The Journal of Neuroscience, March 1993, 13(3): 1010–1021

Pairing the Cholinergic Agonist Carbachol with Patterned Schaffer Collateral Stimulation Initiates Protein Synthesis in Hippocampal CA1 Pyramidal Cell Dendrites via a Muscarinic, NMDA-Dependent Mechanism

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Effects of afferent stimulation on local synthesis of protein in CA1 pyramidal cell dendrites were studied using light microscope autoradiography. Tissue was fixed with paraformaldehyde immediately after 3 min exposure to ^{3}H-leucine in order to trap ^{3}H associated with macromolecules.

The rate of ^{3}H-leucine incorporation into dendrites of resting hippocampal slices was 10% the rate of incorporation into cell somata. Ninety percent of the incorporation into the somata was inhibited by cycloheximide (300 μM); none of the incorporation into dendrites was blocked by cycloheximide. Thus, there is no measurable extramitochondrial synthesis of protein in the dendrites of the resting slice.

Slices were exposed to 50 μM carbachol and the Schaffer collateral afferents to the CA1 pyramidal cells were stimulated intermittently at 10 Hz over a 20 min period. In this case, ^{3}H incorporation into dendrites was increased almost threefold over resting levels, with no effect on label over the cell somata. There was no associated increase in uptake of free ^{3}H-leucine, and the increase in label was completely blocked by cycloheximide. Thus, associating carbachol and afferent stimulation appears to activate *de novo* protein synthesis in the dendrites. Neither the carbachol alone nor the Schaffer collateral stimulation alone increased synthesis.

The activation of dendrite synthesis was completely blocked by 5 μM atropine, and also by 50 μM D-aminophosphonovalerate. It did not occur when carbachol was paired with steady stimulation of the Schaffer collaterals at 1 Hz for 20 min, rather than with the patterned high-frequency stimulation.

Thus, associating a cholinergic agonist with a level of neural activity that occurs in CA3 and CA1 pyramidal cells during exploratory behavior (Muller et al., 1987) initiates local protein synthesis in target dendrites. This effect is dependent on muscarinic cholinergic receptors and NMDA-type glutamate receptors. The possible relationship of this phenomenon to mechanisms of learning is discussed.

[Key words: ACh, dendrities, hippocampus, learning, NMDA, protein synthesis]

The protein composition of postsynaptic dendrites must be a major factor in the determination of neural behavior. While a great deal of attention has been focused on posttranslational modification of dendritic protein (Lisman, 1989; Nelson et al., 1989; Oliver et al., 1989), very little is known about the regulation of turnover of these proteins. The paucity of polysomes in distal dendrites and the demonstration of dendritic flow in a few studies have led to the conclusion that the large bulk of new protein in dendrites originates by flow from the cell somata, as it does in axons (Kreutzberg et al., 1973; Grafstein and Forman, 1980). However, ribosomes and polysomes are found in distal dendrites (Steward et al., 1988), and at least two mRNA species have been observed there (Burgin et al., 1990; Kleiman et al., 1990), suggesting that there may be local synthesis. Indeed, there is now direct evidence that dendrites that are being reinnervated *in situ* (Steward, 1986) and dendrites in cultured neurons (Torre and Steward, 1992), as well as isolated dendritic fragments (Rao and Steward, 1991), do synthesize extramitochondrial protein.

The fact that only a very small fraction of total protein is synthesized in dendrites suggests that this local synthesis has a specific functional role, and the observation that dendritic ribosomes occur largely under dendritic spines (Steward et al., 1988) has led to the suggestion that afferent neural activity might regulate this local synthesis (Lipton and Robacker, 1983; Gordon-Weeks, 1988). Such a mechanism could play a role in neural plasticity, as alterations in synthesis should be restricted to active synapses, as required by Hebbian models (Lisman, 1989). While stimulatory effects of afferent stimulation on protein synthesis have been described in neuronal cell somata (Abdul-Ghani et al., 1980; Hyson and Rubel, 1989; Uenishi et al., 1991), there are no descriptions of effects of afferent stimulation on synthesis of protein in intact dendrites.

In this study, the hippocampal slice was used to examine the effects of afferent pathway stimulation on protein synthesis in CA1 pyramidal cell layer dendrites. The effects of afferent pathway stimulation alone, and in the presence of a cholinergic agonist, were studied to see whether associating two stimuli that may occur together during learning had unique effects. The importance of the cholinergic system to learning is well established (Dunnett et al., 1982; Chrobak et al., 1989; Ridley et al., 1991). A pattern of afferent axon stimulation that resembles that occurring during prolonged exploratory activity by an animal (Muller et al., 1987) was applied, either alone or in combination with the cholinergic agonist carbachol. Synthesis in dendrites

Received June 24, 1992; revised Aug. 31, 1992; accepted Sept. 10, 1992.

We acknowledge the excellent technical assistance provided by Goeffrey Guerts and Randall Massey. This work was supported by NSF Grant BNS9021629.

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was studied using autoradiography, following exposures of slices to precursor amino acid. These exposures were of very brief durations in order to eliminate any contribution from dendritic flow. Associating the stimulus with carbachol had profound effects on protein synthesis in the dendrites.

Materials and Methods

Slice incubation

Transverse hippocampal slices were prepared from guinea pigs as previously described (Feig and Lipton, 1990). Freshly dissected hippocampi were sliced on a Vibratome at a nominal thickness of 500 μm. They were placed in a "preincubation" buffer for 45 min and then transferred to standard buffer for a further 2 hr. Experimental procedures were then carried out between 2.75 and 7 hr of killing the animal. Standard buffer consisted of (in mM) 120 NaCl, 26 $NaHCO_3$, 1.3 $MgSO_4$, 1.2 KH_2PO_4, 1.2 $CaCl_2$, and 10 glucose, with 95% O_2, 5% CO_2 (pH 7.4). Preincubation buffer was the same as standard buffer except 0 mM $CaCl_2$ and 10 mM $MgSO_4$. All incubations were carried out at 36°C. The preincubation allows good morphological and electrophysiological preservation of CA1 pyramidal cells (Feig and Lipton, 1990) (see Results).

Experimental procedure

Electrophysiological studies. A pair of adjacent slices was placed on a nylon bolting cloth grid in an electrophysiological chamber and perfused with standard buffer (Kass and Lipton, 1982). One member of each pair was stimulated with bipolar tungsten electrodes in stratum radiatum of CA2; responses in the CA1 pyramidal cell layer were recorded with a tungsten microelectrode (2–5 MΩ). Slices were stimulated once every 2 min until a steady population spike response was recorded. Transmitter agonists and/or antagonists were added as described in the figure captions, and a new steady state was obtained after a further 15 min. The stimulation paradigm was then begun. In all but one study, 1 min of stimulation is followed by 4 min of silence, repeated four times. During the 1 min, the repeated pattern was 3 sec of 10 Hz stimulation followed by 4 sec of silence. The overall paradigm of 1 min on, 4 min off was chosen to mimic events that might occur during multiple trial learning. Ten hertz was chosen because it is an average firing frequency for activated hippocampal place cells (Muller et al., 1987). The intermittent pattern was chosen to eliminate attenuation of the signal during stimulation. The above paradigm is termed the "standard stimulus paradigm." In one study the stimulation pattern was altered so that the same number of pulses were delivered uniformly throughout the 20 min stimulation time period.

