Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 1611–1614, February 1990
Neurobiology

Contraction of neuronal branching volume: An anatomic correlate of Pavlovian conditioning

(associative memory/morphologic correlates/*Hermissenda*/learning)

D. L. ALKON*, H. IKENO, J. DWORKIN, D. L. MCPHIE, J. L. OLDS, I. LEDERHENDLER, L. MATZEL, B. G. SCHREURS, A. KUZIRIAN, C. COLLIN, AND E. YAMOAH

Laboratory of Molecular and Cellular Neurobiology, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892

Communicated by Sanford L. Palay, October 16, 1989

ABSTRACT **Associative memory of the mollusc *Hermissenda crassicornis*, previously correlated with changes of specific K^+ currents, protein phosphorylation, and increased synthesis of mRNA and specific proteins, is here shown to be accompanied by macroscopic alteration in the structure of a single identified neuron, the medial type B photoreceptor cell. Four to five days after training, terminal arborizations of B cells iontophoretically injected with Ni^{2+} ions and then treated with rubeanic acid were measured with charge-coupled device (CCD)-digitized pseudocolor images of optical sections under "blind" conditions. Boundary volumes enclosing medial-type B-cell arborizations from classically conditioned animals were unequivocally reduced compared with volumes for naive animals or those trained with unpaired stimuli. Branch volume magnitude was correlated with input resistance of the medial type B-cell soma. Such associative learning-induced structural changes may share function with "synapse elimination" described in developmental contexts.**

Associative memory of the mollusc *Hermissenda crassicornis*—i.e., a remembered link between at least two discrete stimuli previously associated in time—has been shown to be accompanied by changes of membrane currents (1–4), changes in protein phosphorylation (5), and changes in synthesis of mRNA and of specific proteins (6, 7). These changes have been measured either in single identified neurons or in their immediate vicinity and can account for storage and recall of a Pavlovian conditioned response by the intact animal. Morphological changes of neurons, unlike those changes just mentioned, have never been directly related to associative memory of *Hermissenda* or any other species, although they have been observed to occur in a variety of developmental (8–13) and training (14–18) contexts. An inherent difficulty with the training experiments is to distinguish those training-induced modifications due to motor activity and sensory stimulation themselves from those that store memory for later recall. Such a distinction is possible with an associative paradigm that includes a control group receiving sensory stimuli not associated in time but with intensity and duration identical to those used to associatively train the animals.

Here we report marked anatomic changes in a single identified neuron (the medial type B cell) of *Hermissenda* in animals (paired group, $n = 8$) that had been associatively trained with paired visual and vestibular stimuli. These changes were measured in comparison with neurons from animals receiving either no training stimuli (naive group, $n = 8$) or to neurons from animals receiving stimuli that were explicitly unpaired at randomly varying temporal intervals (unpaired group, $n = 8$). Because there were no differences between type B cells from naive animals compared with cells from unpaired animals, the anatomic changes described below for the paired group were specific to the temporal association of the training stimuli and, therefore, are not due to the effect of sensory stimulation itself and accompanying nonassociative behavioral activity.

Four days after the last of 4 days of training,† "blind" time-lapse video-based analysis demonstrated the previously observed acquisition of a classically conditioned response: light-elicited contraction of the animal's foot. Analysis of variance (ANOVA) and Scheffé contrasts between groups demonstrated that the paired group clearly differed ($P < 0.02$) in foot contraction from the unpaired and naive groups, which were not different from each other (Fig. 1*A*, Table 1).

All cellular observations were made on a single identified neuron, the medial type B photoreceptor, for which previous studies have shown (1–4) particular K^+ currents (I_A and $I_{Ca^{2+}-K^+}$) are modified for days after classically conditioned behavior is acquired. This cell could be identified in each animal by its location within the *Hermissenda* eye as well as by numerous electrophysiological criteria (1–4). Four to five days after a paired, unpaired, or naive training experience†, medial type B cells were impaled with 30- to 50-MΩ microelectrodes filled with 0.25 M Ni^{2+}/0.25 M lysine/0.5 M KCl solution. After 10 min of dark adaption, input resistance was measured with small (0.1–0.4 nA) negative and positive current pulses. Electrophysiologic measurements of blinded experimental design (Fig. 1*B*, Table 1) revealed that 4–5 days after training, conditioned type B cells had significantly higher ($P < 0.02$ by the ANOVA and post-hoc analyses just mentioned) input resistance (inversely related to K^+ channel conductance) than cells from either unpaired or naive animals (which also showed no between-group differences). These differences in input resistance were measured in the absence

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: EV, enclosing volume; ANOVA, analysis of variance.

