Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 2373–2377, April 1988
Neurobiology

Long-term memory in *Aplysia* modulates the total number of varicosities of single identified sensory neurons

(learning/habituation/sensitization/structural correlates/synapse)

CRAIG H. BAILEY*† AND MARY CHEN*

*Center for Neurobiology and Behavior, The New York State Psychiatric Institute, 722 West 168th Street, New York, NY 10032; and †Departments of Anatomy and Cell Biology and Psychiatry, College of Physicians and Surgeons, Columbia University, New York, NY 10032

Communicated by Eric R. Kandel, January 11, 1988

ABSTRACT **The morphological consequences of long-term habituation and sensitization of the gill withdrawal reflex in *Aplysia californica* were explored by examining the total number of presynaptic varicosities of single identified sensory neurons (a critical site of plasticity for the biochemical and biophysical changes that underlie both types of learning) in control and behaviorally trained animals. Sensory neurons from habituated animals had 35% fewer synaptic varicosities than did sensory neurons from control animals. In contrast, sensory neurons from sensitized animals had twice as many varicosities per sensory neuron compared to controls, as well as enlarged neuropil arbors. These changes suggest that modulation of synapse number may play a role in the maintenance of long-term memory.**

An issue central to the study of learning and memory is the functional relationship between synaptic structure and the prolonged changes in synaptic effectiveness that accompany long-term behavioral modification. It has become clear that synaptic connections in the mature nervous system of both vertebrates and invertebrates exhibit a substantial degree of structural plasticity with normal operation (1) and with learning (2–4). Thus, changes in environmental complexity, social structure, or training in the intact animal (5–11), as well as electrical stimulation *in vitro* (12, 13) and *in vivo* (14–16), have been shown to alter both the number of synapses and their structure. The role these structural alterations may play in learning and memory has been difficult to assess in complex systems, where the contribution of individual synapses to the learning process is largely unknown. The neural parsimony offered by several higher invertebrate preparations has successfully bridged this gap and has proven useful for the cellular analysis of behavioral problems. We have used one such model system—the gill and siphon withdrawal reflex of *Aplysia californica*—to study two simple forms of nonassociative learning (habituation and sensitization) both of which can exist in a short-term form lasting up to 1 hr (17, 18) and a long-term form lasting >3 weeks (19, 20). Several aspects of the biophysical and biochemical mechanisms that underlie short-term habituation and sensitization are understood and involve changes in synaptic effectiveness produced by modulation of the Ca^{2+} current at a common locus—the presynaptic terminals of identified mechanoreceptor sensory neurons (21–24). Less-well characterized are the morphological mechanisms that underlie habituation and sensitization. In particular, it is not known whether structural alterations at sensory neuron synapses contribute to the transition of the short-term form to one of longer duration. To address these issues, we examined the nature and extent of morphological changes at identified sensory neuron synapses that occur following long-term habituation and sensitization.

In the present study we have used horseradish peroxidase (HRP) to label the presynaptic terminals (varicosities) of sensory neurons. We have then used serial 20-μm "slab-thick" sections to count the total number of varicosities per sensory neuron in control and behaviorally modified animals. We have found that sensory neurons from long-term habituated animals have fewer varicosities than do sensory neurons from control animals and that sensory neurons from long-term sensitized animals have more varicosities.

METHODS

Sensory neurons from three groups of animals were examined in this study: control (untrained) animals (n = 16) and animals trained for long-term habituation (n = 10) or long-term sensitization (n = 10).