^{3}H-leucine incorporation. At the end of the stimulus paradigm, the grid was placed into buffer of the same composition used during stimulation, containing ^{3}H-leucine (500 μCi/ml; specific activity, 60 Ci/mmol) added as an aqueous solution. The two slices were removed after 3 min and fixed immediately with ice-cold 4% paraformaldehyde. In one treatment they were fixed in ice-cold 2% glutaraldehyde, 2% paraformaldehyde in order to retain free ^{3}H-leucine in the tissue (Peters and Ashley, 1967; Raley-Susman and Lipton, 1990). After osmication and dehydration, tissue was embedded in Epon; thin (1 μm) sections were cut at 150 μm from the upper surface of the slice. Sections were coated with Kodak NTB2 emulsion, exposed for 7–14 d at 4°C, and developed with D-19 at 14°C for 2.5 min using standard autoradiographic techniques (Cowan et al., 1972). Sections were poststained with toluidine blue (1%, in 1% borax) in order to improve visualization of dendrites. Toluidine blue staining does not affect grain counts over frozen tissue sections (Hendrickson et al., 1972). To determine whether this was the case for the Epon-embedded sections, sample cells with overlying silver grains from unstained 1 μm sections were drawn before staining, stained for 30 sec with toluidine blue, gently washed with distilled water, dried and coverslipped, and redrawn. There was no alteration in the grain numbers. Thus, 1% toluidine blue does not alter emulsion grain density over plastic-embedded sections.

Quantitative autoradiography. ^{3}H incorporation into dendrites or somata was quantified as grains/μm^2 of those tissue elements in a particular field.

The typical location of the CA1 dendritic fields that were analyzed is shown in Figure 1*A*. It begins about 50 μm from the border of stratum lacunosum moleculaire and extends for 150 μm toward the pyramidal layer, ending about 200 μm from the layer; the length extends for 1 mm. Within that region, four equally spaced circular fields with diameters of 150 μm were analyzed. Fields were observed in the light microscope at high power (100×); dendrites and overlying silver grains were drawn using the camera lucida. Only dendrites whose long axes were within 10° of the vertical direction and that had widths between 1.0 and 2.5 μm were drawn and analyzed. (Compare Fig. 1*B,C*; dendrites labeled with arrowheads were those that were drawn.) These orientations and widths are those of dendrites that were observed to be attached to pyramidal layer cells in cases where the entire cell was able to be followed. This criterion undoubtedly excluded short dendritic processes that were parts of pyramidal layer neurons (see Fig. 1*B*), but it meant that the dendrites that *were* analyzed were very likely to be parts of such neurons. The grains within dendrites were counted and the combined areas of the dendrites were measured with a software program (SIGMA SCAN, Jandel Scientific). Final values of grain density for each slice were the means from the four fields. Values for the different fields in the same slice were similar, with SEM values not exceeding 10%. Tests for statistical significance of effects of a treatment used Student's *t* test.

Only grains that were fully within dendrites were counted. This is seen in Figure 1, *B* and *C*. Grain diameter is approximately 0.4 μm, and the half-radius for these grains is approximately 0.3 μm (Rogers, 1973). For these values, it can be calculated graphically that, if a grain within a dendrite is just touching the border of the dendrite, there is a 75% probability that the source of that grain is within the dendrite. This probability increases to 96% if the edge of the grain is 0.4 μm from the border of the dendrite (Rogers, 1973). The average dendrite diameter is 1.5 μm (see Results), so the large majority (between 75% and 96%) of the grains that were counted come from ^{3}H-leucine localized within the dendrite. This estimate rests on the assumption that actual ^{3}H incorporation within and outside dendrites is equal. In fact, the grain density in the region 1.5 μm on either side of resting dendrites was approximately half the grain density within the dendrites (0.011 ± 0.003 vs 0.021 ± 0.002 in six fields that were measured). This will favor intradendritic origin of the ^{3}H even more than the above calculation indicates.

For cell somata, 20 CA1 pyramidal layer somata per slice (five cells with visible nucleoli per field) were drawn and analyzed as described for dendrites. The cells were directly above the analyzed apical dendritic fields. Grain densities for different cells in a slice were similar; the SEM for the 20 cells did not exceed 5%.

Materials

^{3}H-Leucine was from DuPont (NET 135H). All drugs and transmitter agonists or antagonists were from Sigma (St. Louis). Buffer reagents were from Mallinckrodt (Paris, KY).

Results

Integrity of the slice preparation

Previous measurements had shown very low levels of protein synthesis in brain slices (Dunlop et al., 1974). Furthermore, the pattern of incorporation was very different from *in situ*. The ability of neurons to synthesize protein was greatly compromised in the slice. Synthesis was very patchy, and declined greatly in the deeper portion of the slice. Much of the synthesis occurred in capillary endothelial cells (White, 1980). Using a preincubation procedure that greatly improves the morphological preservation of neurons in guinea pig hippocampal slices (Feig and Lipton, 1990) greatly improved synthesis in the slice, as illustrated in Figure 2*A–D*. Overall synthesis of protein is uniform throughout the thickness of the slice (except for the surface 50 μm, which is damaged). Within the plane of the slice, this pattern is very similar to that seen *in situ*, with high levels of label over the principle neuronal cell layers compared with neuropil (Thilmann et al., 1986). The identification of incorporated ^{3}H-leucine with protein is substantiated by its almost complete abolition by cycloheximide (Fig. 2*E*). The pattern of accumulation of free ^{3}H-leucine in the slice (Fig. 2*F*) is strikingly different from that of protein synthesis. The contrast between cell body layers and neuropil is much less. This could result