*To whom reprint requests should be addressed at: Laboratory of Molecular and Cellular Neurobiology, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Room 431 Park Building, 9000 Rockville Pike, Bethesda, MD 20892.

†The paired group received 75 conditioned stimulus–unconditioned stimulus pairing per day for 4 days resulting in a total of 300 classical conditioning trials. The unpaired group received the same number of conditioned stimulus and unconditioned stimulus presentations in an explicitly unpaired manner (i.e., no two stimuli were presented within 30 sec of each other). The naive group received no training and simply remained in their home tubes. The conditioned stimulus was a 3-sec white light (Sylvania 21.5-V, 150-W tungsten halogen projector lamp) that coterminated with 2 sec of high-speed rotation (Labline Instruments Shaker 4600). All animals were tested with the same light 24 hr after the last training session. It was previously shown that these procedures produced significant associative learning for >6 days after the last training session.

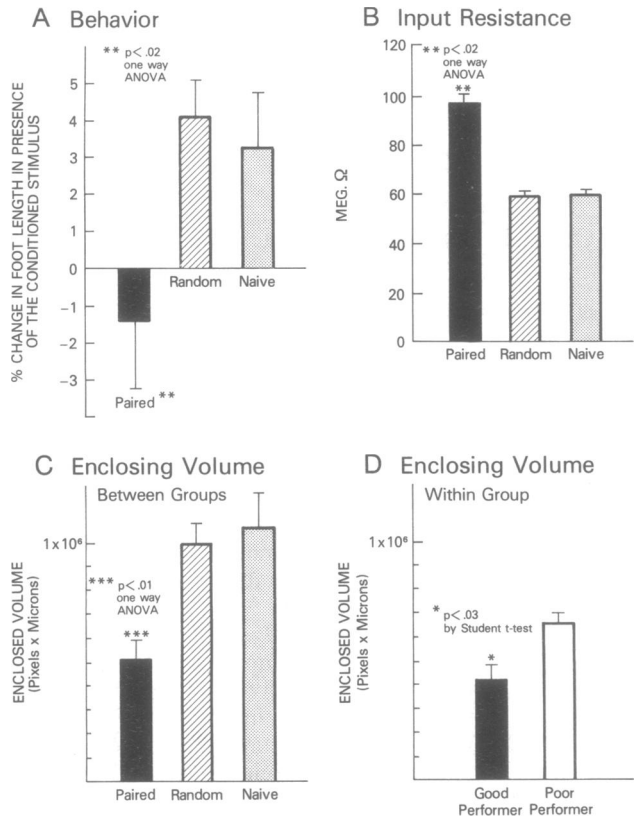

FIG. 1. Bar graphs of behavioral, biophysical, and morphological measurements on type B cells from experimental groups. Statistical significance was assessed by either one-way ANOVA or Student's *t* test. (*A*) Behavior. The paired group ($n = 8$) showed a significant decrease in foot length compared with either control group (random $n = 8$; naive $n = 6$) when exposed to the conditioned stimulus (light). (*B*) Input resistance. The paired group ($n = 6$) showed a significant increase in input resistance as measured by intracellular current injection over both control groups (random $n = 7$; naive $n = 6$). (*C*) EV. The EV was determined as the product of the Feret diameters (*Fx* and *Fy*) and the number of 1-μm steps for which there was <25% decrease in the maximum cross-sectional area of stained branches (*z*). The calculated EV was significantly less in paired type B cells ($n = 8$) than in either control group (random $n = 8$; naive $n = 8$). (*D*) EV, paired subgroups. Animals ($n = 5$) that showed good acquisition of the learned foot contraction (good performers) had significantly reduced EVs compared with a subgroup ($n = 3$) that did not show acquisition (poor performers) of the learned response. Error bars represent SEMs.

