Target-Dependent Structural Changes Accompanying Long-Term Synaptic Facilitation in *Aplysia* Neurons

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The mechanisms underlying structural changes that accompany learning and memory have been difficult to investigate in the intact nervous system. In order to make these changes more accessible for experimental analysis, dissociated cell culture and low-light-level video microscopy were used to examine *Aplysia* sensory neurons in the presence or absence of their target cells. Repeated applications of serotonin, a facilitating transmitter important in behavioral dishabituation and sensitization, produced growth of the sensory neurons that paralleled the long-term enhancement of synaptic strength. This growth required the presence of the postsynaptic motor neuron. Thus, both the structural changes and the synaptic facilitation of *Aplysia* sensorimotor synapses accompanying long-term behavioral sensitization can be produced in vitro by applying a single facilitating transmitter repeatedly. These structural changes depend on an interaction of the presynaptic neuron with an appropriate postsynaptic target.

IN BOTH VERTEBRATES (*1*) AND INVERTEBRATES (*2, 3*), long-term memory involves morphological changes in neurons (*4*). These changes have been demonstrated with anatomical techniques that require fixing the nervous tissue, such as Golgi or horseradish peroxidase staining and electron microscopy. As a result, inferences about the structural changes resulting from learning have been based on comparisons between two different populations of neurons—one that has been subjected to learning-related stimulation and another that has not.

Moreover, because these studies have been carried out on intact tissues, it has not been possible to explore the mechanisms underlying the structural changes. For example, it is not known whether memory-related structural changes in neurons are cell autonomous or require the presence of a target cell. To examine the morphological mechanisms associated with long-term synaptic plasticity more directly, we have taken advantage of the ability of the sensory and motor neurons that mediate the gill-withdrawal reflex of *Aplysia* to survive and form connections in dissociated cell culture (*5, 6*). Utilizing low-light-level video fluorescence microscopy (*7, 8*), we have examined the effect of the facilitating transmitter serotonin (5-HT) on the structure of *Aplysia* sensory neurons when cultured with and without the identified gill and siphon motor cell L7.

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The *Aplysia* sensorimotor synapse undergoes long-term heterosynaptic facilitation in vivo in response to repeated sensitizing behavioral stimuli (*9*). Both in the intact central nervous system (*10*) and in vitro (*11*), this synapse also undergoes long-term facilitation in response to repeated applications of 5-HT, an endogenous facilitating transmitter in *Aplysia* that is important in behavioral dishabituation and sensitization (*12*). We cocultured *Aplysia* sensory neurons with L7 motor cells for 5 days (*13*). We then labeled the sensory neurons with the fluorescent dye 5(6)-carboxyfluorescein and imaged the sensory neurons with low-light-level video fluorescence microscopy (*14*). Twenty-nine sensorimotor cocultures (experimental) were treated with repeated applications of 5-HT (*11, 15*). Another 22 cultures (control) were treated identically to the experimental group except that 5-HT was omitted from the bathing solutions. Approximately 24 hours after treatment we refilled the sensory neurons with dye and imaged them a second time to look for long-term morphological changes. To ascertain the physiological efficacy of the 5-HT treatment, we tested the strength of the sensorimotor connection in nine experimental and nine control cocultures both before and after treatment (*16*).

A rater, who was blind to the experimental treatment of the cocultures, rated the structural changes in sensory neurons on the basis of two sets of fluorescence video micrographs, one made of the cocultures before and another after experimental treatment. The ratings were restricted to the region of the coculture where the processes of the sensory neuron contacted the major axons of L7 or their neighboring neurites (*17*). Each coculture received a single rating of structural change. Positive ratings (0.5 to 2.0) indicated growth of new sensory neuron varicosities or processes, or enlargement of existing varicosities or processes. Negative ratings (−0.5 to −2.0) indicated loss or retraction of varicosities or processes. A score of 0.0 indicated no structural change (*18*). (These values were arbitrarily chosen beforehand. In practice, the lowest rating given was −1.5.) Sensory neurons of 5-HT–treated cocultures exhibited significantly greater structural growth than did control cocultures (Fig. 1A). The mean structural rating for experimental cocultures was 0.88 ± 0.12, whereas that for control cocultures was 0.11 ± 0.15 ($P < 0.003$, two-tailed Mann-Whitney U test). [Values