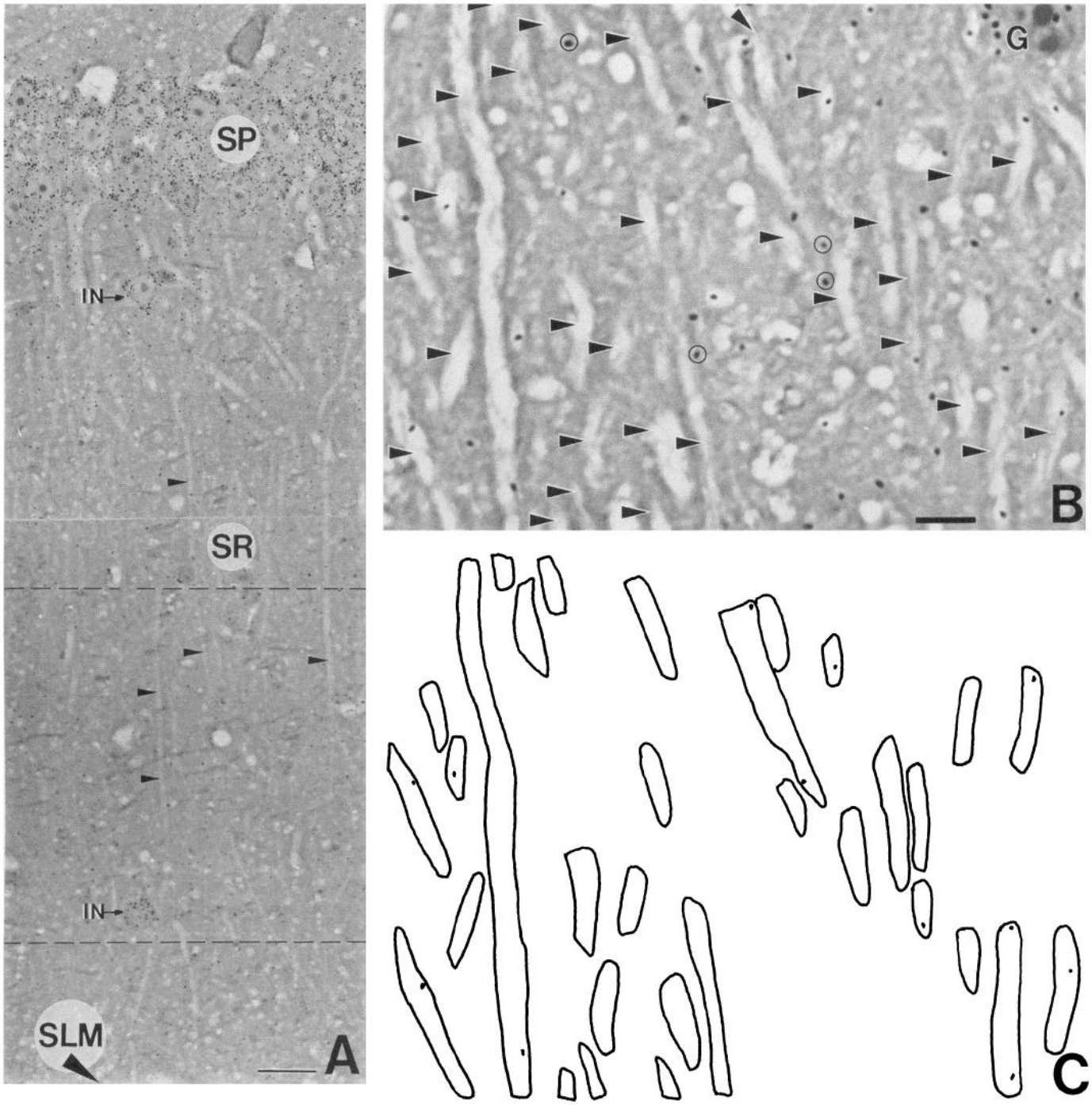


Figure 1. CA1 region of the guinea pig hippocampal slice: analysis of ^{3}H-leucine incorporation into dendrites. *A*, Photomicrograph of CA1 from the stratum pyramidale (*SP*) to the border of stratum lacunosum moleculaire (*SLM*) showing pyramidal cells in stratum pyramidale and their apical dendrites within stratum radiatum (*SR*). Two interneurons (*IN*) are present in this field. Several long apical dendrites within this section are indicated by the *arrowheads*. The region between the *broken lines* indicates the location and size of the fields that were analyzed for ^{3}H incorporation into dendrites. Note that the density of grains (*black dots*) is similar over all neuronal cell bodies, including interneurons and those in the pyramidal layer. *B*, Higher-power photomicrograph from a typical region in CA1 stratum radiatum that was used for analysis. It shows dendritic profiles and the overlying developed silver grains (*black dots*). *Arrowheads* indicate dendritic profiles that were traced for analysis. *Circles* denote silver grains that were not drawn. *G*, glial cell. *C*, Camera lucida drawing of the dendrites in *B* that were selected for analysis (see Materials and Methods for selection criteria). Grains overlying these dendrites are also shown. Drawings such as these were used to calculate grains/μm^2 of the selected dendrites. Scale bars: *A*, 25 μm; *B*, 4 μm.

from a quite uniformly distributed uptake system, or from diffusion of free leucine within the cells following uptake.

Much of this study is focused on protein synthesis in the dendrites. It is thus important to note that not only somatic morphology (Feig and Lipton, 1990) but also dendritic morphology is very well preserved when this preincubation protocol is followed. This is shown in Figure 3. The only notable difference between neuropil in glutaraldehyde-fixed slices and per-