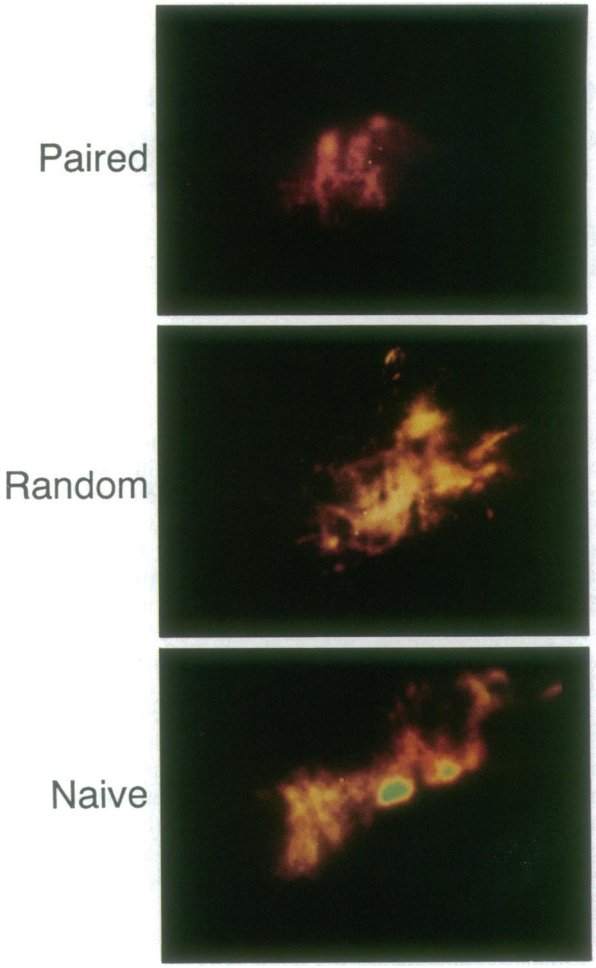

FIG. 2. Charge-coupled device (CCD)-digitized images of representative type B photoreceptors from the three experimental groups: paired, random, and naive. The type B cell was visualized at 440× magnification by the MCID imaging system. Top row, paired; middle row, randomized control; bottom row, naive control.

of any significant between-group differences of resting membrane potential or impulse amplitude. The staining protocol was begun with 8.0- to 10.0-nA 500-msec positive pulses at 1 Hz. Constant negative current (1.0–2.0 nA) injection was maintained to prevent electrode polarization. Over a 13-min injection period, resting potential and the light-elicited gen-

Table 1. Group averages (± SEM) for type B cells: Behavior, input resistance, and EV

	Behavior, % change in footlength (*n*)	Input resistance, MΩ (*n*)	Enclosing volume, μm × pixel (*n*)
Paired	−1.43 ± 1.80 (8)*	97.50 ± 16.60 (6)*	517,922 ± 54,693 (8)†
Random	4.24 ± 1.04 (8)	58.57 ± 8.47 (7)	1,007,928 ± 176,959 (8)
Naive	3.05 ± 1.46 (6)	60.00 ± 9.80 (6)	1,072,447 ± 200,915 (8)

The effect of associative training on biophysical and morphological parameters of type B cells in *Hermissenda crassicornis*. Means (±SEM) of three parameters—behavior, input resistance, and enclosing volume (EV)—were calculated for each experimental group: paired, randomized control, and naive control. In a few cases, behavior or input resistance could not be determined. Statistical significance was assessed with one-way ANOVA across the three groups. Post-hoc contrasts were then made between groups in cases where the overall *F* test was significant ($P < 0.05$). In the case of behavior, the *F* value was 5.34, which was significant at the $P < 0.02$ level. Post-hoc comparisons showed that the paired group was significantly different from both control groups ($F = 9.905$, $P < 0.01$). In the case of input resistance the overall *F* test was also statistically significant ($F = 4.06$, $P < 0.04$). Post-hoc statistical contrasts showed that mean input resistance for the paired group was statistically different from both control groups ($F = 8.064$, $P < 0.02$). Finally, ANOVA on EV was statistically significant overall ($F = 4.22$, $P < 0.03$). Post-hoc contrasts once again showed that the paired group mean was significantly different from either control group ($F = 8.349$, $P < 0.01$).

