only rarely encountered, had a normal morphology. Trophozoites in culture were less ameboid (Fig. 1, B and C) than those in vivo. This ameboid behavior appeared reversible, since the rhesus monkeys inoculated with cultured parasites developed infections where the trophozoites were characteristically highly ameboid (Fig. 1D); when their infected blood was used to reinitiate cultures, the trophozoites developing in vitro were again less ameboid.

Plasmodium cynomolgi differs from previously cultured malaria parasites in its potential for causing relapses. This parasite will be particularly suitable for combined studies in vivo and in vitro on relapsing malaria, since monkeys infected with *P. cynomolgi* show predictable relapses, unlike monkeys infected with *P. vivax* (*13*). Previously cultured malaria parasites include the falciparum-type *P. fragile* (*14*) and the malariae-type *P. inui* (*15*), both of which infect rhesus monkeys. The addition of *P. cynomolgi* underlines the value of *M. mulatta* as a simian model for studies on human malarias.

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8. Trade names and commercial sources are for identification purposes only and do not constitute endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.
9. Prior to being used, human serum samples were tested by overnight incubation with rhesus monkey erythrocytes under routine culture conditions; the occasional samples causing erythrocyte agglutination were rejected. Growth of the parasites was better with human serum than with rhesus monkey serum.
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Long-Term Potentiation in the Hippocampal Slice: Evidence for Stimulated Secretion of Newly Synthesized Proteins

Abstract. *Long-term potentiation of the hippocampal slice preparation results in an increase in the incorporation of labeled valine into the proteins destined for secretion into the extracellular medium. Double-labeling methods established that the increased secretion of the labeled proteins was limited to the potentiated region of a slice; incorporation of labeled valine was increased in the hippocampus if potentiation was through the Schaffer collaterals and in the dentate if potentiation was through the perforant path. Controls for nonspecific stimulation showed no changes. There appears to be a link between long-term potentiation and the metabolic processes that lead to protein synthesis in the hippocampal slice system.*

Long-term potentiation (LTP) of synaptic responses in the rat hippocampus is used as a model system for the neurophysiological study of plasticity (*1–4*). Afferent stimulation of transverse sections of hippocampal slices of rat brain by application of a brief tetanizing train can produce a postsynaptic response that is two to ten times greater than the response before tetanus. The LTP lasts for weeks in the intact preparation (*1*) and for up to 10 hours in the in vitro preparation, its longevity being limited by the slicing technique (*2*). Biochemical effects in brain tissue after electrical stimulation have been demonstrated (*5*), and the effects of LTP on the pattern of protein phosphorylation have been studied (*6*).

We now report a relation between LTP and protein synthesis, explored by techniques that we used to study the pattern of protein synthesis in the goldfish brain (*7*) after training. In the goldfish, we found that two specific glial proteins are rapidly labeled and released into the extracellular fluid after the animals acquire a new pattern of behavior (*8*). Such changes are considered to be part of the sequence of metabolic events that ultimately lead to neuronal plasticity. If LTP in the hippocampal slice is also an indicator of neuronal plasticity, then by analogy to the goldfish data (*8*) some changes in the pattern of synthesis and secretion of proteins might be expected in the rat hippocampal slice after LTP.

Using a double-labeling procedure (*7*) in which the pattern of protein synthesis in a single potentiated slice of rat hippocampus is compared with that of an unpotentiated control slice, we have found a substantial increase in the secretion of labeled proteins into the extracellular fluid after LTP.

Transverse hippocampal slices, cut to preserve the organization of the hippocampus, were prepared from male Sprague-Dawley rats (250 g) (*9*). The slices were placed on nylon nets above a sample chamber containing 200 μl of Earle's incubation medium and maintained at 34°C in a humidified atmosphere of 95 percent O_2 and 5 percent CO_2. Such slices were capable of producing stable orthodromic responses for at least 8 hours. Stimuli were delivered with bipolar electrodes placed on the Schaffer collateral or the perforant path fibers. Monosynaptic excitatory postsynaptic potentials were recorded with electrodes at the CA1 region of the hippocampus or at the dentate gyrus. These electrode configurations record the monosynaptic population spike response to stimulation of afferent fibers.

