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Membrane Changes in a Single Photoreceptor Cause Associative Learning in *Hermissenda*

Abstract. *Single type B photoreceptors in intact, restrained* Hermissenda *were impaled with a microelectrode and exposed to either paired or unpaired presentations of light and depolarizing current to simulate natural stimulus effects during conditioning with light and rotation. Paired, but not unpaired, stimulus presentations produced cumulative depolarization and increased input resistance in type B cells. These membrane changes are similar to those observed after pairings of light and rotation are administered to either intact animals or isolated nervous systems or when light is paired with electrical stimulation of the vestibular system in isolated nervous systems. One and two days after treatment, pairing- and light-specific suppression of phototactic behavior was observed in recovered animals. These findings indicate that the membrane changes of type B cells produced by pairing light with current injections cause acquisition of the learned behavior.*

Previous research with the nudibranch mollusk *Hermissenda crassicornis* has demonstrated striking correlations between the associative suppression of phototaxis and biophysical changes intrinsic to two of the three type B photoreceptors in the animal's eyes during both acquisition and retention of associative learning (*1–8*). We now report that membrane changes of type B cells are causally related to the associative modification of phototaxis that occurs with pairings of light and rotation.

During acquisition of associative learning, type B cells undergo a cumulative depolarization (*2, 3*) and probable increase in intracellular Ca^{2+} (*4, 6*) that arise from a pairing-specific synaptically mediated enhancement of the long-lasting depolarization response of type B cells to light (*2, 5*). This synaptic enhancement is a unique consequence of the temporal convergence of electrical activity in the visual and statocyst (vestibular) sensory pathways upon optic ganglion cells and statocyst hair cells and is responsible for cumulative depolarization of the type B cell (*3*).

During retention of learning, type B cells from conditioned animals exhibit enhanced light-induced generator potentials and impulse activity frequencies, as well as increased input resistances in the dark, both immediately (*7*) and for several days (*8, 9*) after training. Type B cells both encode (change during acquisition) and store (exhibit changes during retention) information concerning pairings of light and rotation (*10, 11*).

Despite these relatively invariant correlations between phototaxic behavioral changes produced by learning and electrophysiological changes of type B cells, it remains to be demonstrated that the membrane changes in type B cells produced by pairing light with rotation are causally related to the learned change in behavior. We sought to establish such a relation by (i) substituting depolarization of a B cell induced by an intracellular