*$P < 0.02$, one-way ANOVA between groups.

†$P < 0.01$, one-way ANOVA between groups.

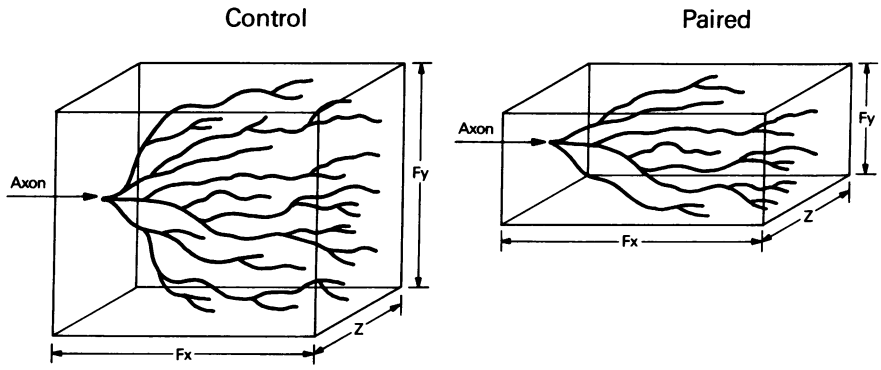

FIG. 3. Schematic diagram of type B cell terminal arborization in paired group and controls demonstrating the significant difference in EV. A reduction in EV may represent a "synapse elimination" that effects the connectivity of the type B cell to its postsynaptic target neurons.

erator potential progressively decreased. The nervous system was immersed in 0.5 M rubeanic acid for 2 hr at 15°C and thereafter fixed in a solution of 3% gluteraldehyde/0.1 M sodium cacodylate/1.5% paraformaldehyde/10.0 mM $MgCl_2$/10.0 nM $CaCl_2$/10% sucrose. After being imbedded in an Epon–Araldite mixture (19) in slide molds (Ladd Research Industries, Burlington, VT) the stained cells were visualized on a Zeiss Axiophot microscope, and, after video enhancement, photographed at 1-μm steps in depth of focus. Images of the terminal arborization of type B cell branches were then visualized and analyzed with a computer-based system for measuring the limits of branching in the x-y plane (MCID System; Imaging Research, Saint Catherines, ON, Canada). For each cell, the maximal cross-sectional area was obtained after the axonal origin of the terminal arborization was visualized. All preparations were imbedded and then optically sectioned in the same sagittal orientation. Charge-coupled device (CCD)-digitized pseudocolor images (Fig. 2) facilitated the quantitation. Extent of branching perpendicular to the sagittal x–y plane was measured as the number of 1-μm steps for which there was <25% decrease in the maximal cross-sectional area of stained branches. A decrement >25% immediately preceded the rapid disappearance of most stained endings. Limiting boundary volume that enclosed the arborization (enclosing volume, EV) was then obtained as the product of x, y, and z values (cf. Figs. 2 and 3) to assess the extent of branching in three dimensions for each cell. EVs of type B cell branches obtained with protocols under which calculation was kept blind throughout the experiments were clearly reduced for the paired as compared with both the unpaired and naive animals (Figs. 1–3). One-way ANOVA and Scheffé between-group post-hoc contrasts demonstrated that both unpaired and naive type B cell branch volumes did not differ from each other (Figs. 1 and 2, Table 1) but differed significantly ($P < 0.01$) in comparison with volumes for the paired animals. The results of these ANOVA and Scheffé analyses suggested a within-group comparison for the paired animals. We predicted and then found that for the five-animal paired subgroup that showed good acquisition of the learned foot contraction (measured by foot shortening of -4.5 ± 2.4 SD) the medial type B cell EVs were significantly reduced ($P < 0.03$) by a Student's t test in comparison with cell EVs (Fig. 1*D*) for the three-animal paired subgroup that did not show acquisition of the learned response (measured by foot lengthening of 3.77 ± 1.66). There was no significant difference between the branching volumes of the "poor performers" and the volumes of the naive or unpaired animals. This lack of significance was apparent from a one-way ANOVA ($P < 0.15$) comparing branching volumes for the three groups. By contrast, the one-way ANOVA between the "good performers" and the two control groups was highly significant ($P < 0.01$). These results (particularly the within-group comparison of good and poor performers) clearly show that better learning performance (and not simply a training paradigm) was related to structural modification of an identified neuron. We would, however, not rule out the possibility that much less striking structural changes might still be occurring within the poor performers, who might be learning something but performing poorly.