Before being stimulated with tetanizing pulses, the slices were incubated for 1 hour and the input-output (I-O) function was determined (Fig. 1). The I-O function describes the relation between the magnitude of the afferent input and the amplitude of the response of the population of cells activated; it is thus a description of the synaptic efficacy or throughput of the system. The tetanus (33 Hz for 3 seconds) was produced at an intensity sufficient to evoke a population spike of 1 mV (*2*). An I-O curve determi-

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nation at 5 minutes and 25 minutes after tetanus was used to assess LTP (*10*). Some slices were tetanized but displayed no LTP; one of these was used as a control. The average level of potentiation was 200 percent, with a range between 100 and 700 percent.

After completion of the stimulation and I-O measurements during a 1-hour period, the electrodes were withdrawn, and each slice was left in the incubation medium without labeling for 120 minutes after tetanus. The period after tetanus was included in the experimental design to allow time for any newly synthesized messenger RNA molecules to move into the cytoplasm and thus become available for influencing the pattern of protein synthesis. Next, 100 μCi of [^{3}H]valine in 5 μl of medium was injected into the incubation medium. For each potentiated slice, a control slice in a separate sample chamber received 10 μCi of [^{14}C]valine. (In one experiment the label was reversed.) At the end of 3 hours of label incorporation, a potentiated slice (^{3}H) and control slice (^{14}C) were extracted together in 0.7 ml of 0.32*M* sucrose containing 1 m*M* nonradioactive valine at 0°C for 10 minutes. The samples were gently vortexed for a few seconds every 2 or 3 minutes. The extract, after being removed with a Pasteur pipette and combined with the incubation media used for the labeling, was the crude extracellular fluid fraction, which was then purified (*11*) and used for the measurement of total ^{3}H and ^{14}C content.

The slices remaining after extraction of the extracellular fluid were homogenized together in 0.32*M* sucrose and used to prepare the cytoplasmic and mixed membrane–nuclear protein fractions (*12*). For studies of the regional distribution of cytoplasmic proteins, the same extraction procedures were used on the combined sections of a control and potentiated slice after the dentate was separated from the hippocampal zones (Fig. 1). Each dentate section contains a small part of the CA4 region, whereas the hippocampus fragment includes the CA1, CA2, and CA3 regions and some of the CA4 region. In these experiments, only the cytoplasmic protein fraction was isolated. The cytoplasmic fraction was obtained after dissected fragments had been washed once with 0.32*M* sucrose at 0°C prior to homogenization. The labeled proteins remaining within the tissue, that is, the nonsecreted components, are measured in this fraction.

Our double-labeling procedure ensures that (i) each potentiated slice is compared with an identically treated control slice from the same hippocampus, (ii) all technical features of the isolation procedure are common to both the experimental and the control slices, and (iii) the ratios of incorporated labels in the extracellular fluid fractions are compared to ratios of incorporated labels in the cytoplasmic or nuclear fractions. Thus, the labeling of the cytoplasmic and nuclear fractions provides an internal control for possible changes in valine uptake in studies of the changes in extracellular fluid protein. If such pool changes occur as a result of LTP, all fractions would have the same specific activity, and no enhanced labeling of the extracellular fluid fraction would be noted. The double-labeling method also removes all problems connected with obtaining quantitative yields of products from the small amounts of tissue used, since any losses during the stages of isolation and purification would be the same for both isotopes.

The average quantity of label incorporated into proteins of the cytoplasmic fraction per slice was 6.5×10^5 and 8×10^4 count/min for the ^{3}H- and ^{14}C-labeled valine, respectively. The average amount of label incorporated into the proteins (nondialyzable products with molecular weight > 5000) of the extracellular fluid fraction was about 23 per-