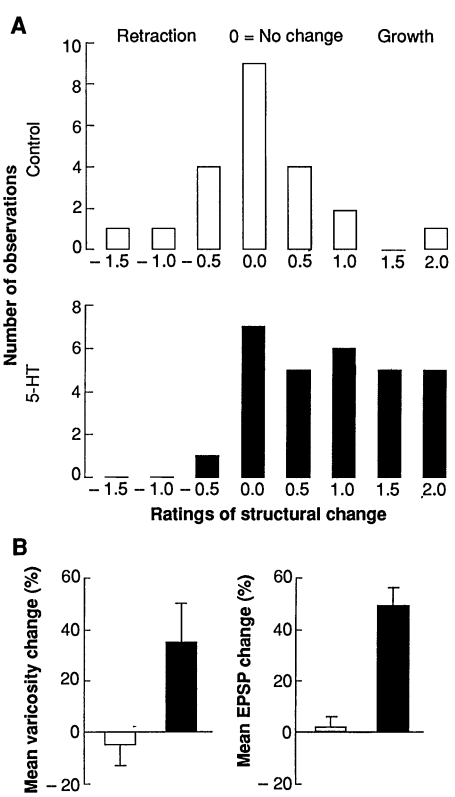

Fig. 1. (**A**) Summary of ratings of structural change in sensory neurons of sensorimotor cocultures produced by 5-HT. Control, $n = 22$; 5-HT, $n = 29$. (**B, left**) Change in the number of varicosities expressed as percentage of the initial number before experimental treatment for nine control cocultures (open bar) and nine 5-HT–treated cocultures (solid bar). These are cocultures for which the physiological change due to the experimental treatment was also measured [see (B, right)]. Error bars represent −(control) or +(5-HT) SEM. (**B, right**) Mean change in the monosynaptic EPSP produced in L7 by firing the sensory neuron, expressed as a percentage of the EPSP before experimental treatment, for nine control cocultures (open bar) and nine 5-HT–treated cocultures (solid bar). [Same cocultures as those used for the varicosity measurements in (B left)]. Error bars represent +SEM.

throughout this report are means ± SEM.]

To further quantify the long-term structural changes in the sensory neurons and to relate the structural changes more directly to any physiological change induced by 5-HT, we counted the number of sensory neuron varicosities before and 1 day after treatment for the nine experimental and nine control cocultures for which we had physiological data (*19*). This was done again by a rater who was blind to the experimental treatment of the cocultures (Fig. 1B, left side). The mean change in number of varicosities on sensory neurons 1 day after 5-HT treatment was enhanced significantly (35 ± 15%, $P < 0.05$, two-tailed test) compared to controls (*20*) (−5 ± 8%, $P > 0.1$). The 5-HT treatment also produced long-term facilitation of the sensorimotor synapse (Fig. 1B, right side). The mean percent change in the excitatory postsynaptic potential (EPSP) amplitude 1 day after 5-HT application was 49 ± 7% for the experimental cocultures ($n = 9$) and 2 ± 4% for the control cocultures ($n = 9$) ($P < 0.001$, two-tailed test) (*21*). Examples of these results are shown in Fig. 2.

Whereas our data indicated that repeated applications of 5-HT increased the number of sensory neuron varicosities, we also wanted to know whether these new varicosities represent new points of synaptic contact. To answer this question, we selected from our sample of sensorimotor cocultures one which had a region where the neurites of a sensory cell clearly contacted the initial segment of the major axon of L7, but where there were no sensory varicosities before 5-HT treatment (Fig. 3, A and B). After 5-HT treatment there were several new sensory varicosities present in this region (Fig. 3C). Electron microscopic examination of this same coculture revealed that some of these new varicosities contained unambiguous ac-