Interestingly, the degree of branching volume reduction was correlated (inversely) with the magnitudes of the blindly obtained input resistance values (Fig. 4). Reduction of cell branching volume cannot directly account for much of the input resistance differences measured in the type B cell body.‡ The cell body is connected to the branch region by a 1-μm axon (60–70 μm long). After axotomy, input resistance in the cell body was still clearly increased for the paired group (20–23), and K^+ currents were still clearly reduced (1–4). Furthermore, the paired group increase of input resistance was approximately the same for axotomized and intact (20–23) type B cells, suggesting that the paired decrease of terminal arborization volume could contribute little to the increase of input resistance measured in the cell body.

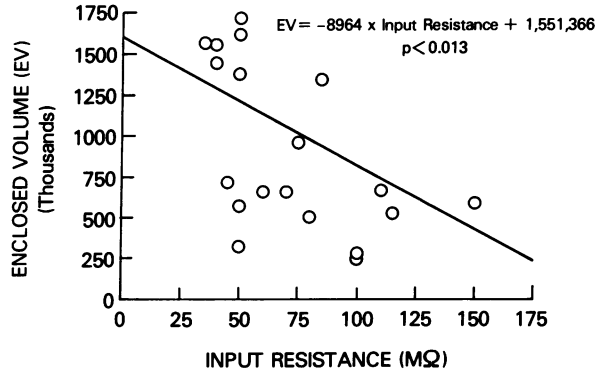

FIG. 4. Graph of EV versus input resistance in type B cells from all experimental subjects. Linear regression analysis of this data demonstrated a significant inverse relationship between the morphological measurement (EV) and the biophysical measure of cell input resistance ($P < 0.013$); the correlation coefficient was 0.57.

‡Because the current injected into the cells was the same for all groups, the charge carried by Ni^{2+} into the cell should be independent of the cellular input resistance. Furthermore, supramaximal currents (8–10 nA) were injected to insure that the voltage across the cell membrane approached a constant asymptotic value that was independent of the cellular input resistance. This asymptote was approximated in all cells due to enormous rectification of the cell membrane for potentials >30 mV. Finally, Ni^{2+} apparently reached the fine terminal branches of cells from all groups in comparable concentrations because there were no measurable between-group differences in the caliber of the stained terminal branches.

Changes of shape or volume of the cell body itself (which could not be assessed with optical sections), however, might still accompany and help effect these input resistance changes. The molecular transformations (including protein kinase C activation) responsible for cell body input resistance increases (24) may, however, also initiate the structural changes observed here in the terminal arborization.

The number of terminal branches and endings was not obviously different between groups, although more detailed analysis will be necessary for a definitive conclusion regarding this issue. We measured no between-group differences in staining intensity of the axons (stain/background ratio of photographic density for paired = 1.27 ± 0.129 SD, $n = 7$; random = 1.33 ± 0.184, $n = 5$; naive = 1.30 ± 0.099, $n = 8$) or in staining of the finest branches (stain/background for paired = 1.15 ± 0.083 SD, $n = 7$; random = 1.12 ± 0.128, $n = 5$; naive = 1.21 ± 0.13, $n = 8$). Thus, diffusion of the Ni^{2+} seemed to be unaffected by any between-group internal resistivity differences that would be expected to cause staining differences in terminal branches.

