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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Table 1. (**A**) Comparison of phosphoinositide turnover measured with [^{3}H]inositol or [^{3}H]cytidine in hippocampal and cerebellar slices (*6*). The data shown are representative of three experiments performed in triplicate. (**B**) Comparison of [^{3}H]CDP-DAG accumulation in hippocampal and cerebellar slices. Transverse hippocampal slices or sagittal cerebellar slices (400 μm thick), identical to those used for autoradiography in Figs. 1 and 2, were prepared from rat brain (*6*). All numbers are expressed as ratios ± SEM of counts per minute in each condition to Li^+ alone. Carb, carbachol; *n*, number of experiments.

Time (min)	[^{3}H]CDP-DAG accumulation				[^{3}H]Inositol phosphates		Tissue	[^{3}H]CDP-DAG accumulation	
	No addition	Li^+	Carb	Li^+ + Carb	Li^+	Li^+ + Carb		Carb	ACPD
A 15	0.8 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	4.7 ± 0.3	1.0 ± 0.2	6.8 ± 0.8	**B** Hippocampus	7.9 ± 2.5 (n = 6)	3.6 ± 1.7 (n = 3)
60	0.8 ± 0.2	1.0 ± 0.1	0.8 ± 0.1	8.2 ± 1.6	1.0 ± 0.1	9.4 ± 2.1	Cerebellum	2.7 ± 1.3 (n = 3)	7.7 ± 2.7 (n = 6)

cursors. In rat hippocampal slices, the same increase of PI turnover was elicited by the cholinergic stimulant carbachol with either [^{3}H]cytidine or [^{3}H]inositol as precursors (*6*) (Table 1A). No augmentation of PI turnover occurred in the absence of lithium or carbachol. In the presence of both lithium and carbachol, the extent of PI turnover stimulation at 15 and 60 min was similar with [^{3}H]cytidine and [^{3}H]inositol. Half-maximal enhancement of [^{3}H]CDP-DAG formation occurs with 100 μM carbachol, a value similar to that seen when PI turnover is monitored with [^{3}H]inositol (*7*). The PI response to carbachol appears to involve M1 or M4 muscarinic receptors, as it is completely blocked by the muscarinic antagonist pirenzipine [inhibitory constant (IC_{50}), 100 nM]. Neomycin, which blocks PI turnover by binding phosphatidylinositol bisphosphate, inhibits PI turnover monitored with [^{3}H]inositol or [^{3}H]cytidine with an almost identical concentration response curve and an IC_{50} of about 0.5 to 0.7 mM. The carbachol-induced augmentation of hippocampal PI turnover was about twice the corresponding increase in the cerebral cortex (*5*).

Within the hippocampus, PI turnover monitored with [^{3}H]cytidine is also increased by *trans*-1-amino-1,3-cyclopentanedicarboxylic acid (ACPD), a derivative of glutamate that, like quisqualate (quis), selectively stimulates glutamatergic synapses associated with PI turnover, which are designated quis-PI responses (*8*). ACPD stimulation of PI turnover was only about half that observed with carbachol (Table 1B). To ensure that PI responses to ACPD involve the quis-PI receptor, we included in incubations with ACPD 2-amino-5-phosphonovaleric acid (APV), a drug that selectively blocks the *N*-methyl-D-aspartate (NMDA) ionotropic glutamate receptors, and 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX), which selectively blocks the ionotropic quis and kainate subtypes of the glutamate receptor. PI turnover augmentation by ACPD was the same in the presence or absence of APV and CNQX. By contrast, ACPD enhancement of PI turnover was blocked by 2-amino-3-phosphonopropionic acid (AP3) (IC_{50}, 0.5 mM), consistent with its known potency in blocking glutamatergic quis-PI responses (*9*). Whereas carbachol enhanced PI turnover more than ACPD in the hippocampus, the pattern is reversed in the cerebellum. Cerebellar ACPD stimulation of PI turnover was about twice that in the hippocampus, while stimulation by carbachol was only about 25% of that in hippocampus. To identify the radiolabeled product in PI turnover experiments with [^{3}H]cytidine, we conducted thin-layer chromatographic (TLC) analysis of the organic extracts and observed a single peak of radioactivity identical to the migration of authentic CDP-DAG (*6*).