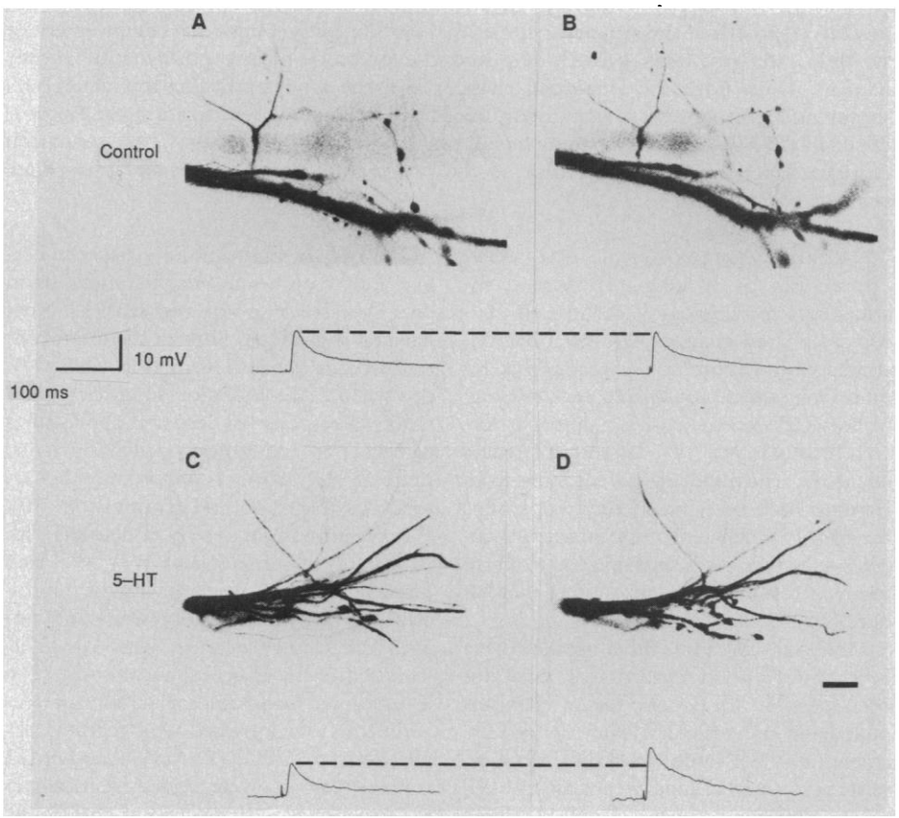

Fig. 2. Representative example of structural and physiological results for control (**A** and **B**) and 5-HT–treated (**C** and **D**) cocultures. Video micrographs made before experimental treatment (A and C) show regions where the processes of the sensory neurons (fluorescence-labeled) contacted the initial segments of the major motor axons. The same regions are shown ~24 hours after treatment in (B) and (D). Below the micrographs are the electrophysiological records of EPSPs produced in the L7 motor neurons by firing the control and 5-HT–treated sensory neurons before and after treatment. The micrographs were printed by a video processor with white reversed to black to enhance resolution of fine details. Scale bar, 25 μm.