Animals were trained for long-term habituation by the protocol of Carew *et al.* (19) and for long-term sensitization by the protocol of Pinsker *et al.* (20). In each case the behavioral memory for the task is retained for several weeks. Animals were individually housed for a minimum of 5 days in circulating seawater before behavioral training. To assess their responsiveness, we delivered two jets of seawater to the siphon with a WaterPik. Animals were accepted for the experiment if the mean duration of their first two test responses (interstimulus interval, 30 sec) was 10 sec or longer. The scores (total of 10 stimuli) were ranked, and the animals were randomly distributed into a control (untrained) group and groups for long-term habituation and long-term sensitization. There were no significant differences among the groups before training. Long-term habituation was produced by giving animals 10 sessions of habituation training per day for 10 days. Long-term sensitization was produced by giving animals training sessions on four consecutive days. Each session consisted of exposure to four electrical stimuli (100 mA for 2 sec), each separated by 1.5 hr. All electrical stimuli were delivered to the neck region through bipolar capillary electrodes.

Retention of habituation and sensitization was tested 1 day after the completion of the respective training sessions. The behavioral performance of animals—control, long-term habituated and long-term sensitized—was estimated by comparing their behavioral scores 24 hr after the completion of training with their pretraining scores. These ratios (after/training/pretraining) were as follows: long-term habituated animals were significantly different from their controls [0.11 ± 0.04 (mean ± SEM, n = 10) vs. 1.18 ± 0.12 (mean ± SEM, n = 8); t = 9.679 sec; $P < 0.0001$, two-tailed test] and long-term sensitized animals were significantly different from their controls [9.08 ± 1.3 (mean ± SEM, n = 10) vs.

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.

Abbreviation: HRP, horseradish peroxidase.

0.95 ± 0.07 (mean ± SEM, $n = 8$); $t = 5.64$; $P < 0.0001$, two-tailed test].

Within 48 hr of the end of training, animals were anesthetized by intracoelomic injection of isotonic $MgCl_2$ (25). The abdominal ganglion was removed from each animal and transferred to a solution of supplemented artificial seawater containing a high concentration of Mg^{2+} (200 mM) and a low concentration of Ca^{2+} (1 mM) to block synaptic transmission during pinning and desheathing. After desheathing, the ganglion was bathed in seawater with a normal concentration of Mg^{2+} (55 mM) and Ca^{2+} (10 mM) for a minimum of 30 min. To label presynaptic varicosities, a single sensory neuron from each animal was chosen at random, identified (26), and intrasomatically pressure-injected with HRP (type VI, Sigma) at a concentration of 20 mg/ml in distilled water. Following a 2-hr incubation period to allow HRP to fill the neuropil arbor of the sensory neuron as well as its axon in the siphon nerve, ganglia were fixed, histochemically processed, and embedded in Epon (27). Each sensory neuron was completely reconstructed by serial 20-μm slab-thick sections, and the total number of HRP-labeled varicosities for each cell was counted through an experimenter-blind procedure using high-resolution light microscopy (27). A total of 36 sensory neurons were analyzed in this fashion.

The axonal branching pattern of individual mechanoreceptor sensory neurons has been described, and the types of varicosities that could be found along their neuropil arbor have been characterized (27). Although the branching pattern is quite complex, relatively few types of varicosities are found. These include (*i*) small relatively symmetrical bead-like varicosities along fine branches; (*ii*) more eccentrically placed varicosities; (*iii*) expansions that occur at some but not all branch points; and (*iv*) terminal varicosities that range from spherical to club-shaped. The first two categories account for ≈90% of the total number of varicosities. All of these are easily and reliably identified in 20-μm-thick sections and can be accurately counted by using long-working-distance high-resolution objectives. The slab-thick section approach offers several advantages over more-conventional whole-mount techniques for quantitative studies of this magnitude and also allows one to re-embed individual thick sections for ultrastructural analysis.

The full extent of the neuropil arbors from nine graphically reconstructed sensory neurons were measured with a Bioquant II digitizing tablet (R & M Biometrics, Nashville, TN) that was interfaced with an Apple IIe microcomputer running the Bioquant II morphometry program. Camera-lucida tracings were made of all the HRP-labeled sensory neuron processes present in each serial 20-μm slab-thick section taken through each abdominal ganglion. The length of each process was determined by a digitized tracing by using the Bioquant II software. The individual values were combined to determine the total extent of all the neuropil branches of single sensory neurons taken from six long-term sensitized animals and three control animals. These totals (for both experimental and control animals) are probably underestimates since no corrections were made of the measured values for projected lengths to account for the inclination of each process throughout the thickness of the tissue slice.

