Neural Correlates of Associative Training in *Hermissenda*

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ABSTRACT Hair cells in *Hermissenda* respond to illumination of the ipsilateral and contralateral eyes. These responses are modified by associative training of the animal. The observed electrophysiological changes appear to result from changes in the photoreceptors' synaptic input to the hair cells.

INTRODUCTION

The nudibranch mollusk *Hermissenda crassicornis* is attracted to light. Repeated exposure of these animals to light associated with rotation removes or reduces this attraction (Alkon, 1974). Such behavioral modification may last several hours depending upon conditions of maintenance, training, and testing. The amount of daily illumination which the animals have previously received, for instance, can critically influence the effect of the associative training.

Considerable information is now available concerning the synaptic interaction of the statocyst with the visual pathway (Alkon, 1973 *a*, *b*; Alkon and Bak, 1973; Detwiler and Alkon, 1973). In the present study, changes in the synaptic interaction of these two pathways are found to result from associated visual and rotational training only under conditions which also produce the previously described behavioral modification.

No attempt will be made here to demonstrate that the electrophysiologic changes observed are the main cause of the behavioral modification. The principal objective of this report is to demonstrate that repeated association of two sensory stimuli causes changes in the synaptic interaction between two pathways responsive to such stimuli. The relations between electrophysiologic changes and the behavioral modification should be further investigated separately.

In earlier histologic and electrophysiologic investigations, cells have been studied in the three paired structures, the eyes, the optic ganglia, and the statocysts, located with the circumesophageal nervous system of *Hermissenda*. There are 5 cells in each eye, 14 cells in each optic ganglion, and approximately 13 cells in each statocyst.

It was shown that photoreceptors in the eye (Dennis, 1967; Alkon and Fuortes, 1972) respond to light with a depolarizing generator potential and that the hair cells of the statocyst depolarize in response to mechanical stimulation (Alkon and Bak, 1973). From intracellular recording and current passage in more than 400 pairs of cells in the three structures mentioned, it was possible to determine the principal interactions within and between the visual and statocyst pathways (Alkon and Fuortes, 1972; Alkon, 1973 *a*, *b*; Detwiler and Alkon, 1973).

The synaptic interactions between these two pathways were shown to account for two types of hair cell responses to visual stimulation: a hyperpolarizing wave with cessation of spontaneous hair cell activity, and a hyperpolarizing wave of variable magnitude followed by a prolonged increase in hair cell impulse activity often accompanied by a slight depolarization. This second hair cell response to visual stimulation will be referred to as the "depolarizing response." This depolarizing response was shown to result largely from illumination of the ipsilateral eye and the hyperpolarizing response from illumination of the contralateral and the ipsilateral eyes.

Simultaneous recordings from hair cells and photoreceptors (Alkon, 1973 *b*) demonstrated that a train of impulses in a Type A photoreceptor was followed by a delayed long-lasting increase in firing and often a small depolarization in some ipsilateral hair cells. More recently a train of impulses in a Type A photoreceptor was often observed to cause an initial hyperpolarizing wave followed by a delayed long-lasting increase in firing in an ipsilateral hair cell (Alkon, in press). Impulse trains in Type B photoreceptors were seen to produce a small hyperpolarizing wave (with cessation of impulse activity) in ipsilateral and/or contralateral photoreceptors.

In this report data are presented showing that associative training of the animals changes the two types of hair cell responses to visual stimulation. Additional data suggest that the observed changes cannot be explained by a change in the hair cells themselves, but rather by a change in the synaptic interaction of photoreceptors with the hair cells.

METHODS

Recording and Stimulation Techniques

Hermissenda were provided by Dr. Rimmon Fay of the Pacific Bio-marine Supply Co., Venice, Calif. The eyes, statocysts, and optic ganglia of *Hermissenda* are located symmetrically under the integument at the junction between the pedal and cerebropleural ganglia. A transverse cut immediately beneath the anterior portion of the animal causes the integument to retract exposing the entire circumesophageal nervous system. This nervous system (ganglia and sensory organs) was then dissected and immersed in artificial seawater at 15.5–16.5°C.