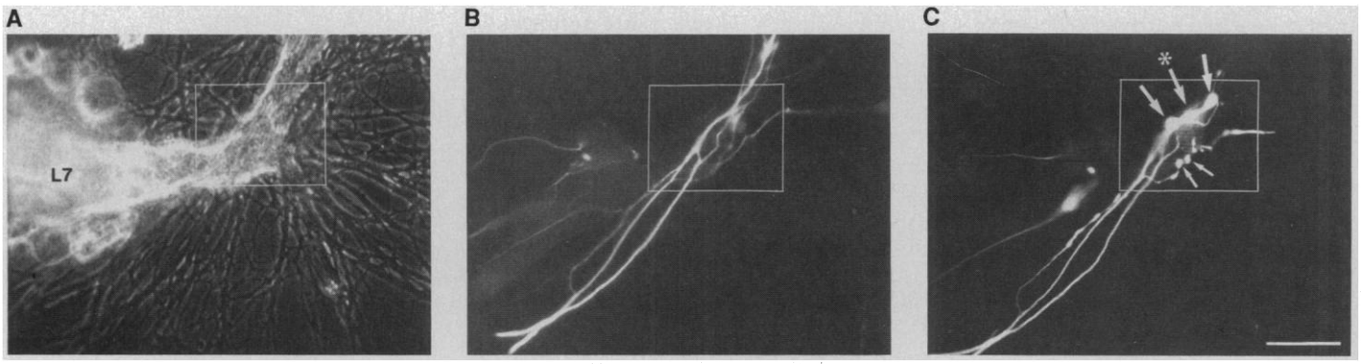

Fig. 3. Phase-contrast (**A**) and fluorescence (**B** and **C**) video micrographs made of a 5-HT–treated coculture. The same region of the coculture is shown so that the relation between presynaptic and postsynaptic structures can be seen. All of the neurites visible in (A) belong to the motor neuron L7. The sensory processes are finer and run along the motor neurites; they are not apparent in the phase-contrast micrograph. In (A) one can see a portion of the soma of L7 and the large initial segment of the major motor axon, which in this coculture has an elbow-like shape. The axon was removed from the abdominal ganglion along with the soma during dissociation. (B) Fluorescence micrograph of the processes of a single, dye-labeled sensory neuron before 5-HT treatment. By comparing (A) and (B) one can see where the sensory neuron contacts the initial segment of L7. In the outlined region there are no sensory varicosities. (C) Fluorescence micrograph of the same sensory neuron ~24 hours after 5-HT treatment. Note the presence of several new varicosities in the outlined region (arrows) and the disappearance of a sensory growth cone evident above the outlined region in (B). This same coculture was fixed after reimaging the sensory neuron and prepared for electron microscopy. The varicosities in (C) could be unambiguously identified in thin sections cut through the coculture. One of the three larger varicosities (* in C) contained a fully developed active zone, as did two of the smaller varicosities (small arrows in C). The other large varicosities had small or immature active zones. Scale bar, 50 μm.

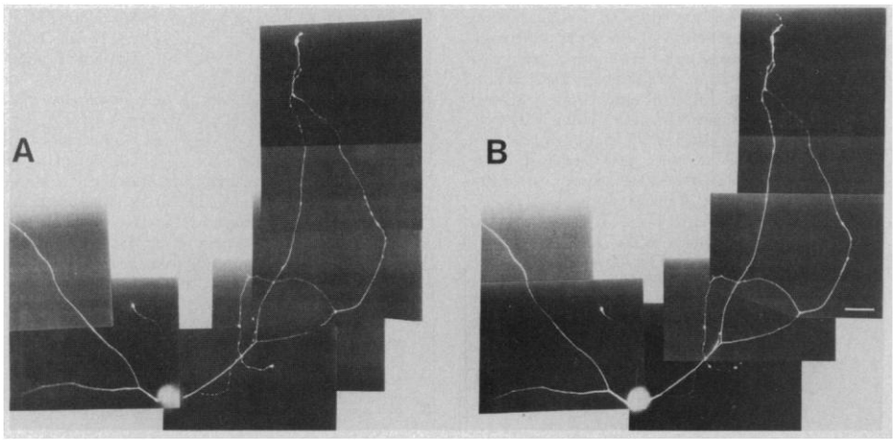

Fig. 4. Composite fluorescence micrographs of a sensory neuron in culture alone. (**A**) The sensory neuron before 5-HT treatment. (The soma was only partially imaged.) (**B**) The same sensory neuron ~24 hours after 5-HT treatment. Scale bar, 50 μm.

tive zones. Because we have found that active zones in sensorimotor cocultures are associated with sensory varicosities (although not every varicosity contains an active zone) (*6*), the varicosities shown in Fig. 3C appear to represent new, 5-HT–induced synapses.