RESULTS

In an earlier study (2), we combined selective intracellular labeling techniques with serial reconstruction approaches to analyze the fine structure of identified sensory neuron presynaptic varicosities in control and behaviorally modified animals. Our results indicated that the number, size, and vesicle complement of sensory neuron active zones were larger in animals showing long-term sensitization than in control animals and were smaller in animals showing long-term habituation. To maximize the chances of finding labeled sensory neuron processes in thin sections, multiple cells in each animal were injected with HRP, but it was not feasible to examine if long-term training had any effect on the total number of presynaptic varicosities per sensory neuron.

In the present study, we have addressed this question directly by quantitatively analyzing the total axonal arbor of single HRP-filled sensory neurons. The total number of varicosities per sensory neuron are summarized in Fig. 1 and show a trend parallel to our initial observations on active-zone morphology. However, the present result is even more dramatic, because it involves modulation of each neuron's entire synaptic field. Sensory neurons from control animals had on average 1291 varicosities; this was reduced 35% to 836 varicosities per sensory neuron in long-term habituated animals. In contrast, sensory neurons from long-term sensitized animals had approximately twice as many varicosities (2697 varicosities) compared to their controls (1320 varicosities).

The alterations in synapse number that accompany long-term memory in *Aplysia* suggest a number of parallels with the sequence of structural events that has been described throughout the normal development of the nervous system in other animals. For example, the reduction in varicosity number during long-term habituation may be similar to the phenomenon of synapse elimination. In contrast, the dra-

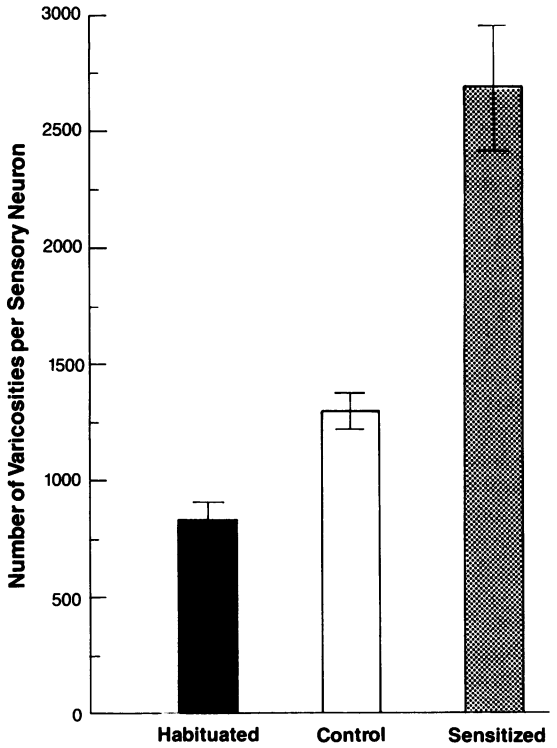

FIG. 1. Total number of varicosities per sensory neuron in control and long-term behaviorally modified animals. Each bar represents the mean ± SEM. Control groups from both experiments were not significantly different from one another and have been combined (1306 ± 82 varicosities, mean ± SEM, $n = 16$). The total number of varicosities per sensory neuron from each group was as follows: for long-term habituation animals, 836 ± 75 varicosities (mean ± SEM, $n = 10$) and, for their controls, 1291 ± 138 varicosities (mean ± SEM, $n = 8$); and, for long-term sensitized animals, 2697 ± 277 varicosities (mean ± SEM, $n = 10$) and, for their controls, 1320 ± 99 varicosities (mean ± SEM, $n = 8$). The mean number of varicosities for long-term habituated animals is significantly less than the mean for their controls ($t = 3.05$, $P < 0.01$), and the mean for long-term sensitized animals is significantly larger than the mean for their controls ($t = 4.25$, $P < 0.01$).