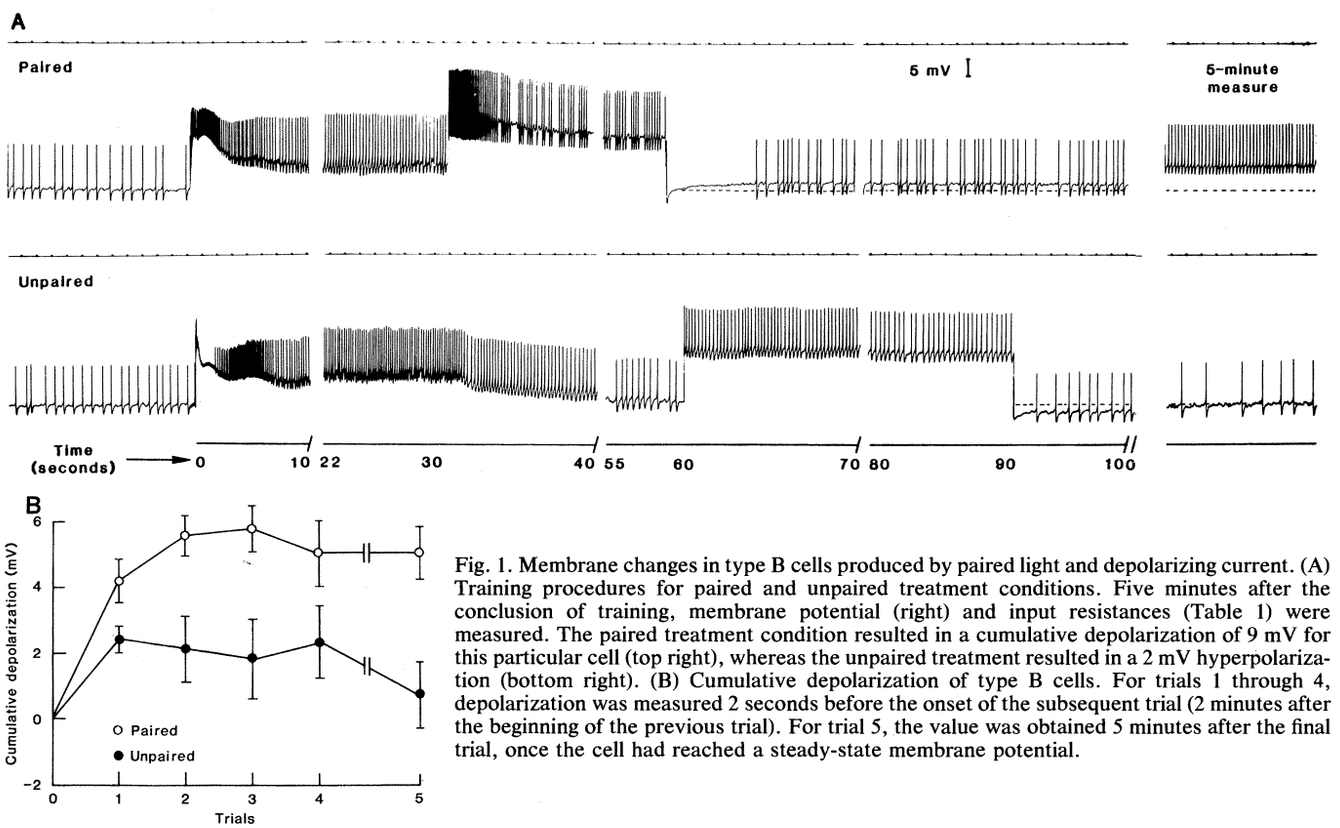

Fig. 1. Membrane changes in type B cells produced by paired light and depolarizing current. (A) Training procedures for paired and unpaired treatment conditions. Five minutes after the conclusion of training, membrane potential (right) and input resistances (Table 1) were measured. The paired treatment condition resulted in a cumulative depolarization of 9 mV for this particular cell (top right), whereas the unpaired treatment resulted in a 2 mV hyperpolarization (bottom right). (B) Cumulative depolarization of type B cells. For trials 1 through 4, depolarization was measured 2 seconds before the onset of the subsequent trial (2 minutes after the beginning of the previous trial). For trial 5, the value was obtained 5 minutes after the final trial, once the cell had reached a steady-state membrane potential.

microelectrode for the synaptic depolarization of the B cell normally produced by rotation and (ii) subsequently testing animals for long-term reductions in phototaxis. By synthesizing the changes in the B photoreceptors that have previously been correlated with learning and restricting our stimulation to a single neuron, we arranged a strong test of the hypothesis that training-induced membrane changes in type B cells are sufficient to cause long-term associative suppression of phototaxic behavior (*12*).

Baseline phototaxic and negative geotaxic latencies were first measured for intact animals ($N = 66$) by methods described previously (*8, 13*). A single type B cell in a restrained intact animal (*14*) was then impaled with a single microelectrode (30 to 60 megohm; 3*M* KCl); after stabilization of recording conditions and measurement of input resistance (R_m) (*15*), the cell was exposed to one of three treatment conditions (Fig. 1A). In one, the B cell was exposed to five successive pairings (at 2-minute intervals) of 30 seconds of light (5.0×10^3 erg cm^{-2} sec^{-1}) followed by 30 seconds of depolarizing (15 mV) positive current presented through the balanced bridge circuit (*16*). In the second condition, the type B cell was exposed to five explicitly unpaired presentations (at 2-minute intervals) of 30 seconds of light and 30 seconds of positive depolarizing current. The unpaired presentation trials differed in only one way from paired trials: a delay of 30 seconds, as opposed to no delay, intervened between the offset of light and the onset of current stimulation. For a third, sham treatment, intracellular penetration of type B cells lasted for less than 5 minutes, with no stimulation delivered. Measurements of changes in membrane potential (V_m) and R_m were then obtained 5 minutes after the conclusion of paired and unpaired training conditions. Animals were removed from the bath and allowed to recover in individual aquaria. Subsequently, they were retested for changes in phototaxis.