A connective tissue sheath enveloping the circumesophageal nervous system was partially digested with Pronase (Calbiochem, San Diego, Calif.), a nonspecific pro-

tease, to facilitate insertion of the microelectrodes. The micropipettes were filled with 4 M potassium acetate and had a resistance of 70–100 mΩ. Conventional methods were used to record electrical potentials of the penetrated cells. A bridge circuit was employed in the experiments involving use of extrinsic currents. Hair cell resistances were measured by passage of negative current pulses through a balanced bridge circuit. Illumination was provided by a quartz iodide incandescent lamp. The intensity of light between 4,000 and 8,000 Å which reached the preparation from this source was about 6×10^3 ergs cm^{-2} s^{-1}.

MAINTENANCE TRAINING AND ASSOCIATIVE TRAINING General conditions of maintenance, training, and testing of *Hermissenda* are as described previously (Alkon, 1974). The animals used for the experiments reported here were not the same as those animals used in a previous related study on behavior (Alkon, 1974).

Both of the two test groups consisted of animals exposed to 3 h of rotation associated with light; one group was maintained with 6½ h of daily light (A, Fig. 2) while the other was exposed to at least 18 h of daily light (B, Fig. 2). The three control groups consisted of animals taken directly from the aquarium (D, Fig. 2), animals exposed to 3 h of rotation in darkness (C, Fig. 2), and animals exposed to 3 h of the same light intervals used for associative training (E, Fig. 2). All control animals had been previously maintained for 3–5 days with 6½ h of daily light. All test and control animals were taken from the aquarium at the end of the dark period of the light cycle with the exception of the animals in one control group (D, Fig. 2). One-half of this control group was taken at the end of the light period.

In a previous study it was shown that 95% of animals rotated with light (as were animals in test groups A and B) took longer than 90 min to reach a test light spot after the training period. Seventy percent of the animals rotated in darkness reached a test light spot within 90 min after the training period. Fifty percent of animals taken directly from the aquarium and 50% of animals given 3 h of light intervals reached the test light spot within 30 min (Alkon, 1974). Because most animals under test conditions took longer than 90 min and most animals under control conditions less than 90 min to reach the test light spot, all those hair cell light responses recorded within the first 90 min after the training period (which training period includes the protease incubation described below) were analyzed. Occasionally hair cell light responses were recorded after that time interval and examined (see Results). These are not included in Fig. 2.

For experiments on all the groups except those taken directly from the aquarium, the animals were dissected after approximately 3 h of the training regimen and the isolated nervous system was returned to the training conditions while incubating in protease (0.3–0.5 mg/cc) for 15–25 min. Hair cell light responses were recorded after at least 10 and not more than 19 min of dark adaptation for each cell. The entire experiment was automatically recorded on a polygraph recorder (including the electrical recording from the hair cell, time, events, such as flashes, currents, etc.). The data were then taken from records automatically obtained throughout the experiment.

Measurement of the depolarizing responses of hair cells after flashes (6×10^3 ergs/cm^2-s) illuminating only the ipsilateral eye was obtained from a ratio of hair

cell firing frequency for 10 s immediately after, to the frequency for 10 s immediately before a 1-s test flash. The presence of a hyperpolarizing response of hair cells in response to flashes (6×10^3 ergs/cm^2-s) illuminating only the contralateral eye was indicated by a measurable hyperpolarizing wave and/or the cessation of spontaneous firing of the hair cells during the test flash.

Statistical Methods

An approximate nested analysis of variance (Brownlee, 1960; Snedecor and Cochran, 1967) was used in conjunction with the ratio method (see above) for detecting changes in spike rate to evaluate the depolarizing response of hair cells with illumination of the ipsilateral eye. This test was utilized to determine whether or not there were differences between animals within the same group as well as differences between hair cells of animals of different groups (e.g., test vs. control). The same analysis was used for the data of Fig. 3, Table III, and Table IV.