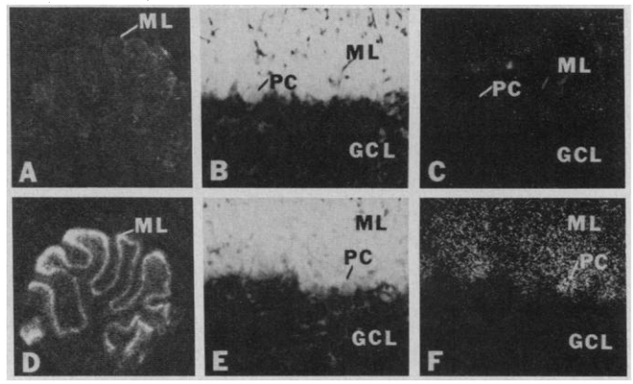

Fig. 1. Imaging of ACPD-stimulated [^{3}H]CDP-DAG accumulation in rat cerebellar slices (*10*). The effect of 5 mM LiCl alone imaged by (**A**) autoradiographic film (×20) and by emulsion-coated cover slip autoradiography under (**B**) light-field and (**C**) dark-field (×400). (**D**, **E**, and **F**) The effect of LiCl plus 100 μM ACPD. ML, molecular layer; PC, Purkinje cell and Purkinje cell layer; and GCL, granule cell layer. Experiments were repeated a minimum of five times with similar results.

For autoradiographic studies, we incubated brain slices with [^{3}H]cytidine for 1 hour followed by treatment with lithium in the presence or absence of neurotransmitters and drugs. Tissue slices were embedded and cut in 16-μm frozen sections. In a permeabilizing buffer containing saponin, the sections were treated with deoxyribonuclease and ribonuclease to destroy nucleic acids that may have incorporated [^{3}H]cytidine. The sections were exposed to film or photographic emulsion, and autoradiographic grains were monitored. Only [^{3}H]CDP-DAG remained in the treated sections, as TLC analysis of the nuclease-treated sections revealed a single radioactive peak corresponding to authentic CDP-DAG (*10*). Addition of exogenous *myo*-inositol (20 mM) reversed the accumulation of [^{3}H]CDP-DAG, as monitored by both TLC and autoradiography, providing further evidence that the incorporated cytidine reflects the PI cycle (*6*).

Autoradiographic analysis revealed an enhancement of PI turnover elicited by ACPD in the cerebellum (Fig. 1) and by both carbachol and ACPD in the hippocampus (Fig. 2). In the cerebellum only negligible amounts of CDP-DAG–associated silver grains are observed in the absence of ACPD, whereas grain density is markedly increased after ACPD treatment. Grains are localized to the molecular and Purkinje cell layers with negligible densities over the granule cell layer or white matter. Carbachol (1 mM) did not augment grain density compared with lithium controls. Since APV and CNQX were included in all incubations, ACPD stimulation probably involves the quis-PI receptor system.

In the hippocampus, carbachol increased grain density as compared to the negligible amounts in lithium controls (Fig. 2). Grain density was similar in the subiculum, CA1, CA3, and CA4 and was diminished at the borders between CA1 and CA3 and between CA3 and CA4. Grains were most concentrated over the pyramidal cell layer with an extension at a somewhat lesser density in the subjacent dentritic field of pyramidal cells. The grain pattern in the pyramidal cell layer was not uniform, but consisted of discrete patches. The patch-like

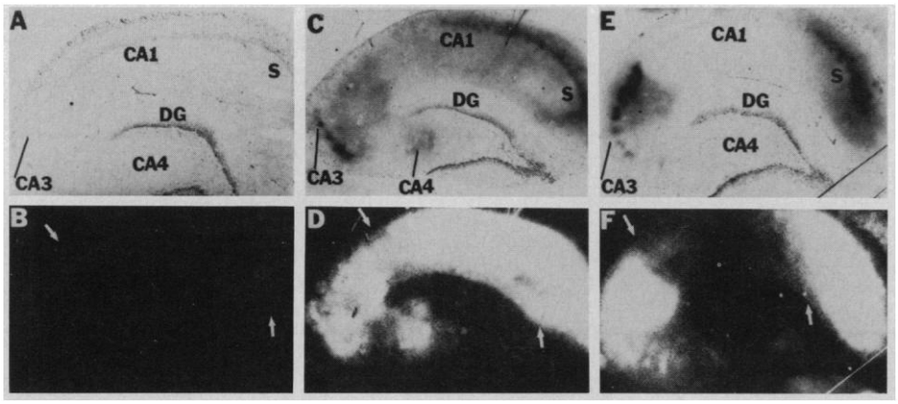

Fig. 2. Autoradiographic imaging of [^{3}H]CDP-DAG accumulation in rat hippocampal slices in the presence of 5 mM LiCl alone (**A** and **B**), LiCl plus 1 mM carbachol (**C** and **D**), or LiCl plus 100 μM ACPD (**E** and **F**), viewed under light-field and dark-field microscopy (×50). Procedures used were identical to those in Fig. 1. DG, dentate gyrus and S, subicular area. Arrows indicate CA3-CA1 and CA1–subicular area borders. Experiments were repeated a minimum of three times with similar results.

appearance was apparent in all the pyramidal cell layers, but was more prominent in CA3. Grain densities were substantially lower in the granule cell layer and the molecular layer of the dentate gyrus.