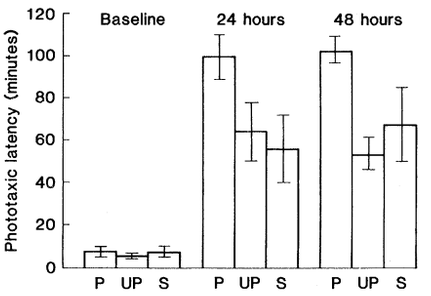

Fig. 2. Phototaxic mean (± standard error) latency before (*Baseline*) and after (24 and 48 hours) paired (*P*), unpaired (*UP*), or no (*S*) conditioning treatment.

Pairings of light and positive current produced a cumulative depolarization (Fig. 1B and Table 1) in type B cells that was significantly greater [$t(44) = 3.55$; $P < 0.001$] than the negligible change that occurred for cells exposed to the unpaired treatment. Input resistance was also significantly increased for the paired [$t(23 = 2.05$; $P < 0.01$], but not unpaired [$t(18) = 0.09$] treatment condition by 48 percent (Table 1). These results demonstrate that pairings of light and depolarizing current in type B cells reproduce the essential acquisition-related electrophysiological changes in these cells that occur with pairings of both light with rotation (*2*) and light with positive current stimulation of the caudal hair cell (*3*).

Tests of phototactic behavior for the animals that recovered (*17*) revealed a pairing-specific suppression of phototactic behavior (Fig. 2). One and two days after electrophysiological training, all animals exhibited phototactic latencies longer than initial baseline values. Between-group comparisons of phototactic behavior revealed significantly longer test latencies for paired than unpaired ($P < 0.01$) and sham ($P < 0.01$) treatments, which did not differ from each other (*18*). The pattern of electrophysiological changes in type B cells for the subset of animals that recovered was essentially identical to that of the larger population (Table 1). These data therefore indicate that the production of electrophysiological changes in a single type B photoreceptor is sufficient to cause a retained suppression of phototactic behavior (*19*).

A limited case might also be made for the causal necessity of these B cell changes as well. Since unpaired presentations of light and positive current did not differ from sham treatment in their effect on phototactic latency, since they produced no sustained membrane changes in B cells, B cells must change if the normal associative training is to result in phototaxic suppression.

Electrophysiological correlates of various associative training procedures have been reported in a number of invertebrate (*20*) as well as vertebrate (*21*) preparations. Moreover, lesions that specifically interfere with the associative learning component of a classically conditioned response—and that therefore may implicate brain regions causal for learning—have also been described (*22*). To the best of our knowledge, our results represent the first direct demonstration of a causal relationship between membrane changes of an identified class of neurons and associative learning for any preparation in which a neurobiological understanding and memory is currently being pursued.

Table 1. Membrane changes (mean ± standard error) in type B cells produced by five presentations (paired or unpaired) of light and depolarization.

Category	*N*	Membrane potential changes (ΔmV)	Input resistance (megohm)	
			Before	After
All preparations				
Paired	24	5.48 ± 0.78*	31.6 ± 1.37	47.05 ± 2.25†
Unpaired	22	0.66 ± 1.00	38.53 ± 1.13	39.65 ± 1.69
Recovered animals				
Paired	13	4.82 ± 1.21*	29.4 ± 7.58‡	46.24 ± 12.53†
Unpaired	8	1.42 ± 0.80	42.19 ± 9.60¶	36.58 ± 15.74

*$P < 0.05$; one-tailed *t*-tests, independent samples, paired versus unpaired. †$P < 0.05$; one-tailed *t*-tests, correlated samples, before versus after. ‡$N = 10$. ¶$N = 6$.

