D. L. Alkon, *J. Gen. Physiol.* **65**, 46 (1975); *ibid.* **66**, 507 (1975).
8. D. L. Alkon, *ibid.* **67**, 197 (1976).
9. Techniques of intracellular recording and details of animal supply and maintenance have been described (*2*). All records presented here were made on a Brush pen recorder (Gould, Inc., Cleveland, Ohio). Light stimuli were provided by an incandescent lamp 2 cm from the eye (intensity, ~ 10^4 erg cm^{-2} sec^{-1}). The effect of each stimulus paradigm was assessed beginning with the same state of dark adaptation and the same resting membrane potential of the type B photoreceptor. For a given orientation, each of three stimulus patterns (except for two cases; see Table 1) were presented to each photoreceptor. Order of presentation of stimulus patterns was randomized independently for each B photoreceptor, and each cell was allowed to return to its initial membrane potential level after being exposed to a given stimulus pattern. No effects of presentation order could be detected, and in the subsequent statistical analysis, presentation order was ignored. Following intracellular penetration and 15 minutes of dark adaptation, 30-second light steps occurred at 90-second intervals.
10. This analysis was an unweighted-means, mixed-design analysis of variance in which the orientation (cephalic versus caudal) was a between-subjects factor. Stimulus patterns (paired, unpaired, light-alone) and trials (1 and 2) were within-subjects factors [R. E. Kirk, *Experimental Design: Procedures for the Behavioral Sciences* (Brooks/Cole, Belmont, Calif., 1968), pp. 276–283. The main effect of neither the orientation factor [F (1, 8) = 3.29, $P > .10$] nor trials [F (1, 8) = 1.36, $P > .10$] was significant ($\alpha = .05$). Three of the four interaction terms were significant ($P < .01$): orientation by stimulus pattern [F (2, 16) = 7.33], stimulus pattern by trials [F (2, 16) = 36.62], and the triple interaction of stimulus pattern by trials by orientation [F (2, 16) = 565.81]. These justify the subsequent multiple comparisons of individual pairs of means reported in the text. The orientation by trials interaction was not significant [F (1, 8) = 1.26; $P > .10$]. All conclusions regarding the significance of Table 1 mean differences were based upon Scheffé multiple comparisons (1 degree of freedom per comparison; $\alpha = .05$ experiment-wise. The error term was the appropriate analysis of variance mean-square error term used in assessing the overall F for the main effect or interaction term from which the two means compared were drawn.
11. D. L. Alkon and M. G. F. Fuortes, *J. Gen. Physiol.* **60**, 631 (1972); D. L. Alkon, *ibid.* **61**, 444 (1973); *ibid.* **62**, 185 (1973); *ibid.* **66**, 507 (1975); ———, T. Akaike, J. Harrigan, *ibid.* **71**, 177 (1978).
12. L. J. Stensaas, S. S. Stensaas, O. Trujillo-Cenoz, *J. Ultrastruct. Res.* **27**, 510 (1969).
13. The results reported here are consistent with a previous study on the type A photoreceptor (*8*). This cell (there are two in each *Hermissenda* eye) responded with fewer impulses to a light step after presentation of repeated light steps paired (but not unpaired) with rotation of the isolated circumesophageal nervous system in the caudal orientation. The type A photoreceptor was thought to receive increased inhibition from the type B photoreceptors (there are three in each *Hermissenda* eye) as a result of the same central nervous system stimulation which here caused cumulative membrane depolarization (Fig. 1).
14. M. Tabata and D. L. Alkon, *Invest. Ophthalmol. Vis. Sci.* (Suppl.) (1979), p. 77.
15. J. Farley and D. L. Alkon, *Science* **210**, 1373 (1980).
16. I thank J. Farley, I. Lederhendler, J. Shoukimas, and W. Corson for their helpful discussions.

22 April 1980; revised 11 July 1980