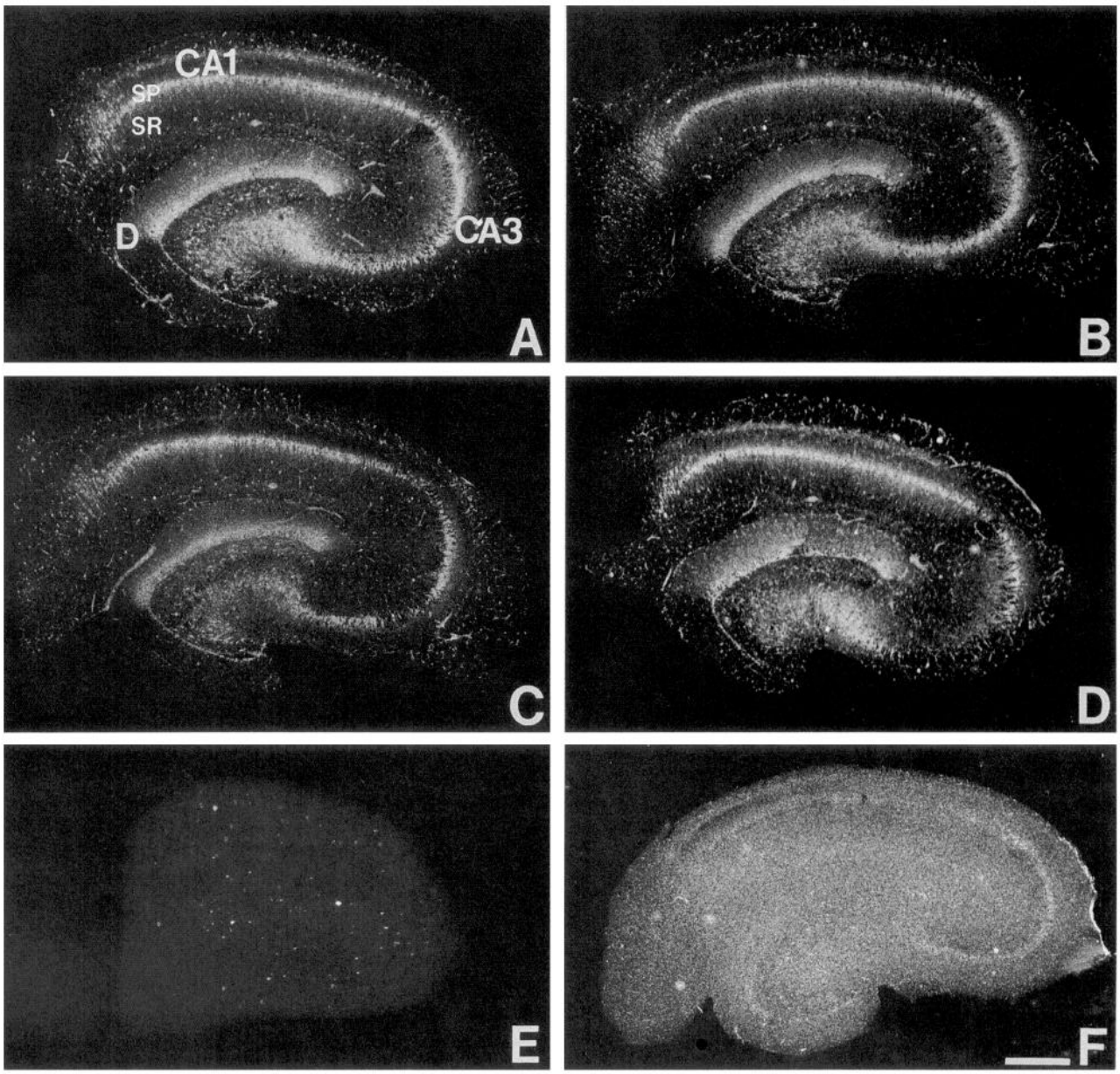
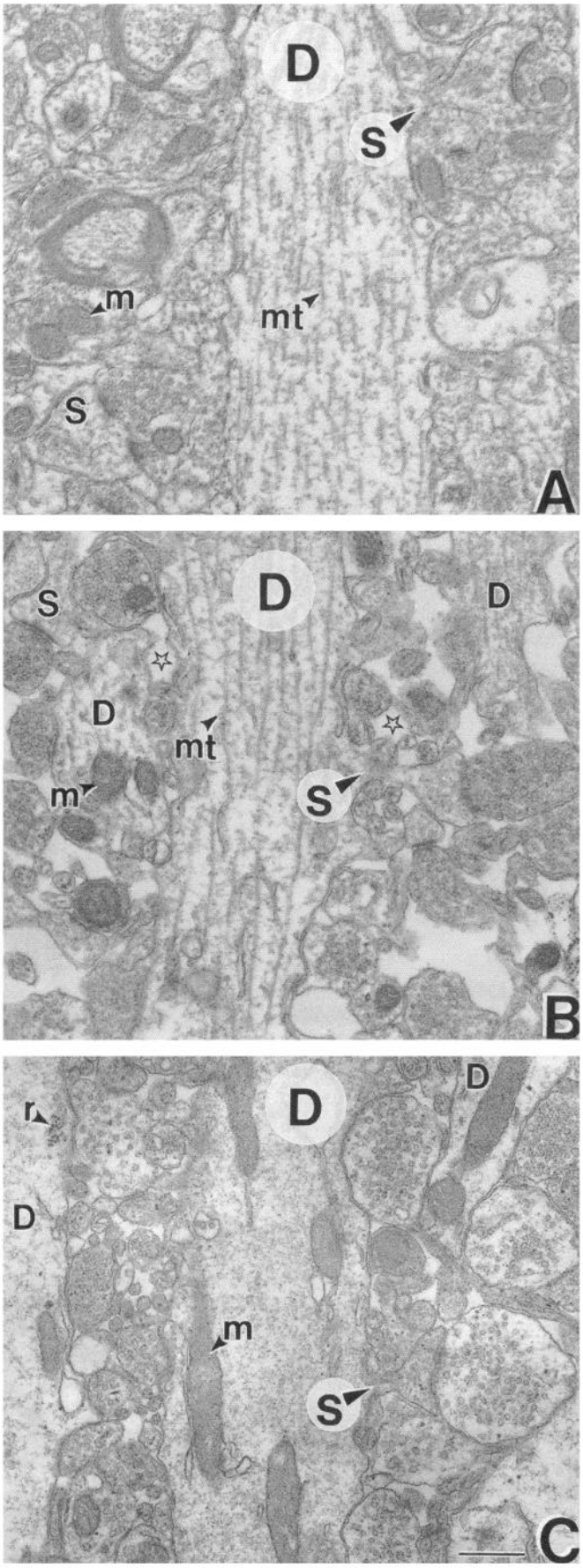


Figure 2. Protein synthesis in guinea pig hippocampal slice. *A–D*, Dark-field photomicrographs taken at different depths in a hippocampal slice (*A*, 80 μm from upper surface; *B*, 200 μm; *C*, 320 μm; *D*, 440 μm). This slice was incubated for 30 min in ^{3}H-leucine and immediately fixed in ice-cold 4% paraformaldehyde. Frozen 40 μm sections were taken through the slice and processed (see Materials and Methods) to reveal silver grains due to ^{3}H-leucine incorporated into macromolecules. Note uniform labeling of major cell populations in CA1, CA3, and dentate gyrus (labeled *D* in *A*) of each section. The punctate labeling in the stratum radiatum (*SR*) denotes synthesis in the interneurons, glia, and capillary endothelial cells. The cells in the pyramidal cell layer (*SP*) of CA1 are uniformly labeled throughout the slice. *E*, Dark-field photomicrograph of a 40 μm frozen section 120 μm from the surface of a slice that had been incubated with ^{3}H-leucine in the presence of (300 μM) cycloheximide, which inhibits protein synthesis. There is very little labeling over this slice (the scattered *bright spots* are artifacts), confirming that the label in *A–D* represents newly synthesized protein. *F*, A dark-field photomicrograph of a 40 μm section, 120 μm from the surface of a slice incubated as in *E* with cycloheximide but fixed in 2% glutaraldehyde, 2% paraformaldehyde to retain free ^{3}H-leucine. In this case, diffuse label is present over all regions of the hippocampus. Scale bar, 670 μm.

fusion-fixed brain is the increased extracellular space (Fig. 3*A,B*). Organelles, spines, and synapses in the slice are not notably different from their counterparts *in situ*. In the studies of protein synthesis, tissue had to be fixed in paraformaldehyde so that free ^{3}H-leucine would not be retained. In this case, the fixation led to loss of microtubules in the dendrites with a concomitant alteration in the morphology of the dendritic cytoplasm (Fig. 3*C*). This fixation of course occurred after the experimental procedures were complete.

Protein synthesis in dendrites and somata of resting slices

Incorporation of ^{3}H-leucine into dendrites of unstimulated cells was approximately 10% the level of incorporation into the cell somata (Fig. 4, first and third bars). Cycloheximide reduced the label over cell somata by 90%, indicating that almost all the incorporation is *de novo* synthesis of protein. In contrast, cycloheximide had no effect on label over dendrites (Fig. 4). Thus, in the resting state there is no measurable extramitochondrial



protein synthesis in the distal dendrites of the CA1 pyramidal layer cells.

As shown in Figure 4, the cycloheximide-insensitive label is approximately the same in dendrites and cell somata. Its composition is unknown. The most likely components are mitochondrial protein synthesis, and formation of leucyl-tRNA. About half this label disappears when fixation is delayed by 4 min following the 3 min exposure period. The rest remains for at least 30 min (S. Feig and P. Lipton, unpublished observations)

Effects of stimulation and of carbachol on ^3H incorporation into protein

Stimulating the Schaffer collaterals with the standard stimulus paradigm slightly decreased label over paraformaldehyde-fixed dendrites. Exposure of slices to 50 μM carbachol for 35 min had a similar effect. However, when the standard stimulus paradigm was applied in the presence of 50 μM carbachol, the result was quite different. There was a nearly threefold increase in silver grains per unit area of dendrite (Fig. 5*A*). There was no accompanying effect of the associated stimuli on label over cell somata; in fact, there was a small decrease in label when the two conditions were combined (Fig. 5*A*). The micrographs (Fig. 5*B,C*) show portions of stratum radiatum from a pair of unstimulated and stimulated slices that were exposed to carbachol. The only notable difference is the increased number of grains over dendrites in the stimulated slice.

Relationship of increased ^3H incorporation to increased protein synthesis

Three further studies were done to determine whether the enhanced ^3H incorporation due to carbachol and Schaffer collateral stimulation was an activation of protein synthesis.