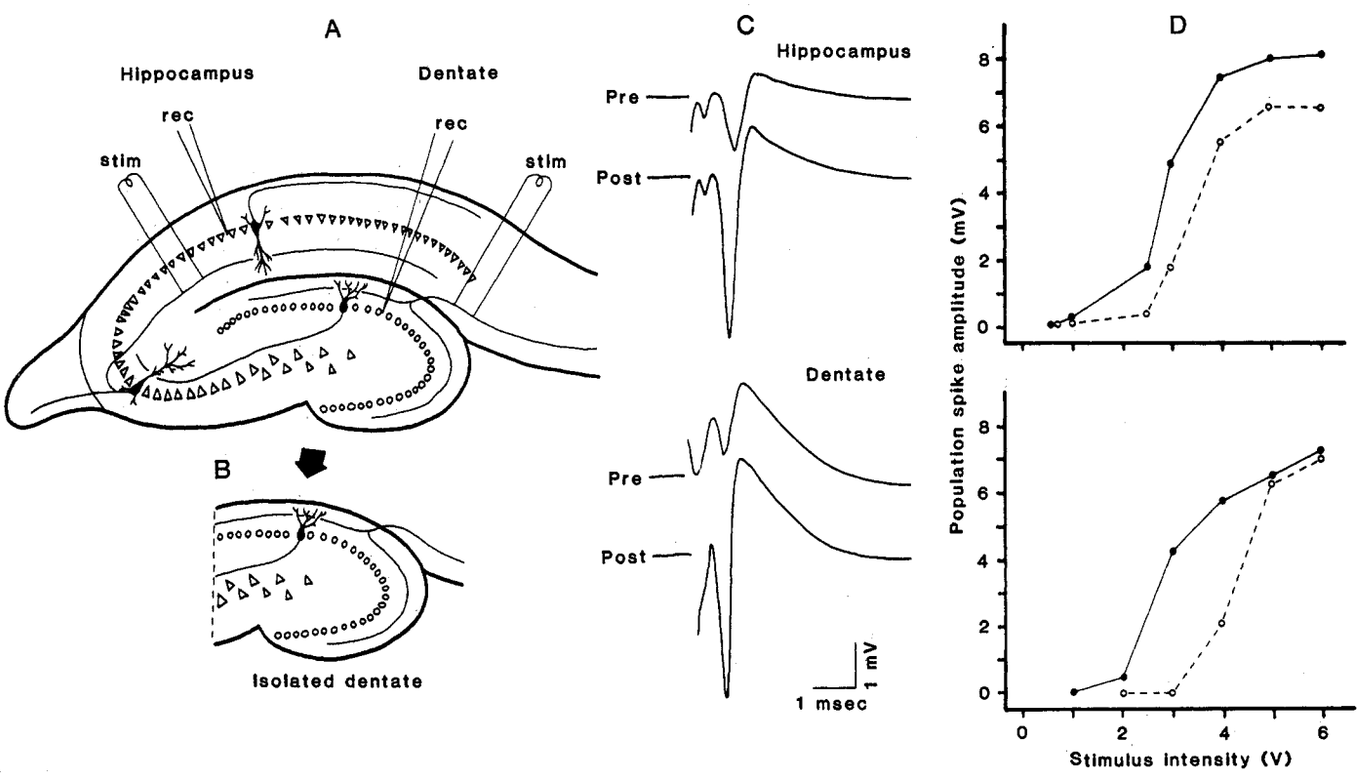

Fig. 1. (A) Hippocampal slice preparation showing the placements of the stimulating (*stim*) and recording (*rec*) electrodes for LTP of the hippocampus and dentate. (B) The segment of the slice removed in the analysis of the isolated dentate. (C) Oscilloscope tracings for the responses before (*Pre*) and after (*Post*) tetanus at a stimulus intensity of 3 V for two experiments in the hippocampus and dentate, respectively. (D) The corresponding I-O curves for the two experiments, showing responses before (- - -) and after (——) tetanus.

cent of the amount present in the cytoplasmic components for a potentiated slice and about 9 percent for a control slice.