matic increase in synapse number we have observed during long-term sensitization may represent a residual growth capacity of sensory neurons that has been carried over from development. A further consequence of this growth potential that is expressed during long-term sensitization is illustrated in Fig. 2. Graphic representations of completely reconstructed cells indicate that in addition to an increase in varicosity number, sensory neurons from long-term sensitized animals also display enlarged neuropil arbors.

DISCUSSION

The present findings, combined with those of our earlier ultrastructural study (2), indicate that long-term memory in *Aplysia* is accompanied by morphological alterations on two levels of synaptic organization: (*i*) changes in focal regions of membrane specialization (active zones) of the synapse and (*ii*) more global alterations involving a modulation of the total number of synaptic varicosities. Each mechanoreceptor sensory neuron in *Aplysia* appears to possess a profound capacity for structural plasticity that is differentially expressed depending upon the type of long-term training it receives (Fig. 3). The persistent depression that accompanies long-term habituation is reflected not only by a decrease in the total number of synaptic varicosities but also by a reduction in the incidence and extent of their active zones. In contrast, the morphological changes that accompany long-term facilitation in *Aplysia* appear to involve an element of growth, resulting in an enlarged neuropil arbor, a near doubling of the total number of varicosities, and an increase in the number and size of their active zones. The percent increase in the number of active zones revealed in our ultrastructural studies (41% to 65%), combined with the near doubling of varicosities observed in the present study, are both consistent with the possibility that virtually every new varicosity that is induced by long-term sensitization training comes along with an active zone.

The nature and extent of these structural alterations at sensory neuron synapses are consistent with both the known behavioral efficacy of long-term habituation and sensitization in *Aplysia* (19, 20) and with electrophysiological studies indicating an enduring alteration in the strength of sensory-