The increased incorporation may have resulted from increased free ^3H-leucine uptake into the dendrites, and a resulting rise in the specific activity of the precursor amino acid pool. This was tested by including cycloheximide in the incubation medium prior to and during incubation with ^3H and then fixing the tissue in glutaraldehyde/paraformaldehyde rather than para-

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Figure 3. Comparison of dendrite morphology *in situ* and in the slice. *A*, Electron micrograph of a hippocampal CA1 dendrite (labeled *D*) in stratum radiatum from a guinea pig perfused with fixative containing 2% glutaraldehyde, 2% paraformaldehyde. This is a presumed pyramidal cell dendrite as it has a dendritic spine (*S with arrowhead*). Note numerous microtubules (*mt*). Mitochondria (*m*) are indicated as well as a dendritic spine that is not attached to a dendrite in this section (*S without arrowhead*). *B*. Electron micrograph of hippocampal CA1 dendrite (labeled with *large D*) from the stratum radiatum of a hippocampal slice immersion fixed in the same fixative used in *A*. This is also a presumed pyramidal cell dendrite because of the dendritic spine (*S with arrowhead*); note the well-preserved microtubules (*mt*). Sample mitochondria (*m*) are indicated for comparison with those in *A*. Note also other small dendritic (also labeled *D*) profiles and spine (*S without arrowhead*) present in the field. While the morphological preservation of the cellular elements is remarkably similar to that of perfusion-fixed tissue, a notable characteristic of these immersion-fixed slices is the appearance of extracellular space (*stars*). *C*, Electron micrograph of hippocampal CA1 dendrite (*D*), from the stratum radiatum of a hippocampal slice, prepared and incubated exactly as the slice for *B* but immersion fixed in solution containing 4% paraformaldehyde and no glutaraldehyde. This is also a presumed pyramidal cell dendrite because of the dendritic spine. Note the absence of microtubules; the dendrite is now filled with an amorphous flocculent material. However, mitochondria (*m*) are well preserved. There is a cluster of ribosomes (*r*) present in a neighboring dendrite (*smaller D*). Scale bar, 0.5 μm.

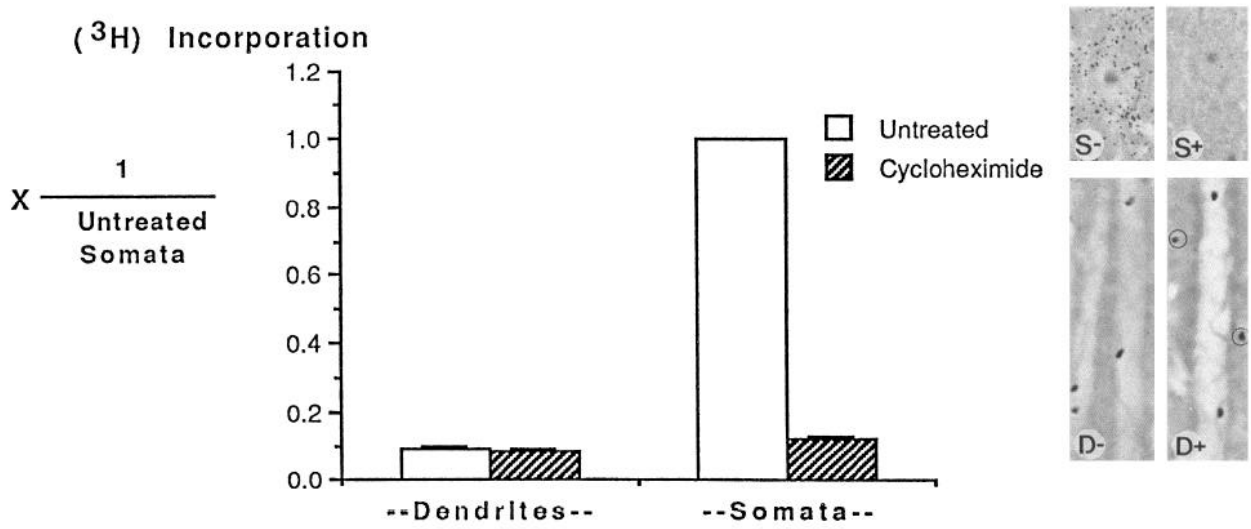
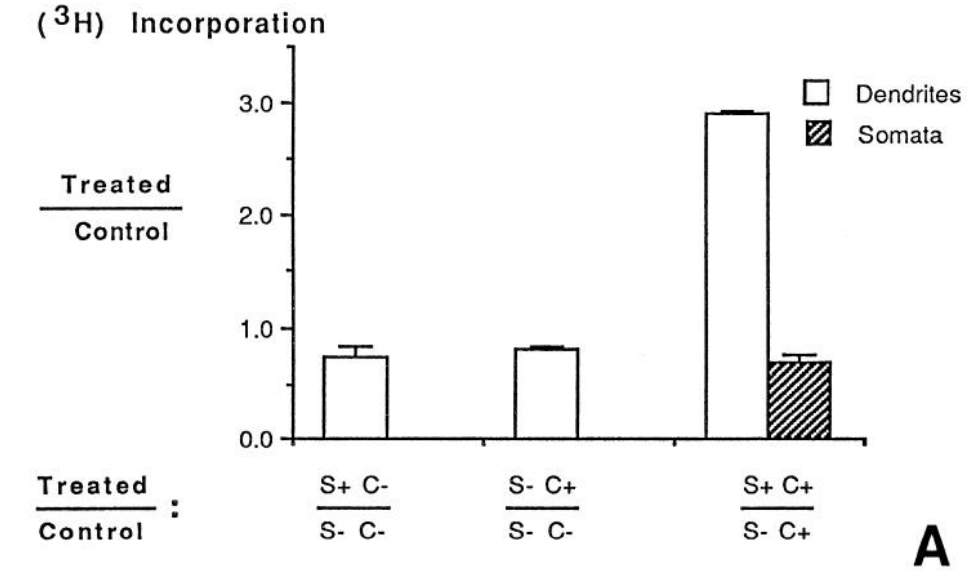


Figure 4. Cycloheximide blocks ^{3}H incorporation into cell somata but not into dendrites in unstimulated slices. Cycloheximide-treated slices were incubated with cycloheximide (300 μM) for 15 min prior to and during exposure to ^{3}H-leucine. Untreated slices were maintained in standard buffer. All slices were exposed to ^{3}H-leucine for 3 min prior to fixation in paraformaldehyde. In histogram, *bars* are means of 11 pairs of slices from five experiments. Background grain density, measured in dendrites of slices unexposed to ^{3}H, averaged 15% (±4%) of the value in resting dendrites. This was not subtracted in these histograms. Photomicrographs show cell somata (*S*) and dendrites (*D*) from both control (−) and cycloheximide-treated (+) groups. *Dots* are developed silver grains. Note the dramatic drop in label over somata in the cycloheximide treated slices, and the apparent lack of any difference over the dendrites.