The secretion of labeled proteins into the extracellular environment was enhanced by potentiation (Table 1). In experiments 1 to 5, isotope ratios were determined for the labeled proteins in the extracellular fluid (R_{ECF}) derived from a ^{3}H-labeled potentiated slice and a ^{14}C-labeled control slice of CA1. In experiment 6, similar data were obtained for a slice potentiated at the dentate region via the perforant pathway. The ratios of ^{3}H- to ^{14}C-labeled proteins remaining in the cytoplasmic fraction (R_{cyto}) were also determined (Table 1). The ratio of R_{ECF} to R_{cyto} normalizes the values for secreted proteins to those remaining in the cytoplasmic fraction of the same experimental and control slices. This ratio varies from 1.8 to 3.5, suggesting that more labeled proteins become associated with the extracellular fluid fraction after potentiation (Table 1). The average ratio is 2.75 ± 0.64 as compared to the ratio of 0.8 ± 0.17 obtained for the control experiments in which no stimulation of secretion occurs. Comparisons of the data with a *t*-test indicate that results are highly significant ($P < .0005$). Thus, LTP causes an average increase of 175 percent in the amount of secreted labeled proteins into the extracellular fluid.

In experiment 7, a value of 0.9 was obtained for the R_{ECF}/R_{cyto} ratio for an unstimulated ^{3}H-labeled control slice compared to an unstimulated ^{14}C-labeled control slice (within 10 percent of the theoretical value of 1.0). In experiment 8, a comparison of ^{3}H/^{14}C for the nuclear proteins with ^{3}H/^{14}C for the cytoplasmic proteins gave a value of 1.0; this can occur only if the procedures for protein isolation and the specific activity of the amino acid precursor pools are identical for the two fractions in each of the stimulated and control hippocampal slices.

Experiments 9 and 10 indicate that protein secretion is not enhanced if no long-term potentiation develops in a slice. Thus, in experiment 9, application of the same number of pulses as the tetanizing train (33 Hz for 3 seconds) distributed over a 20-minute period did not cause an increase in R_{ECF}/R_{cyto}. In experiment 10, a tetanizing train was used, but the individual slice did not exhibit LTP when tested 25 minutes later; this slice showed no enhanced secretion, and R_{ECF}/R_{cyto} decreased to 0.6, suggesting that an inhibition of secretion might be occurring. These controls indicate that LTP rather than stimulation produces enhanced secretion of labeled protein.

In another series of experiments, we examined the effect of the locus of potentiation on the pattern of secretion (Table 2). If there is coupling between LTP and the amount of secreted protein, then potentiation of the CA1 region should enhance secretion from that region in comparison with secretion from the dentate in the same slices, and the inverse changes should occur if the dentate is potentiated. Because of the difficulty of identifying the source of the secreted protein from different regions of the same slice, we used alterations in the pattern of labeling of the proteins that remained in the cytoplasmic fraction after removal of the secreted proteins. Thus, in experiments 11 to 15, the CA1 regions of the hippocampal slices were potentiated. After being labeled with [^{3}H]valine for 3 hours, the CA1 regions were separated from the dentate (Fig. 1) and combined with a similar dissected region of CA1 from a control slice labeled with [^{14}C]valine. The labeled hippocampal cytoplasmic proteins from these fragments were isolated after extraction of the extracellular fluid. Similar procedures were used to isolate the proteins from the remaining dentate fragments from the same two slices. Thus, the ratios R_H and R_D represent the ^{3}H/^{14}C ratios of the cytoplasmic proteins from the potentiated hippocampal CA1 and the unpotentiated dentate regions, respectively. A stimulated secretion

Table 1. Enhanced secretion of labeled proteins after potentiation. After potentiation, each slice was incubated for 2 hours before being labeled for 3 hours. In all experiments except experiment 6, [^{3}H]valine (specific activity, 5 Ci/mmole) was used for the potentiated slice, and [^{14}C]valine (specific activity, 280 mCi/mmole) was used for controls. In experiment 6, the labels were reversed. R_{ECF} is the ratio of labeled proteins in the extracellular fluid of a potentiated slice to that in a control unstimulated slice; R_{cyto} is the ratio of labeled proteins in the cytoplasmic fraction of a potentiated slice to that in a control slice after removal of the extracellular fluid fractions; H and D refer to the hippocampal and dentate regions, respectively. Comparisons with a *t*-test of experimental versus control data give $P < .0005$.