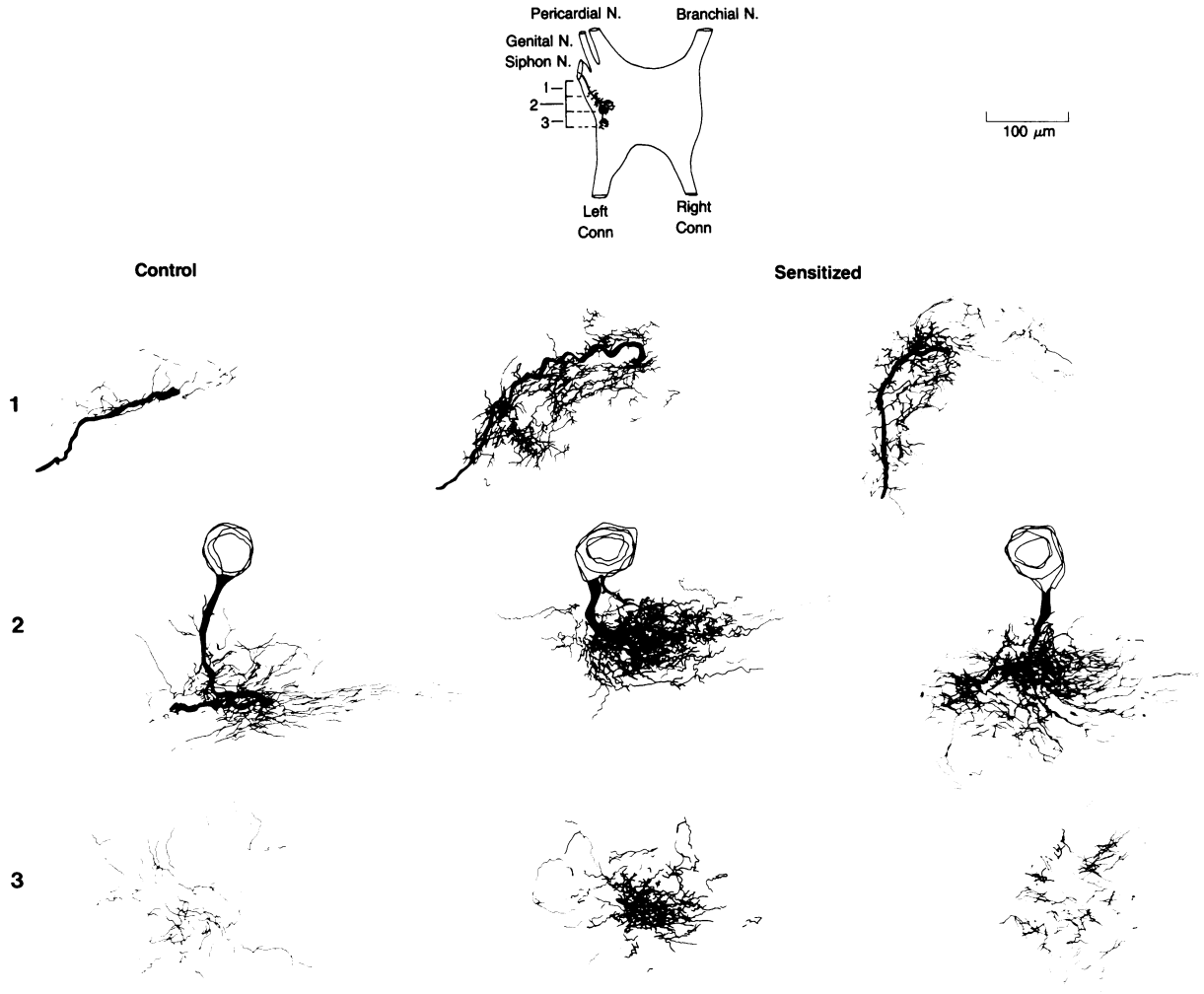

FIG. 2. Serial reconstruction of sensory neurons from long-term sensitized and control animals. Total extent of the neuropil arbors of sensory neurons from one control and two long-term sensitized animals are shown. In each case the rostral (row 3) to caudal (row 1) extent of the arbor is divided roughly into thirds. Each panel was produced by the superimposition of camera-lucida tracings of all HRP-labeled processes present in ≈17 consecutive slab-thick sections and represents a linear segment through the ganglion of roughly 340 μm. For each composite, ventral is up, dorsal is down, lateral is to the left, and medial is to the right. By examining images across each row (rows 1, 2, and 3), the viewer is comparing similar regions of each sensory neuron. In all cases, the arbor of long-term sensitized cells is markedly increased compared to control. Quantitative estimates indicate that the total neuropil arbor of sensory neurons from long-term sensitized animals is 2.7 times greater in extent [22,254 ± 2415 μm (mean ± SEM, n = 6)] than that from control animals [8415 ± 1640 μm (mean ± SEM, n = 3); t = 3.746; P < 0.01, two-tailed test]. Siphon N., Genital N., etc., are various peripheral nerves of the abdominal ganglion; Left and Right Conn are left and right connectives, fiber tracts connecting the abdominal ganglion with other ganglia.