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11. Persistent increases in light responsiveness of

type B cells can at least partially explain the reduced phototaxic behavior through increased inhibition of type A photoreceptors which, via interneurons, excite motoneurons involved in phototaxis [Y. Goh and D. L. Alkon, *Soc. Neurosci. Abstr.* **8**, 825 (1982); I. Lederhendler, Y. Goh, D. L. Alkon, *ibid.*, p. 825].
12. Similar experimental approaches involving the substitution of electrical brain stimulation for the natural unconditioned stimulus have been explored in mammalian classical conditioning paradigms [F. W. Mis, I. Gormezano, J. A. Harvey, *Science* **206**, 473 (1979); G. K. Martin, T. Land, R. F. Thompson, *J. Comp. Physiol. Psychol.* **94**, 216 (1980); G. M. Powell and J. W. Moore, *Physiol. Behav.* **25**, 205 (1980)].
13. For phototactic tests, animals were initially confined to one end of a clear plastic tube (16 cm long, 5/8 inch inside diameter) filled with seawater (15°C); they underwent dark adaptation for 15 minutes. A light of 1×10^2 erg cm^{-2} sec^{-1} was then turned on and focused on the distal end of the tube. Measurements of phototactic latencies required for animals to traverse the complete distance of the tube were obtained by one or more observers with no knowledge of the experimental histories of the animals. Animals were dark-adapted again and tested for negative geotactic behavior. For both light (phototactic) and dark (negative geotactic) tests of locomotor behavior, the tubes were oriented vertically (*8*). Cut-off scores before and after training, were: light test, 15 and 120 minutes; dark test, 30 and 120 minutes.
14. Relaxation and restraint of animals were aided by the addition of 10^{-4} *M* nicotine to the seawater bath, and the nervous system was exposed and then secured on a stainless steel substage attached to a micromanipulator.
15. Input resistances were estimated from linear regression curves fitted to the current-voltage data for hyperpolarizing current injections (range, −0.2 to −0.8 nA) obtained just before and 5 minutes after training, once V_m had reached a steady state.
16. This set of stimulation parameters was based on our previous (*3*) in vitro simulation of the effects of light-rotation pairings in the isolated nervous systems, which had shown (i) five paired (but not random) presentations of 30 seconds of light and 30 seconds of electrical stimulation of the caudal hair cell (at 2-minute intervals) produced a persistent cumulative depolarization of 9 mV and (ii) the synaptic enhancement of the type B cell long-lasting depolarization was most pronounced during the 30 seconds immediately after the offset of light.
17. Animals were considered recovered only if they clung to the side of the testing tube or locomoted around the base of the tube during both post-training light tests. Decisions as to recovery were made without knowledge of the previous treatment condition. Of the 24 preparations for which we successfully conducted the paired training, 13 (54 percent) recovered. For unpaired and sham treatments, the numbers were 8 of 24 and 7 of 18 (33 and 39 percent), respectively. These differences were not statistically significant. The major factor preventing recovery in animals was the prolonged restraint necessary to accomplish the electrophysiology and apparently not exposure to the dilute nicotine. A separate group of animals dissected (but not restrained) and exposed to nicotine for comparable durations recovered fully (six of six).
18. A one-way repeated-measured analysis of variance revealed a significant main effect of treatment [$F(2, 23) = 4.75$; $P < 0.05$] and interaction of treatment with training day [$F(4, 46) = 2.83$; $P < 0.01$]. Group P differed significantly from group UP at 24 and 48 hours after training [$t(17) = 1.89$ and 2.53, respectively], as well as from group S at these same times [$t(18) = 1.92$ and 1.96]. Group UP did not differ from group S at either 24 or 48 hours [$t(11) = 0.46$ and 1.49]. Similarly, there were no significant differences among any of the groups in terms of original baseline latencies.
19. The pairing-specific differences in phototactic suppression were only partially attributable to treatment-produced differences in light-evoked behavior, since "dark" latencies were also significantly increased for all groups (means: P, 109; UP, 118; and S, 110 minutes). Analysis of the distances traveled within this time, however, revealed that animals did in fact move. There were no significant differences among treatment conditions, however. Thus, some degree of light-specificity was demonstrated.
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Selective Recording and Stimulation of Individual Identified Neurons in Freely Behaving *Aplysia*