Experiment	Locus of potentiation or stimulation	^{3}H/^{14} Ratios		R_{ECF}/R_{cyto}	Remarks
		R_{ECF}	R_{cyto}		
1	H	11.8	4.5	2.6	
2	H	12.6	5.2	2.4	
3	H	13.2	4.8	2.8	
4	H	12.3	3.4	3.5	
5	H	21.1	6.2	3.4	
6	D	3.4	1.9	1.8	
Average				2.75 ± 0.64	
			Control experiments		
7	None	7.6	8.4	0.9	^{3}H control versus ^{14}C control
8	H	8.0*	8.0	1.0	Membrane-nuclear versus cytoplasmic
9	H	3.3	4.1	0.8	Activation control
10	H	3.7	5.9	0.6	Stimulation but no potentiation
Average				0.8 ± 0.17	

*Ratio of labeled proteins in the membrane-nuclear fraction of a potentiated slice to that in a control slice after removal of extracellular fluid.

Table 2. Labeling of cytoplasmic fractions of potentiated versus unpotentiated regions of the same slice. R_H is the ^{3}H/^{14}C ratio of the labeled cytoplasmic proteins for the hippocampal fragment of a potentiated (^{3}H) to a control (^{14}C) slice . R_D is the ratio of the labeled cytoplasmic protein for the remaining dentate segments of the same two slices. H and D denote the potentiation locus at Schaffer collaterals to CA1 and perforant path to dentate, respectively. Comparisons of the data with the controls in Table 1 give $P < .005$. R_P and R_C are the ratios for the potentiated and control regions of the slices, respectively.

Experiment	Locus of potentiation	R_H	R_D	R_P/R_C
11	H	2.5	6.3	0.39
12	H	7.1	18.2	0.39
13	H	2.9	7.1	0.43
14	H	3.5	4.7	0.74
15	H	3.6	5.3	0.68
16	D	4.5	1.2	0.27
17	D	8.0	5.7	0.74
18	D	4.0	1.5	0.38
19	D	4.6	2.9	0.62
Average				0.52 ± 0.18

from the CA1 zone should result in a $^3H/^{14}C$ ratio that is smaller for the CA1 fraction than that for the dentate fraction; that is, R_H should be less than R_D. This type of result was observed in five experiments, where a decrease in the cytoplasmic proteins for the potentiated section was between 26 and 61 percent. Experiments 16 to 19 give the results for the slices in which the dentate was potentiated through the perforant pathway. Again there was a substantial decrease for the cytoplasmic proteins of the potentiated region of the slice, between 26 and 63 percent. These results are consistent with the concept that enhanced secretion from the locus of potentiation gives rise to a decrease in the amount of labeled proteins remaining in the cytoplasmic fraction.

Our studies indicate that LTP is linked with the metabolic process that leads to protein synthesis in the slice preparation. A coupling between neurophysiological events and the local secretion of newly synthesized proteins is observable 2 hours after tetanizing trains are applied, suggesting that the biochemical changes remain long after the neurophysiological events have been completed. The development of LTP appears to be essential for the protein changes since nonspecific stimulation or stimulation without potentiation does not produce enhanced secretion. In this respect, LTP is similar to the effects of training on the pattern of protein synthesis in vivo (*8*) in the goldfish brain and focuses attention on the proteins that are released into the extracellular fluid.

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10. Changes in the synaptic efficacy were expressed as a percentage of the I-O determination before tetanus. Only slices that showed no changes in their population excitatory postsynaptic potential thresholds and significant potentiation (200 percent or more) at the 5- and 25-minute I-O determinations were considered valid for biochemical studies.
11. The crude extracellular fluid fraction was purified by centrifugation at 90,000*g* for 30 minutes at 0°C to yield a supernatant free from particulate matter. This was mixed with an equal volume of brain protein solvent buffer containing 2 percent sodium lauryl sarcosinate, 0.08*M* EDTA, and 5*M* urea in 0.02*M* phosphate buffer, *p*H 10. The mixture was incubated for 2 hours at 37°C to hydrolyze any labeled aminoacyl transfer RNA molecules that might be present and then dialyzed four times against 1 liter of 0.03*M* phosphate, *p*H 7.0, containing 1 m*M* nonradioactive valine, and twice against 1 liter of distilled water. Each dialysis step required 18 hours. The products were then concentrated to 0.1 ml, and 10-μl portions were used for the radioactivity determinations in a liquid scintillation counter. All products, including glycoproteins with molecular weight > 5000, are measured by this procedure.
12. Each experimental slice was homogenized with a control slice in 0.7 ml of 0.32*M* sucrose containing 1 m*M* unlabeled valine, in a Teflon glass homogenizer (0.05-mm gap) at 0°C with 31 strokes at 300 rev/min. The mixture was separated by centrifugation at 90,000*g* at 0°C for 30 minutes into a supernatant cytoplasmic fraction and a sediment containing the mixed membrane-nuclear fraction. The supernatant and sediment were mixed with an equal volume and 3 ml of brain protein solvent, respectively, and purified according to the procedure for the extracellular fluid fraction (*11*) for analysis of ^3H and ^{14}C content.
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Lysosomal Cathepsin B: Correlation with Metastatic Potential