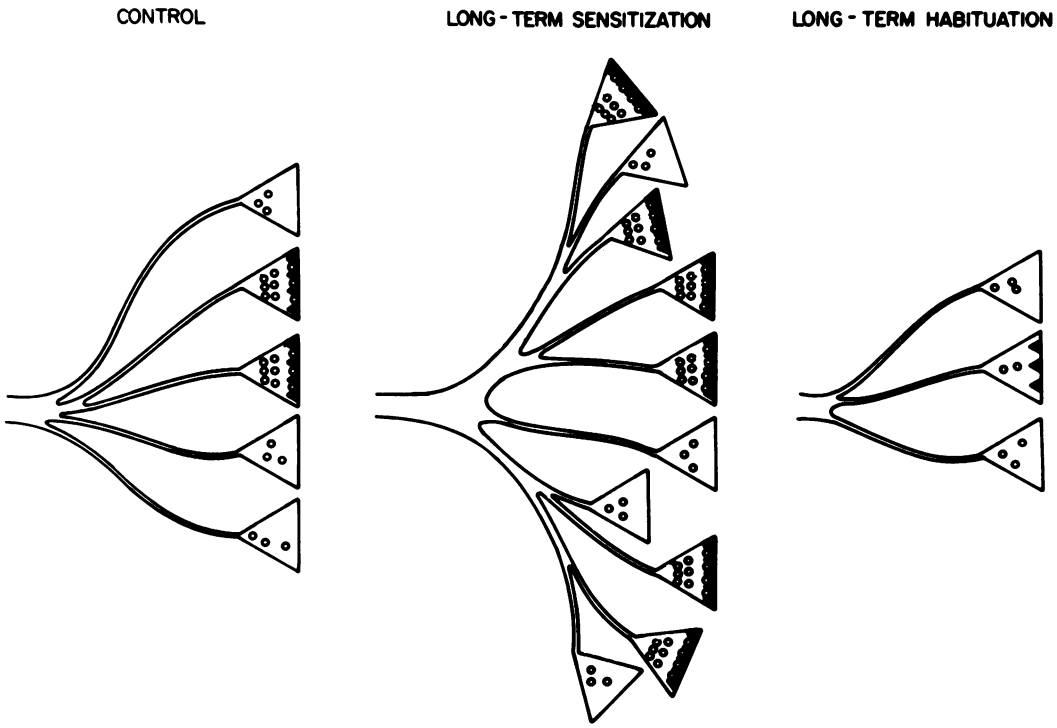

FIG. 3. Morphological model of long-term habituation and sensitization in *Aplysia*. Long-term memory is accompanied by alterations in both the number of sensory neuron varicosities and their active zones (solid triangles). These changes are differentially expressed depending upon the type of training. Habituation leads to a decrease in these morphological features, whereas sensitization leads to an increase. Open circles, synaptic vesicles.

to-follower cell connections following long-term training (25, 28). In addition, the increase in synapse number that we have observed during long-term sensitization in *Aplysia* is similar to results from studies in the mammalian central nervous system that indicate increases in the number of synapses following various forms of environmental manipulations and learning tasks (for review, see refs. 4 and 29). These emerging parallels from studies on vertebrates and invertebrates suggest that synapse formation may be a feature of long-term memory that is highly conserved throughout the animal kingdom.

Montarolo *et al.* (30) have reconstituted the monosynaptic component of the gill-withdrawal reflex of *Aplysia* in dissociated cell culture and found that long-term facilitation of the sensory–motor neuron connection can be blocked by inhibiting either protein or RNA synthesis. These findings combined with our morphological evidence suggest that the retention of long-term sensitization may involve the induction of new proteins or the increased synthesis of preexisting proteins, perhaps by the activation of specific genes, and that this may be reflected by structural rearrangements at sensory neuron synapses. If true, this hypothesis would be consistent with the proposal of Cajal and others that learning may resemble processes of cellular growth and differentiation (31) and would suggest that the timekeeping steps for a long-term memory trace may be specified, in part, by morphological changes at the synapse.

This work was supported by Grant MH37134 from the National Institute of Mental Health, Scope B of Grant GM23540 from the National Institute of General Medical Sciences, and the McKnight Endowment Fund for Neuroscience. We thank Drs. E. R. Kandel and V. F. Castellucci for critical review of the manuscript and H. Ayers for typing the manuscript.