A variety of *Hermissenda* neurons (e.g., hair cells, interneurons, and motorneurons) show no intrinsic changes of electrophysiologic characteristics with conditioning but do show response changes that passively reflect modification of extrinsic input from the medial type B cell (1–4, 20–23). The clear correlation of the observed structural change with the increased input resistance intrinsic to the conditioned medial B cells decreases the likelihood that other *Hermissenda* neurons showing no intrinsic electrophysiologic changes with conditioning would show structural changes. The structure of these neurons should, however, be analyzed in the future as well as the structure of other identified neurons known to be postsynaptic to the medial B cells (e.g., the optic ganglion cells). Structural, biophysical, and molecular transformations, then, work in concert to change the excitability of the type B cell and, thus, the synaptic signals this cell sends to postsynaptic neurons. Learning-induced reduction of cell-branching volume may serve to further bias the neural system by enchancing certain synaptic interactions at the expense of others for which fewer terminal branches are available. Thus, these changes correlated with associative memory may share important functions with synapse elimination observed in developmental contexts (8–13).

1. Alkon, D. L., Lederhendler, I. I. & Shoukimas, J. J. (1982) *Science* **215**, 693–695.
2. Alkon, D. L. (1984) *Science* **226**, 1037–1045.
3. Alkon, D. L., Sakakibara, M., Forman, R., Harrigan, J., Lederhendler, I. I. & Farley, J. (1985) *Behav. Neural Biol.* **44**, 278.
4. Collin, C., Ikeno, H., Harrigan, J., Lederhendler, I. I. & Alkon, D. L. (1988) *Biophys. J.* **54**, 955–960.
5. Neary, J. T., Crow, T. & Alkon, D. L. (1981) *Nature (London)* **293**, 658–660.
6. Nelson, T. & Alkon, D. L. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 7800–7804.
7. Nelson, T., Collin, C. & Alkon, D. L. (1989) *Science*, in press.
8. Huttenlocher, P. R., de Courten, C., Garey, L. J. & Van der Loos, H. (1982) *Neurosci. Lett.* **33**, 247–252.
9. Proctor, W. & Roper, S. (1982) *J. Physiol.* **326**, 189–200.
10. Cowan, W. M. (1981) *Studies in Developmental Neurobiology: Essays in Honor of Viktor Hamburger* (Oxford Univ. Press, New York).
11. O'Kusky, J. R. (1985) *Brain Res.* **354**, 81–91.
12. Purves, D., Voyvodic, J. T., Magrassi, L. & Yawo, H. (1987) *Science* **238**, 1122–1126.
13. Rakic, P., Bourgeois, J. P., Eckenhoff, M. F., Zecevic, N. & Goldman-Rakic, P. S. (1986) *Science* **232**, 232–235.
14. Rosenweig, M. R., Mollgaard, K., Diamond, M. C. & Bennett, E. L. (1972) *Psychol. Rev.* **79**, 93–96.
15. Horn, G., Rose, S. P. & Bateson, P. P. (1973) *Science* **181**, 506–514.
16. Greenough, W. T., Juraska, J. M. & Volkmar, F. R. (1979) *Behav. Neural Biol.* **26**, 287–297.
17. Scheich, H. (1987) *J. Comp. Physiol.* **161**, 605–619.
18. Bailey, C. & Chen, W. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2373–2377.
19. Geiselman, C. W. & Burke, C. N. (1973) *J. Ultrastruct. Res.* **43**, 220–227.
20. Crow, T. J. & Alkon, D. L. (1980) *Science* **209**, 412–414.
21. West, A., Barnes, E. J. & Alkon, D. L. (1982) *J. Neurophysiol.* **48**, 1243–1255.
22. Farley, J. & Alkon, D. L. (1982) *J. Neurophysiol.* **48**, 785–807.
23. Goh, Y., Lederhendler, I. I. & Alkon, D. L. (1985) *J. Neurosci.* **5**, 536–543.
24. Alkon, D. L. & Rasmussen, H. (1988) *Science* **239**, 998–1012.