One long-term functional change produced by 5-HT in *Aplysia* sensory neurons—an increase in membrane excitability ascribable to modulation of the S-type potassium current—is independent of the motor neuron's presence, since it can be produced in isolated sensory neurons (*22*). We therefore asked whether the growth of the sensory neurons produced by 5-HT depended on the presence of the postsynaptic target. Isolated sensory neurons in vitro were treated with repeated applications of 5-HT or application of control solution and were inspected for growth 24 hours after treatment. We used the same blind rating procedure that we had previously used to quantify growth in sensorimotor cultures, except that now we inspected the entire sensory neuron (*6*). We found no evidence for 5-HT–induced structural change in isolated sensory neurons (Fig. 4). The mean rating for sensory neurons of control cultures was 0.31 ± 0.17 ($n = 16$), whereas that for sensory neurons of experimental cultures was 0.07 ± 0.26 ($n = 15$). This difference was not statistically significant ($P > 0.1$, Mann-Whitney U test). Therefore, in the absence of a target motor neuron, 5-HT does not produce long-lasting growth in sensory neurons (*23*).

By means of quantal analysis, Dale and colleagues (*24*) found that long-term facilitation of the *Aplysia* in vitro sensorimotor synapse involves an increase in transmitter release by the sensory neuron. This increased transmitter release could, in principle, be due to two different mechanisms: an increase in the amount of transmitter released from preexisting presynaptic sites or the growth of new release sites. Our experiments suggest that at least part of the long-term facilitation of this synapse involves growth of new points of synaptic contact between the sensory and motor neurons (*25*).

These experiments extend in several ways results from previous structural studies of long-term memory in *Aplysia* (*2*). (i) By using dissociated cell cultures, we have been able to directly image the memory-related growth of additional varicosities on the processes of living sensory neurons. (ii) We were able to demonstrate that the structural changes can be induced by 5-HT, an endogenous facilitating transmitter that contributes to behavioral dishabituation and sensitization in *Aplysia* (*12, 26*). (iii) The growth of the presynaptic neuron appears to require the presence of the postsynaptic target. (iv) The postsynaptic regulation of the growth of the sensory neuron produced by 5-HT resembles that which occurs during the development of the connections between the sensory and motor neuron in vitro (*6*). These results therefore suggest that learning and development in *Aplysia* may utilize one or more common molecular interactions.

Recent evidence suggests that long-term potentiation (LTP) in the hippocampus may involve, in part, a change in presynaptic terminals leading to enhanced transmitter release, which depends upon some retrograde postsynaptic signal (*27*). The parallel between these findings and our results is intriguing. Possibly, LTP of some hippocampal synapses and long-term facilitation of *Aplysia* sensorimotor synapses both require interaction between the presynaptic and postsynaptic cells for their expression (*28*).

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13. See (*5*) for methodological details on cell culturing. Cells were dissociated from central ganglia with the initial segments of their major axons attached and placed into cell culture. The identified gill and siphon motor neuron L7 was the postsynaptic target cell. We used siphon sensory neurons from the abdominal ganglion or tail sensory neurons from pedal ganglia as presynaptic cells. We saw no differences in our results if we used abdominal or pleural sensory neurons. Each coculture contained one motor neuron and one or two sensory neurons. The sensory and motor cells were allowed to grow together in cell culture for 5 days before the start of the experiment. During this time the synaptic connections gradually increased in strength. After day 5 in culture, the connections normally remained stable during the next 24 hours, as demonstrated by the fact that there was no significant difference change in the mean EPSP for the control group of cultures in our experiments, which were analyzed during days 5 to 6 in culture (see the text and Fig. 1B, right side). Montarolo *et al.* (*11*) also found no significant change in EPSP amplitude for in vitro sensorimotor synapses between days 5 and 6 in culture.
14. When there were two sensory neurons in a coculture, both were labeled with dye and imaged (*6*). Sensory neurons were imaged with a Hamamatsu C2400-08 silicon-intensified target (SIT) video camera coupled to a Zeiss IM 35 inverted microscope, or with a Dage-MTI SIT 66 video camera coupled to a Nikon Diaphot inverted microscope. The video images were recorded with either an optical disc recorder (Panasonic TQ-2025F) or a video cassette recorder (Panasonic NV-9240 XD).
15. After initially imaging the sensory neurons, the experimental cocultures received five 5-min applications of 5-HT. Serotonin (Sigma, creatinine sulfate) was added to the bath to a final concentration of 5 μM. After 5 min the 5-HT was gradually washed out by perfusion with 20 ml of 50% L15 (Flow Laboratories) culture medium and 50% artificial seawater (Instant Ocean), pH 7.6. Washout of each 5-HT application took 15 min. Thus, the intertrial interval for the 5-HT applications was 20 min. After treatment, the cocultures were returned to culture medium and placed into an incubator at 18°C until subsequent imaging.
16. See P. G. Montarolo, E. R. Kandel, S. Schacher, *Nature* **333**, 171 (1988) for details on the electro-