The ratio method for detecting changes in spike rate was not directly applicable to the responses of hair cells to illumination of the contralateral eye. These responses were usually all-or-none. Rarely did a cell only decrease its firing and not entirely cease firing for a brief interval after the onset of a contralateral flash. For this reason the proportions of hair cells with the hyperpolarizing response (to contralateral illumination) not present were calculated for individual animals and for animal groups (cf. Table II). The standard error of the combined sample proportion for each group was calculated taking into account the differences between animals within that group (Snedecor and Cochran, 1967).

Finally, it should be noted that the number of hair cells per animal almost always varied between one and three. In one animal four hair cells were tested and in another five hair cells were examined.

RESULTS

Responses of Hair Cells to Visual Stimulation

In these experiments, two clear differences appeared between the hair cell light responses of the control and test animals. (*a*) Depolarizing response (Figs. 1 and 2, Table I). In hair cells of test animals, statistically (cf. Table I) there was little or no increase in firing frequency after flashes illuminating only the ipsilateral eye, provided the hair cells were penetrated during the first 80 min of recording. In fact, the majority of hair cells in the test animals showed a decreased firing frequency after an ipsilateral flash.

By nested analysis of variance procedures, the five groups of Table I, Fig. 2 were found to differ statistically ($P < 0.001$). It is clear that this difference arises from comparison of control and test groups, since an examination of Table I indicates the strong homogeneity of the three control groups with respect to their means and variability. The two test groups were similarly homogeneous.

Thus, what is often, at least in part, a depolarizing response to light (indfcated by increased firing) in control animals becomes a hyperpolarizing

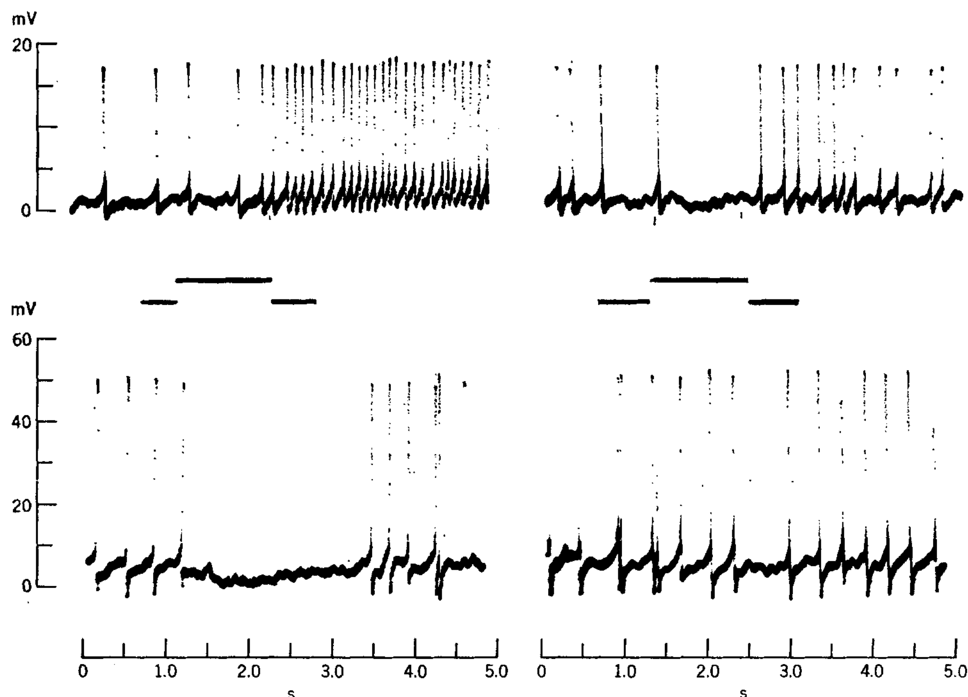

FIGURE 1. Hair cell responses to light. Upper records are of hair cells in untrained animals. Left marker indicates duration of light flash to ipsilateral eye. Right marker indicates flash to contralateral eye. The hair cell of associatively trained animal (lower records) responds with an isolated hyperpolarization to illumination of the ipsilateral eye and shows no response with illumination of the contralateral eye. Intensity of flashes: 6×10^3 ergs/cm^2-s. Base-line activity of lower hair cell: 210 impulses per minute; of upper hair cell: 140 impulses per minute.