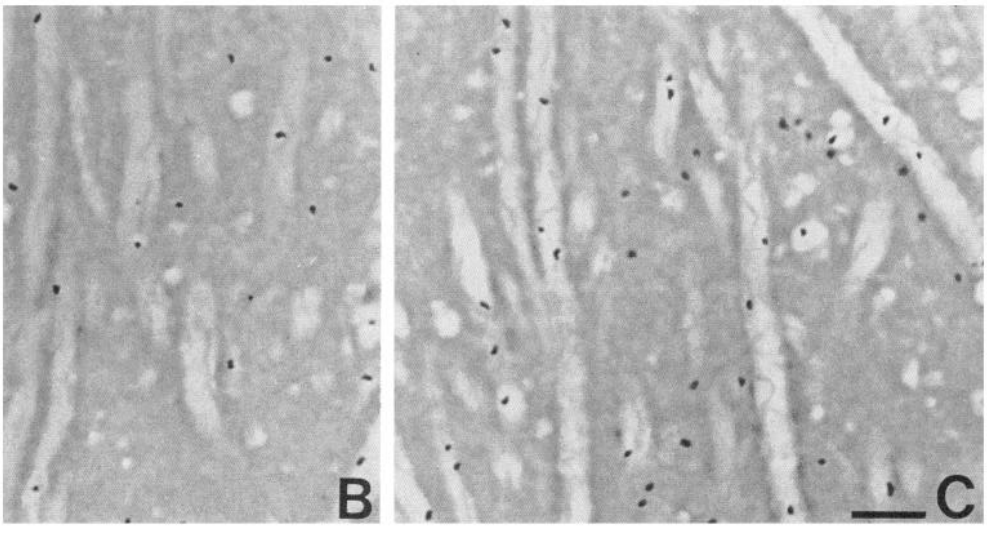


Figure 5. Effects of Schaffer collateral stimulation and carbachol on ^{3}H-leucine incorporation into dendrites and somata. *A*, *S* denotes standard stimulus paradigm; *C* denotes carbachol. Members of a pair of slices were subjected to either the treated or control conditions shown, and then both slices were exposed to ^{3}H-leucine for 3 min. Ratios of ^{3}H incorporation in the two conditions were averaged and the results are shown in the four bars. Stimulus alone (*first bar*) decreased incorporation into dendrites ($p < 0.05$); carbachol alone (*second bar*) decreased incorporation into dendrites ($p < 0.005$). Combining the two conditions (*third bar*) increased incorporation into dendrites threefold ($p < 0.005$) and decreased incorporation into cell somata ($p < 0.01$). (n = seven pairs for each group). *B* and *C*, Light micrographs of regions from stratum radiatum of the unstimulated (*B*) and stimulated (*C*) slices that were members of an experimental pair that was exposed to carbachol. Note the increased density of grains over dendrites in *C* compared to *B*. Scale bar, 3 μm.

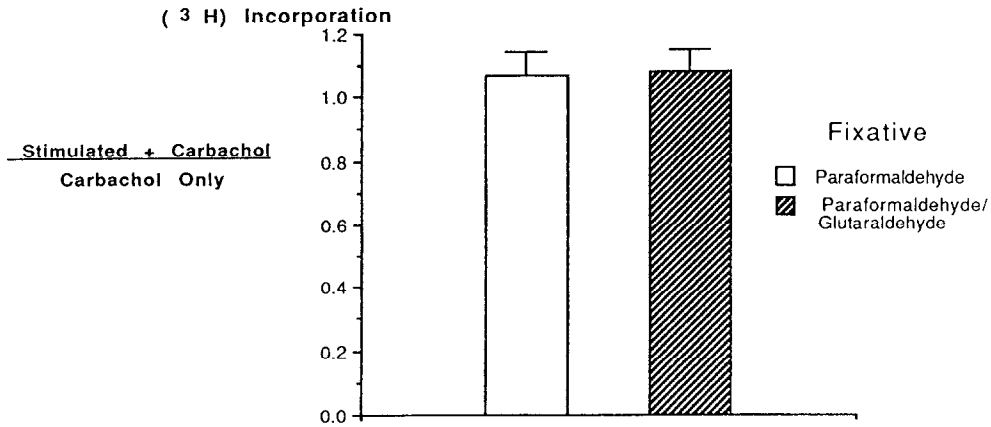


Figure 6. The effects of stimulation and carbachol on (^{3}H) incorporation in the presence of cycloheximide. All slices were exposed to carbachol (50 μM) and cycloheximide (300 μM). Cycloheximide was added 10 min prior to ^{3}H-leucine. Fixatives were 2.5% glutaraldehyde, 2% paraformaldehyde, which measures the accumulation of precursor, and paraformaldehyde alone, which allows the washout of free ^{3}H-leucine.

formaldehyde alone (see Materials and Methods). The combined stimuli did not increase label in these conditions (Fig. 6), showing that the stimuli do not enhance precursor uptake.

The observed increase may have been ^{3}H incorporation into a fraction other than newly synthesized protein. However, there was no increase in label over paraformaldehyde-fixed tissue when cycloheximide was included in the buffer (Fig. 6).

Synthesis is measured as grains per unit dendritic area, so the measured increase may have resulted from a narrowing of dendrites rather than an increase in protein synthesis. Thus, the mean width of dendrites in stimulated and nonstimulated slices was compared. Width was calculated by dividing the area of a dendrite by its longest dimension. Six pairs of slices were analyzed. Eight to ten dendritic profiles in each slice were measured. There was no difference between the mean width of dendrites in the two groups (1.50 ± 0.04 μm in carbachol only vs 1.53 ± 0.03 μm in carbachol + stimulation).

Effects of a different stimulus pattern and of muscarinic and NMDA receptor antagonists on the increased protein synthesis

The effect was tested of altering the stimulus pattern to eliminate the high-frequency episodes while maintaining the same net number of stimuli. A steady stimulation at 1 Hz was applied throughout the 20 min period. In contrast to the effect of the intermittent higher-frequency trains, the steady lower-frequency activity did not enhance ^{3}H-leucine incorporation into the tissue (Fig. 7*C*).

The receptor types involved in the activation of synthesis were examined by using the general muscarinic receptor antagonist atropine and the NMDA receptor antagonist D-aminophosphonovalerate (D-APV). Each of these inhibitors completely blocked the synthesis activated by combined carbachol and Schaffer collateral stimulation (Fig. 7*A,B*).

Effects of different conditions on field potentials during patterned stimulation

It is important to establish the extent to which any of the observed effects on protein synthesis might be explained by actions on electrophysiological responsiveness of the pyramidal neurons. If a treatment alters the magnitude of the population response, or the ability of the slice to maintain the response throughout the stimulation interval, protein synthesis might be affected.

Except for one instance, the different treatments had no effect on the four parameters of the response that were examined (Table 1). These four parameters are most easily understood by reference to Figure 8, which is a diagrammatic representation of the standard stimulus paradigm.