ACPD stimulation elicited a markedly different pattern than carbachol, with dense grain accumulations in the subicular area and in CA3 and negligible densities in CA1 and CA4, the areas most stimulated by carbachol. ACPD-elicited grains displayed sharp borders between CA3 and CA1 and between the subicular area and CA1. The border was maintained both in the pyramidal layer and in the subjacent area of pyramidal cell dendrites. Negligible grain densities occurred in the dentate gyrus in ACPD-treated sections.

In the cerebellum, the localization of ACPD-stimulated turnover in Purkinje cells and their dendrites fits with abundant evidence that the major PI-associated pathway involves the quis-PI synaptic responses of Purkinje cells to glutamate released from parallel fibers of the granule cells. In the hippocampus, the localization of carbachol-stimulated PI turnover to the subiculum, CA1, CA2, CA3, and CA4 pyramidal cell layers is consistent with the cholinergic neuronal input to these areas reflected in cholinesterase staining and muscarinic cholinergic receptor autoradiography (*11*). We observed much lower densities of carbachol-stimulated PI turnover in the dentate gyrus than in the pyramidal cell layer. Of M1 and M4, the two muscarinic receptor subtypes linked to the PI system, mRNA associated with M1 receptors is concentrated in the dentate gyrus as well as the pyramidal cell layer, whereas M4 concentrations are substantially lower in the dentate gyrus (*12*). This localization suggests that the PI responses to carbachol we have seen reflect a greater influence of M4 receptors than M1 receptors.

Our visualization of ACPD-enhanced PI turnover localizes the glutamatergic PI system in the hippocampus to CA3, and sheds light on issues that have not been resolved in immunohistochemical studies of glutamate (*13*) and autoradiography of glutamate receptors (*14*). Long-term potentiation (LTP) in CA1 of the hippocampus utilizes NMDA receptors (*15*), whereas the pertussis toxin–sensitive, NMDA-resistant LTP of CA3 probably involves quis-PI glutamatergic synapses. This distinction fits with the high density of ACPD-stimulated PI turnover in CA3 but not CA1 regions.

The patch-like arrangement of autoradiographic grains in the pyramidal cell layer of the hippocampus cannot be explained by the disposition of pyramidal cells or their synaptic inputs. A patch-like pattern of the commissural pathways connecting pyramidal cell layers of the two hemispheres may determine the patchy pattern of PI turnover. This element of synaptic responsivity would not be apparent from patterns of neuronal input or receptor binding sites.

Imaging PI responses to neurotransmitters at a microscopic level may help clarify the nature of synaptic interactions. The slice preparations we used are identical to those utilized for electrophysiologic analysis, permitting studies of differentially localized PI response to discrete neuronal inputs. Although we have focused on the hippocampus and cerebellum and influences of cholinergic and glutamatergic stimulation, our technique can be applied to numerous brain regions and peripheral tissues in response to neurotransmitters, hormones, drugs, and physiologic and pathologic stimuli.

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6. Brain slices (400 μm) were prepared from male Sprague-Dawley rats and allowed to recover for 1 hour at 20°C in an interface chamber (95% O_2:5% CO_2). Slices were labeled on Whatman filter paper circles (2.1 cm in diameter) immersed in 0.1 ml of Krebs-bicarbonate buffer containing either 0.3 μM *myo*-2-[^{3}H]inositol (Du Pont, Biotechnology Systems; 17 Ci/mmol) or 0.4 μM 5-[^{3}H]cytidine (Du-Pont, Biotechnology Systems; 27.8 Ci/mmol) for 1 hour at 30°C. Ten minutes before adding drug (final concentration, 100 μM APV, 100 μM CNQX, and 1 mM carbachol or 100 μM ACPD) we evenly dispersed LiCl (final concentration, 5 mM) in the 0.1-ml volume of buffer under the filter paper. [^{3}H]Inositol phosphate and [^{3}H]CDP-DAG accumulations were determined at 15 or 60 min (*4, 5*). More than 90% of the lipid-extracted radioactivity comigrated with authentic CDP-DAG on TLC, and the ratio of stimulated to basal values was conserved when the spots corresponding to CDP-DAG were cut out and the radioactivity was determined. Addition of 20 mM *myo*-inositol reversed the accumulation of [^{3}H]CDP-DAG (P. M. Hwang, D. S. Bredt, S. H. Snyder, unpublished data).
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