TABLE I

DEPOLARIZING RESPONSE WITH IPSILATERAL ILLUMINATION

	N′*	N‡	Mean	SEM	SD
Control D	22	39	1.6741	0.1194	0.7456
Control C	9	20	1.6190	0.2028	0.9069
Control E	6	12	1.6300	0.2641	0.9150
Test A	9	20	0.9719	0.0523	0.2341
Test B	10	19	0.8689	0.0497	0.2166

* N′ = number of animals.
‡ N = number of hair cells.

response (indicated by a hyperpolarizing wave and/or decrease of firing) in test animals. As mentioned above, the depolarizing response in control animals is preceded by a hyperpolarizing wave of variable magnitude. In test and control animals, this hyperpolarizing wave is accompanied by a cessation

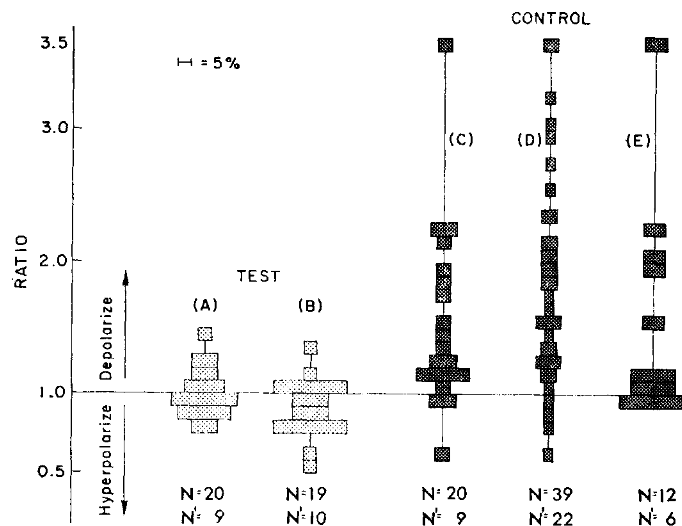

FIGURE 2. Hair cell responses to illumination (6×10^3 ergs/cm²-s) of ipsilateral eye measured by the ratio of hair cell firing frequency for 10 s immediately after to the frequency for 10 s immediately before a 1-s test flash. Each measurement was the average of two to four responses. Test group A, B: Animals exposed to 3h of rotation associated with light; A: maintained with 6½ h of daily light. B: maintained with 18 h of daily light. Control group C: Animals exposed to 3 h of rotation in darkness, maintained as Group A. Control group D: Animals taken directly from aquarium, maintained as Group A. Control group E: Animals, maintained as Group A, exposed to 3 h of light intervals (identical to those used for the test groups but without associated rotation). Horizontal extent of bars is proportional to the percentage of animals within a given ratio interval. N = Number of hair cells. N′ = Number of animals.

of impulses in hair cells. In test animals this wave may be followed by a small decrease in firing of hair cells in response to an ipsilateral flash.

The depolarizing response often did appear in hair cells of test animals provided the hair cell was penetrated after the first 80 min (and thus tested after the first 90 min of recording). In addition, an intense light (3.0×10^5 ergs/cm²-s) illuminating both eyes often elicited some depolarizing response in hair cells penetrated within the first 80 min. Finally, no significant difference was found between the hair cell light responses of control animals (Group D, Fig. 2) taken from the aquarium at the end of the dark period and responses of animals taken at the end of the light period.

(*b*) Hyperpolarizing response. Considering the standard errors in Table II, it is clear the overall proportions for combined samples of hair cell hyperpolarizing responses (to contralateral illumination) not present in the two test groups are greater than the proportions within control groups C and D. A similar tendency is indicated when control group E is compared to the test groups. In summary, then, the hyperpolarizing response of hair cells to

TABLE II

NUMBER OF HAIR CELLS WITHOUT HYPERPOLARIZING RESPONSE TO ILLUMINATION OF CONTRALATERAL EYE/NUMBER OF HAIR CELLS PER ANIMAL