(1) None of the treatments, including carbachol, carbachol + atropine, and carbachol + D-APV, significantly altered the magnitude of the population spike prior to the patterned stimulation, although 50 μM carbachol did produce a transient depression in some cases (Fig. 9*A*). Cycloheximide (300 μM) was added after the termination of the second stimulation period (Fig. 8), so its

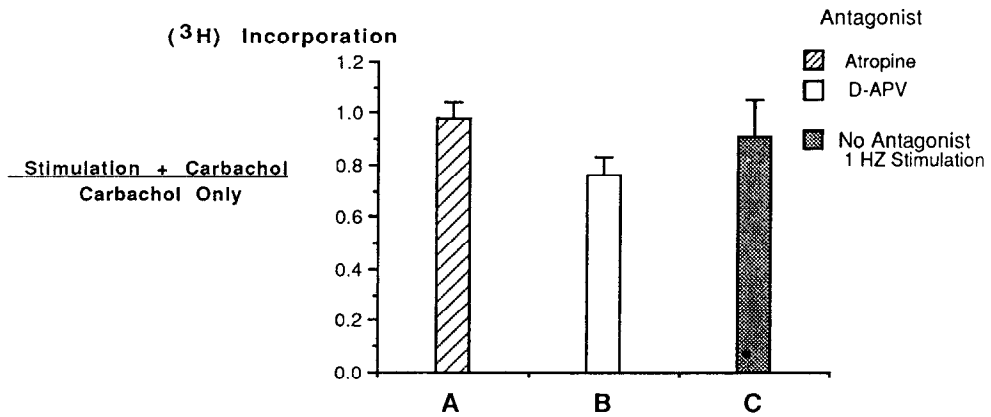


Figure 7. Effects of transmitter antagonists and low-frequency stimulation on protein synthesis in dendrites. Pairs of slices were exposed to 50 μM carbachol in the electrophysiological chamber and one of the slices was stimulated. For *A* and *B* either atropine (5 μM; $n = 5$) or D-APV (50 μM; $n = 6$) was added 15 min before beginning the standard stimulus paradigm. For *C*, no drug was added but a steady stimulus of 1 Hz was applied throughout the 20 min stimulation period ($n = 4$) instead of the standard stimulus paradigm. Three pairs of slices using the standard stimulus paradigm in the absence of any drugs were run as parts of these experiments and the ratio for these was 2.15 ± 0.32. For these treatments the only significant effect is a decrease in ratio for *B* ($p < 0.05$).

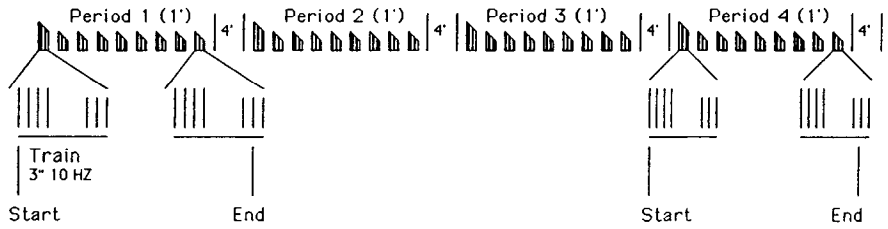


Figure 8. Diagrammatic representation of standard stimulus paradigm. *Vertical lines* represent population spike responses. *Periods 1–4* are 1 min long, during which stimulus trains of 3 sec duration are separated by 4 sec of silence. These are shown by the nine groups of population spikes in the *top row* and the expanded scale insets in the *second row*. These periods are separated from each other by 4 min, during which no stimulus is applied. The stimulus train is shown in some detail; the start period and end period data in Table 1 are averages of the first four and last four population spikes of the first and last stimulus trains in that period, respectively.

effect on the population spike could not be measured. However, cycloheximide, added at this time, did not affect the ratio of the spike height at the beginning of the fourth minute of stimulation to that at the beginning of the first (Table 1), indicating it did not affect the basal response.

(2) In none of the cases did the response decrement between the start of the first stimulus period and that of the fourth (Table 1).

(3) The events during each 1 min stimulus period are diagrammed in Figure 8. Except for D-APV (see below), there was only a slight decrement with time in the magnitude of the first spike of successive trains. Within a train, after an initial increase in height and addition of a second spike to the response, there was a small gradual decrement in spike height (Fig. 9*B,C*). The overall effects of these two changes are documented in the last two columns in Table 1 (see also Fig. 8). Table 1 shows that this parameter is similar for all the treatments except D-APV.

(4) The decrement during a stimulus period was very pronounced when D-APV was present (Table 1). This occurred because of an abrupt drop in the height of all the spikes in a train, not an increased decrement within the train. This took place between the second and fourth trains. The drop did not occur in all slices. In four out of six cases there was a large fall (Fig. 9*D*), while in two cases there was no fall (Fig. 9*E*). Thus, in some cases NMDA receptor activation appears necessary for maintenance of prolonged stimulation at moderate frequencies. This phenomenon was not explored further in the present study.

Discussion

Associating patterned Schaffer collateral stimulation with carbachol increased incorporation of ^{3}H-leucine into macromolecules in dendrites of cells in the CA1 region of the guinea pig hippocampal slice. It is very likely that this represents activation of *de novo* protein synthesis. (1) It is completely blocked by cycloheximide at concentrations that blocked synthesis in the resting pyramidal cell somata by 90% in this study and that are known to inhibit protein synthesis severely in many cell populations (Pestka, 1971), including hippocampal slices (Lipton and Heimbach, 1977). (2) It is very unlikely that the increased ^{3}H incorporation into newly synthesized protein is due to an increase in precursor specific activity. Although specific activity cannot be measured in the dendrites, several considerations argue against this possibility. Most importantly, uptake of ^{3}H-leucine was unaffected by the combined stimuli. Thus, increased

Table 1. Effects of different treatments on population spike responses

	Population spike amplitude ratios			
Treatment	Treated[a]/ control	Start period 4/ start period 1	End period 1/ start period 1	End period 4/ start period 4
None	—	1.44 ± 0.25 (4)	0.95 ± 0.06	0.66 ± 0.20
Carbachol (50 μM)	1.05 ± 0.10 (5)	1.15 ± 0.14	0.68 ± 0.12	0.71 ± 0.09
D-APV[b] (50 μM)	0.92 ± 0.04 (6)	1.05 ± 0.02	0.35 ± 0.13**	0.34 ± 0.15**
Atropine[b] (5 μM)	0.88 ± 0.12 (4)	1.00 ± 0.11	0.83 ± 0.15	0.67 ± 0.10
Cycloheximide[b] (100 μg/ml)	—	0.98 ± 0.09[c] (4)	—	0.54 ± 0.19

Steady population spike amplitudes were attained while stimulating once every 2 min. Drugs were then added (except for cycloheximide) and a new steady state attained after 15 min at the same stimulation rate. The standard stimulation paradigm (SSP) was then started. In the case of cycloheximide, the drug was added 10 min after the start of SSP. The first column refers to events before beginning SSP. The last three columns refer to events during SSP. "Start period" (1 or 4) refers to the average magnitude of the first three spikes of the first or fourth minute of stimulation. "End period" (1 of 4) refers to the average magnitude of the last three spikes of the first or fourth minute of stimulation. Results are means ± SEM. Numbers in parentheses refer to number of slices for that treatment.

[a] "Treated" is 15 min after adding drug; "control" is immediately before adding drug.

[b] Added in presence of carbachol.

[c] Cycloheximide added immediately after period 1.