Abstract. *Although lysosomal enzymes are implicated in the processes of tumor invasion and metastasis, their cellular origin within the tumor is unclear. The activity of the lysosomal proteinase cathepsin B is significantly elevated in a variant of the B16 melanoma with high metastatic potential. The cathepsin B activity is localized to the lysosomes of the tumor cells.*

Metastasis is the result of an intricate sequence of events initiated when the primary tumor cells invade normal tissue, detach, and disseminate to distant sites, and culminated when the neoplastic cells invade and establish secondary tumors. The malignancy of a tumor depends on its ability to invade normal tissue both at the primary and secondary sites of growth. Increases in lysosomal enzyme activities and in lysosomal enzyme release have been correlated with the ability of primary tumors to invade normal tissue as well as with the occurrence of metastatic tumors (*1–3*). Up to 11 times more of the lysosomal proteinase cathepsin B is released from malignant human breast tumors than from normal breast tissue or nonmalignant tumors (*1*). Further evidence linking cathepsin B to tumor malignancy is the positive correlation between serum cathepsin B levels and vaginal clear-cell adenocarcinoma (*4*). Because solid tumors contain heterogeneous cell types (macrophages, lymphocytes, and stromal cells as well as tumor cells), the cellular source of elevated or released enzymes has not been demonstrated definitively. Using the technique of centrifugal elutriation (*5*) to separate the actively growing tumor cells from other cell types dispersed from the solid tumor, we have determined the activities of five lysosomal enzymes in metastatic variants of the mouse B16 melanoma (*6*).

Variants of the B16 melanoma with low ($B16F_1$) and high ($B16F_{10}$) metastatic potential (*6*) were propagated in vivo by subcutaneous injection into syngeneic C57BL/6J male mice (Jackson Laboratory). The $B16F_{10}$ cells when injected intravenously into the tail vein of syngeneic mice consistently produced a greater number of metastatic foci in the lungs than did the $B16F_1$ cells (*7*). The lysosomal enzymes, cathepsin D, β-*N*-acetylglucosaminidase, β-glucuronidase, acid phosphatase, protein, and DNA were assayed fluorometrically as described by Sloane and Bird (*8*). Cathepsin B was assayed fluorometrically (*4*) with carbobenzyloxy-Ala-Arg-Arg-4-methoxy-β-naphthylamine (Enzymes Systems Products, Livermore, California) as substrate (Ala, alanine; Arg, arginine). Enzyme assays were standardized to ensure linearity. Subcutaneous $B16F_1$ and $B16F_{10}$ tumors (0.5 to 1.5 g) were removed, homogenized, and assayed for lysosomal enzyme activities. Cathepsin B activity was threefold higher in homogenates of the $B16F_{10}$ tumor than in those of the $B16F_1$ tumor (Table 1). Cathepsin B activity in these homogenates was inhibited more than 99 percent by the cysteine proteinase inhibitor leupeptin (1 μg/ml). No differences in activity were detected between the $B16F_1$ and $B16F_{10}$ tumors for cathepsin D, β-*N*-acetylglucosaminidase, β-glucuronidase, or acid phosphatase when expressed per gram of wet weight, per milligram of protein, or per microgram of DNA (not illustrated). The threefold elevation in cathepsin B activity in the $B16F_{10}$ tumor with its higher metastatic potential may simply reflect a difference in the macrophage content of the two tumors. Possible changes in the number of macrophages have been suggested as contributing to changes with age in lysosomal

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