	Test A	Test B	Control C	Control D		Control E
	1/1	1/2	1/3	0/1	0/2	0/2
	1/1	1/2	0/1	0/3	1/1	0/1
	2/2	2/2	0/1	0/1	1/2	0/2
	1/2	2/2	1/2	2/2	2/3	3/5
	1/2	0/2	1/3	1/1	0/1	1/2
	0/2	0/1	0/3	1/1	0/3	0/2
	3/3	2/2	1/2	0/1	1/1	
	3/3	1/1	1/4	0/1	1/2	
	3/3	2/3	0/1	0/2	0/2	
		0/1		1/3	1/3	
				0/2	0/1	
Proportion (P) of combined sample	0.79	0.61	0.25	0.31		0.28
Standard error of P	0.12	0.12	0.06	0.08		0.15
No. of animals	9	10	9	22		6

illumination of the contralateral eye occurs more frequently in control animals.

Hair Cell Properties

There were no significant differences in base-line activity or spike amplitude of hair cells in the control (Group A) group when compared to hair cells of a test group (Group C) (Table III). Nor were there significant differences in resting membrane potential observed between the control and test group hair cells. The hair cell light responses, however, were usually examined at different levels of membrane potential (using steady intracellular currents) to rule out the possibility that small differences in hair cell membrane potential might account for the differences in the hair cell light responses mentioned above. Finally, reference to Table III reveals no significant difference in membrane resistance between test and control hair cells.

A depolarizing wave in hair cells was shown to be produced by a train of impulses in Type A photoreceptors as well as by a hyperpolarizing current pulse (Alkon, 1973 *b*). The effect of a standard negative current pulse, therefore, on hair cells of control and test animals was examined. Analysis of the data in Fig. 3 reveals no significant difference between depolarizing waves produced by a negative current pulse in hair cells of control and test animals.

Photoreceptor Responses

Thus far, we have seen that the synaptic input received by hair cells from the visual system has been altered in the test animals. No large alterations were

TABLE III
HAIR CELL PROPERTIES

Spike amplitude		Resting membrane potential		Base-line activity		Resistance	
Control	Test	C	T	C	T	C	T
mV		*mV*		*impulses/s*		*m*Ω	
53	60	40	36	1.0	0.9	145	159
46	28	40	41	1.1	0.9	115	85
38	47	38	25	1.7	2.0	99	132
31	50	30	40	3.1	1.7	143	148
34	46	20	40	3.0	1.9	137	110
46	33	30	35	1.5	0.9	90	116
38	42	33	32	1.6	0.9		
42	52	40		4.6	1.8		
33	39	30		1.2	2.1		
41	36			1.5	2.4		
				2.5	1.5		
				0.6	2.3		
				3.2	0.6		
				1.9			
				1.5			
Average value:							
40.2	43.3	33.4	35.6	2.00	1.53	121.5	125.0
Number (N) of hair cells:							
10	10	9	7	15	13	6	6
Number (N') of animals:							
5	6	5	5	7	7	4	4

observed, however, in the hair cells themselves (e.g. spontaneous activity, resting membrane potential, membrane resistance, rebound excitation effect of a hyperpolarizing pulse). What of photoreceptors themselves? For the same intensity 1.0-s flashes used to test the hair cell light responses, the photoreceptor responses were measured using the same conditions of maintenance training, testing, and recording described under methods. In Table IV, are listed the amplitudes of the response peaks of photoreceptors from animals exposed to associative training and those taken directly from the aquarium. No significant difference was found between the responses in the two groups.

DISCUSSION

Intracellular recording from hair cells in animals taken from groups trained not to go into a light spot for at least 90 min revealed certain changes in the responses of hair cells to light (Figs. 1, 2, Tables I, II). These changes were not observed to occur without this training. They also did not occur in animals which had been rotated in darkness or exposed to 3 h of light intervals. The observed changes, then, occur only when an animal was exposed to as-