1. Purves, D., Hadley, R. D. & Voyvodic, J. T. (1986) *J. Neurosci.* **6**, 1051–1060.
2. Bailey, C. H. & Chen, M. (1983) *Science* **220**, 91–93.
3. Greenough, W. T. (1984) *Trends Neurosci.* **7**, 229–233.
4. Greenough, W. T. & Bailey, C. H. (1988) *Trends Neurosci.*, in press.
5. Globus, A., Rosenzweig, M. R., Bennett, E. L. & Diamond, M. C. (1973) *J. Comp. Physiol.* **82**, 175–181.
6. Greenough, W. T. & Volkmar, F. R. (1973) *Exp. Neurol.* **40**, 491–504.
7. Garey, L. J. & Pettigrew, J. D. (1974) *Brain Res.* **66**, 165–172.
8. Greenough, W. T., Juraska, J. M. & Volkmar, F. R. (1979) *Behav. Neural Biol.* **26**, 287–297.
9. Vrensen, G. & Nunes Cardozo, J. (1981) *Brain Res.* **218**, 79–97.
10. Turner, A. M. & Greenough, W. T. (1985) *Brain Res.* **329**, 195–203.
11. Camel, J. E., Withers, G. S. & Greenough, W. T. (1986) *Behav. Neurosci.* **100**, 810–813.
12. Chang, F.-L. F. & Greenough, W. T. (1984) *Brain Res.* **309**, 35–46.
13. Bailey, C. H. & Chen, M. (1988) *J. Neurosci.*, in press.
14. Fifkova, E. & van Hareveld, A. (1977) *J. Neurocytol.* **6**, 211–230.
15. Lee, K. S., Schottler, F., Oliver, M. & Lynch, G. (1980) *J. Neurophysiol.* **44**, 247–258.
16. Lnenicka, G. A., Atwood, H. L. & Marin, L. (1986) *J. Neurosci.* **6**, 2252–2258.
17. Pinsker, H., Kupfermann, I., Castellucci, V. F. & Kandel, E. R. (1970) *Science* **167**, 1740–1742.
18. Carew, T. J., Castellucci, V. F. & Kandel, E. R. (1971) *Int. J. Neurosci.* **2**, 79–98.
19. Carew, T. J., Pinsker, H. M. & Kandel, E. R. (1972) *Science* **175**, 451–454.
20. Pinsker, H. M., Hening, W. A., Carew, T. J. & Kandel, E. R. (1973) *Science* **182**, 1039–1042.
21. Castellucci, V. F. & Kandel, E. R. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 5004–5008.
22. Klein, M. & Kandel, E. R. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3512–3516.
23. Castellucci, V. F., Kandel, E. R., Schwartz, J. H., Wilson, F. D., Nairn, A. C. & Greengard, P. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 7492–7496.
24. Klein, M. & Kandel, E. R. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6912–6916.
25. Castellucci, V. F., Carew, T. J. & Kandel, E. R. (1978) *Science* **202**, 1306–1308.

26. Byrne, J., Castellucci, V. F. & Kandel, E. R. (1974) *J. Neurophysiol.* **37,** 1041–1064.
27. Bailey, C. H., Thompson, E. B., Castellucci, V. F. & Kandel, E. R. (1979) *J. Neurocytol.* **8,** 415–444.
28. Frost, W. N., Castellucci, V. F., Hawkins, R. D. & Kandel, E. R. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 8266–8269.
29. Greenough, W. T. & Chang, F.-L. F. (1985) in *Synaptic Plasticity,* ed. Cotman, C. W. (Guilford, New York), pp. 335–372.
30. Montarolo, P. G., Goelet, P., Castellucci, V. F., Morgan, J., Kandel, E. R. & Schacher, S. (1986) *Science* **234,** 1249–1254.
31. Cajal, S. R. (1911) *Histologie du Système Nerveux de l'Homme et des Vertébrés* (Maloine, Paris), Vol. 2; Azoulay, Z. L., trans. (1955) *Consejo Superior de Investigaciones Cientificas* (Inst. Ramon y Cajal, Madrid).