Abstract. *A neuroethological technique is described for selective recording and stimulation of an individual neuron in freely behaving* Aplysia *by means of a fine wire glued into the connective tissue sheath above the identified cell body. A whole-nerve cuff electrode simultaneously monitored functionally related multiunit axon activity. For biophysical analysis the soma was impaled with a microelectrode when the ganglion was subsequently exposed. The technique is illustrated for several identified neurons involved in different behaviors.*

Three criteria are currently used to establish the behavioral importance of an identified neuron (*1*). To establish that a cell is "necessary" for a given behavior, it must be selectively destroyed (or prevented from firing) in an intact animal to show that the normal behavior is abolished or severely disrupted (*2*). To establish that a cell is "sufficient," it must be selectively stimulated in an intact animal to show that the behavior is elicited. However, a cell could be both necessary and sufficient by these criteria but not fire appropriately during a normal behavior. To establish that a cell is "appropriate," its normal pattern of activity must be recorded when the behavior occurs spontaneously, which also specifies the appropriate pattern of stimulation to establish the sufficiency criterion. Thus, a

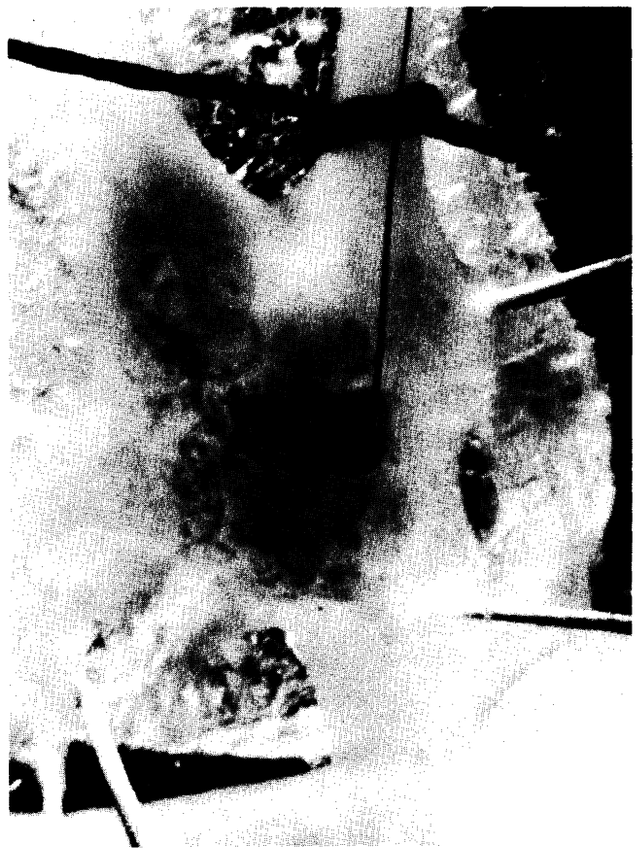

Fig. 1. Dorsal abdominal ganglion of *Aplysia californica* during surgical implantation of fine wire electrode to selectively stimulate and record from an identified neuron during normal behavior. The ganglion is pinned to a subilluminated stage. The fine wire, loosely sutured to the right connective (top right), extends caudally across the surface of the ganglion; the uninsulated tip is inserted into the connective tissue to lie directly above the large, darkly pigmented cell body of a giant neuron, cell R2 (diameter, 600 μm).