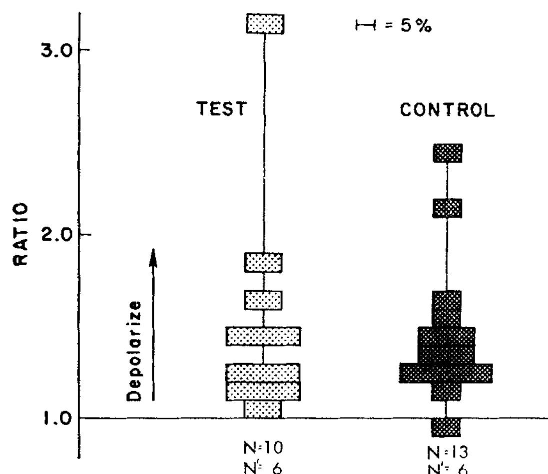

FIGURE 3. Hair cell responses to a hyperpolarizing current pulse (0.6 nA, 1.0 s), measured by the ratio of hair cell firing frequency for 10 s immediately after to frequency for 10 s immediately before a 1-s current pulse. Each measurement was the average of two to four responses. Test responses taken from cells in test group A of Fig. 2. Control responses taken from cells in Control Group C of Fig. 2. Horizontal extent of bars is proportional to the percentage of animals within a given ratio interval. N = Number of hair cells. N' = Number of animals.

TABLE IV

AMPLITUDE OF PEAK PHOTORECEPTOR RESPONSES TO FLASH (6×10^3 ergs/cm^{-2} s^{-1})

	Associative training	Control
	mV	*mV*
Type A	27.5	20
	17.5	18
	32	16
	14	22
	26	
Type B	15	16
	20	17.5
	19	29
	16	16
		27
Average:	20.7	20.2
Number of photoreceptors (N)	9	9
Number of animals (N')	3	5

sociated visual and rotational stimulation which would cause the animal to cling and subsequently not go to a test light spot for at least 90 min.

There were two clear changes in the hair light responses to test animals: the depolarizing response caused by illumination of the ipsilateral eye was

not present and the hyperpolarizing wave caused by illumination of the contralateral eye was present less frequently than in hair cells of untrained animals. These changes in the responses of hair cells to visual stimulation cannot be explained by a change in the hair cells themselves, but rather by a change in the synaptic interaction of photoreceptors with the hair cells.

One change in the synaptic interaction of photoreceptors with hair cells is the reduction of excitation of hair cells produced by ipsilateral Type A photoreceptors. This excitation must be reduced because it has been previously shown (Alkon, 1973 *b*) that a Type A photoreceptor is by itself capable of exciting ipsilateral hair cells. The observed reduction in excitation might also be due, however, to additional changes in the disinhibition effect of optic ganglion cells on hair cells or of other unknown synaptic influences on the hair cells.

In associatively trained animals, the less frequent occurrence of a hyperpolarizing response caused by illumination of the contralateral eye could arise in at least two ways. One synaptic change could be the reduction of inhibition of hair cells produced by firing of contralateral photoreceptors. Alternatively, the absence of the hyperpolarizing response to contralateral illumination could be a consequence of the absence of the depolarizing response to ipsilateral illumination. Hair cells of one statocyst were observed (Detwiler and Alkon, 1973) to inhibit hair cells of the contralateral statocyst. Thus, if hair cells do not give a depolarizing response when the ipsilateral eye is illuminated they will not inhibit hair cells of the contralateral statocyst, thus reducing the probability of observing a hyperpolarizing response in hair cells of the contralateral statocyst.

A more precise understanding of the photoreceptor-hair cell interactions will help pinpoint what synaptic changes underlie the electrophysiologic changes produced by associative training. Insight into these synaptic changes would also be aided by studying the effect associative training has on the hair cell responses to impulse trains in simultaneously impaled photoreceptors and optic ganglion cells.

SUMMARY

(*a*) Hair cells in the statocysts of *Hermissenda* hyperpolarize in response to illumination of the contralateral eye and depolarize (often with a brief preceding hyperpolarizing wave) in response to illumination of the ipsilateral eye.

(*b*) Changes in the hair cells' responses to ipsilateral and contralateral flashes occur after associative training of the animal.

(*c*) These changes appear to result from changes in the photoreceptor synaptic input to hair cells and not from changes in the hair cells themselves.

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