physiological techniques.
17. In a pilot study we found that the 5-HT treatment produced growth of sensory neurons, but only in those regions of the coculture where sensory neurons contacted major neurites of L7, and particularly the initial segments of the original axons of L7. This finding reinforced an earlier one from our normative study of the *Aplysia* sensorimotor synapse in vitro where we found that only those sensory varicosities apposed to major neurites of L7 appeared to contain unambiguous synaptic profiles.
18. Assessments of structural changes in these experiments were subject to potential artifacts resulting from differences in the amount of dye injected on the two consecutive days, differences in the amount of illuminating light, or differences in focal plane. Although the same injection protocol was used throughout the experiments, the extent to which sensory neurons were labeled with dye varied. However, the experimenter always used the minimum intensity of illumination necessary to clearly visualize a cell's processes. The effect of differences in focal plane was more problematic. Typically, two or three different focal planes were required to image all of the sensory outgrowth within a given region of the initial segment of the major axons of L7. We were careful to match as closely as possible the view of a given region of a coculture upon reimaging with the equivalent view recorded on the previous day. Also, we always imaged the same number of focal planes through a given region on the 2 days to minimize any effect of experimenter bias on the results.
19. We included varicosities on the initial segments of the original axons of L7 or on motor neurites within 100 μm of the initial segments without correcting for varicosity size. Any discrete, round fluorescent structure ~3 μm in diameter or larger was counted as a varicosity unless it was clearly a growth cone, in which case it was ignored.
20. The mean initial number of varicosities per coculture before treatment was 24.4 ± 5.5 for the experimental group and 33.3 ± 6.8 for the control group. This difference was not statistically significant ($P > 0.2$). The mean change in varicosity number was 4.4 ± 1.2 for the experimental group and −3.8 ± 2.6 for the control group.
21. The mean amplitude of the EPSP before treatment was 13.5 ± 2.2 mV for experimental cultures and 16.6 ± 3.6 mV for controls. These results replicate those of Montarolo *et al.* (*11*). Cocultures were randomly assigned to either the experimental or control groups. The difference between the mean EPSPs of the two groups before treatment was not statistically significant ($P > 0.2$). The difference between the mean percent change in EPSP size for the control and experimental groups cannot be attributed to a ceiling effect: the mean EPSP size for the experimental group after 5-HT treatment was 20.1 ± 1 mV.
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23. It is not, strictly speaking, correct to compare the rating scores for the sensorimotor cocultures and for the cultures of sensory neurons alone, since the scores were derived from somewhat different measurements. However, we had no a priori justification for confining our observations to a specific region of the sensory neuron for the cultures of sensory neurons alone as we had for the sensorimotor cultures.
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25. How much of the physiological change induced by 5-HT in the sensorimotor cultures can be accounted for by the structural changes? The mean synaptic drive per varicosity for the experimental group before treatment was ~0.6 mV. The 5-HT treatment produced a mean increase of 4.4 varicosities (*20*). By extrapolation, the change in varicosities would be expected to produce a mean increase in EPSP strength of ~2.6 mV in the experimental group. The actual mean increase in EPSP strength produced by 5-HT treatment was 6.6 mV (*21*). Thus, the additional varicosities induced by 5-HT might have contributed substantially to the observed facilitation. Studies of central synapses [for example, H. Korn, A. Triller, A. Mallet, D. S. Faber, *Science* **213**, 898 (1981)] have suggested that a presynaptic bouton corresponds to a single physiological release site. According to this model, a single bouton would be expected to release either zero or one quantum of transmitter. Quantal analysis indicates that release of a single quantum of transmitter from a sensory neuron produces approximately a 100-μV depolarization of L7 (*24*); our estimate of the synaptic drive per varicosity may therefore seem unrealistic. However, we have found that some sensory varicosities can contain several active zones (*6*).
26. Injections of adenosine 3′,5′-monophosphate (cAMP), an intracellular second messenger that is elevated by 5-HT, produce a long-term increase in the number of varicosities for sensory neurons in the intact *Aplysia* pleural ganglion [F. Nazif, J. H. Byrne, L. J. Clearly, *Soc. Neurosci. Abstr.* **15**, 1283 (1989)].
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28. See D. L. Glanzman *et al.* [in *Neuromuscular Junction*, L. C. Sellin, R. Libelius, S. Thesleff, Eds. (Elsevier, Amsterdam, 1989), pp. 489–497] for further discussion of this idea.
29. We thank E. Masurovsky for assistance with the electron microscopy; K. Hilten, S. Mack, and R. Woolley for preparing the figures; H. Ayers for typing the manuscript; and R. D. Hawkins and R. K. S. Wong for their comments on an earlier draft of the manuscript. Supported by the Howard Hughes Medical Institute, and by grants GM 323099 and NS 19595 from the National Institute of General Medical Sciences and the National Institute of Neurological Disorders and Stroke, respectively (to S.S.).

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Autoradiographic Imaging of Phosphoinositide Turnover in the Brain

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With [^{3}H]cytidine as a precursor, phosphoinositide turnover can be localized in brain slices by selective autoradiography of the product [^{3}H]cytidine diphosphate diacylglycerol, which is membrane-bound. In the cerebellum, glutamatergic stimulation elicits an increase of phosphoinositide turnover only in Purkinje cells and the molecular layer. In the hippocampus, both glutamatergic and muscarinic cholinergic stimulation increase phosphoinositide turnover, but with distinct localizations. Cholinergic stimulation affects CA1, CA3, CA4, and subiculum, whereas glutamatergic effects are restricted to the subiculum and CA3. Imaging phosphoinositide turnover in brain slices, which are amenable to electrophysiologic studies, will permit a dynamic localized analysis of regulation of this second messenger in response to synaptic stimulation of specific neuronal pathways.

THE PHOSPHOINOSITIDE (PI) second messenger system mediates numerous neurotransmitter effects in the brain, which, with some exceptions, have not been readily assigned to specific cellular sites (*1*). Localization of neurotransmitter synaptic responses in the brain has been explored by autoradiographic mapping of receptor binding sites, but these sites sometimes do not reflect known synaptic input (*2*). Imaging functional, second messenger responses to neurotransmitters at specific loci in the brain has been difficult to accomplish with immunohistochemical mapping of endogenous adenosine 3′,5′-monophosphate (cAMP) and guanosine 3′,5′-monophosphate (cGMP) (*3*); no localizations of PI responsivity have been attempted.

Phosphoinositide turnover in tissue slices is usually assessed biochemically by labeling phospholipid precursors with [^{3}H]inositol and monitoring the generation of [^{3}H]inositol phosphates in response to neurotransmitter agonists, a procedure that is not compatible with anatomical localization because of the solubility of the products (*4*). Godfrey (*5*) has measured PI turnover in brain slices with [^{3}H]cytidine as a precursor. In this technique, the generation of [^{3}H]cytidine diphosphate diacylglycerol ([^{3}H]CDP-DAG) reflects PI turnover rate. Since CDP-DAG is membrane-bound, we attempted to localize [^{3}H]CDP-DAG by autoradiography, rinsing away water-soluble metabolites and removing [^{3}H]cytidine-containing nucleic acids by enzymatic digestion. By using [^{3}H]cytidine as a precursor, we produced selective autoradiographic images of PI turnover, identifying discrete localizations of PI turnover within the hippocampus and cerebellum that were elicited differentially by muscarinic cholinergic and glutamatergic stimulation.

Godfrey (*5*) found similar enhancement of PI turnover in cerebral cortical slices with either [^{3}H]inositol or [^{3}H]cytidine as pre-

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