** Different from carbachol, $p < 0.05$

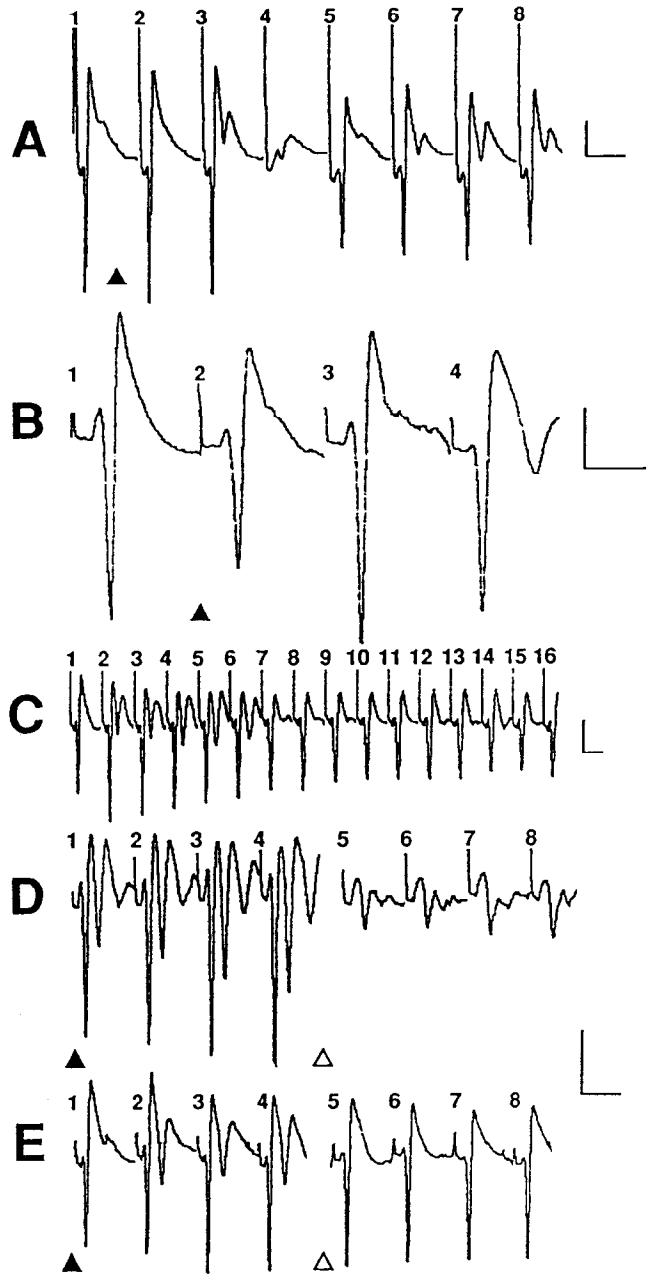


Figure 9. Effects of the various treatments upon field potentials. Field potentials were recorded in CA1 pyramidal layer following stimulation of Schaffer collaterals in CA2. All field potential responses are numbered. In all cases the time scale refers to points during the field response. Intervals between field responses were different in the different traces. *A*, Effect of carbachol (50 μM) on field potential prior to standard stimulus paradigm. Carbachol (50 μM) was added after *trace 1* (*arrowhead*), and the following traces were taken subsequently every 2 min. There is often an appearance of a second spike, and almost always a transient decrease in the response, with a return to steady levels that vary with respect to the original level (see Table 1). *B*, The first three field potentials of a 10 Hz train that is part of the standard stimulus paradigm. The *first trace* is after 15 min in carbachol, immediately prior to delivery of the train pulse. Train begins at *arrowhead*; the population spike increased in amplitude (labeled *3*) after the first trace and developed a second population spike (labeled *4*) by the *third trace*. This pattern occurred in almost every case, including the cases when atropine and D-APV were added to the bath. *C*, First 15 field potentials from a train at 10 Hz; standard stimulus paradigm began at *second trace*. Response *1* is after 15 min in carbachol. Response grows at first, and includes a second spike, and then falls off slightly to steady level (the level shown in *16* is maintained throughout the train). *D* and *E*, Effects of D-APV on stimulus trains. In both cases, stimulation was in presence of carbachol and D-APV. *Traces 1–4* are from first stimulus train during the first minute of stimulation (*solid arrowhead*). *Traces 5–8* are from last stimulus train during that minute (*open arrowhead*). The marked decrease during the minute, shown in *D*, occurred in four out of six slices. In the other two slices, illustrated by *E*, there was no significant decrement during the minute. Calibration: 1 mV, 10 msec.

specific activity could only explain the increased incorporation if *endogenous* leucine in the dendrites were *decreased* to about one-third its control value by the stimuli. There is no apparent reason for this to occur. Furthermore, an increased specific activity of intracellular leucine should lead to increased incorporation into the cycloheximide-insensitive fraction, as it too presumably utilizes a pool of free cell leucine. No increase was seen, even though this basal incorporation represents a sizeable fraction of total incorporation. Finally, there is no incorporation into the cycloheximide-sensitive pool prior to stimulation, and a large incorporation into the pool afterward. Thus, an altered specific activity due to decreased endogenous leucine would have to initiate incorporation into a previously inaccessible pool. This seems extremely unlikely.

Absolute values of protein synthesis could not be measured. Average rates of synthesis in the dendrites did rise to approximately 20% of those in the cell somata as a result of the associated stimuli (combining data from Figs. 4, 5). This is sizable, especially considering the fact that there is no measurable synthesis in the resting state.

Cell population affected

The Schaffer collaterals form synapses with apical dendrites of pyramidal cells and dendrites of interneurons (Schwartzkroin and Kunkel, 1985; Ishizuka et al., 1990; Miles, 1990). The population of dendrites that was analyzed included those from pyramidal cells but may also have included those from interneurons throughout CA1, as these each send small numbers of vertically oriented dendrites into stratum radiatum. The latter appear to comprise about 20% of the dendritic field (Schlander and Frotscher, 1986; Sloviter, 1989). Since a large activation of synthesis in a small population of dendrites was not observed, it is very likely that activation of synthesis was not restricted to dendrites of interneurons. Therefore, while the activation of synthesis may have occurred in interneuron dendrites, it almost undoubtedly occurred in the apical dendrites of the pyramidal cells.

Dendritic protein synthesis

The relative contributions of dendritic flow and local synthesis to turnover of dendritic protein is poorly understood (Kreutzberg et al., 1973; Kiss, 1977; Grafstein and Forman, 1980). The density of polysomes in dendrites is far less than in cell somata (Peters et al., 1976; Steward et al., 1988), but the relative contributions of different components of flow and of local synthesis to supply of new protein have not been determined.

There is, however, good evidence that local synthesis of protein occurs in dendrites in certain instances. Polysomes are localized in dendrites of several neuronal types *in situ*, and their number is greatly increased during regeneration of synaptic connections following deafferentation (Steward, 1986), as is the amount of ribosomal RNA (Phillips et al., 1987), suggesting an active local protein synthesis. Furthermore, ^{3}H-leucine is in-

corporated into a cycloheximide-sensitive fraction of dendrites after they have been isolated from cultured hippocampal neurons (Torre and Steward, 1992), providing a very clear demonstration of nonmitochondrial synthesis of protein in dendrites.

In the present study, the incorporation time for ^{3}H-leucine was limited to 3 min in order to minimize any opportunity for a contribution from dendritic flow in the intact tissue. The most rapid dendritic flow has been measured at a rate of about 50 μm/min (Schubert et al., 1972; Kiss, 1977). At this rate it would take protein 4 min to flow to the dendritic region being examined once it had been synthesized and fully processed in the soma. The time required for synthesis in the soma and processing through the Golgi apparatus is not known for these cells but is approximately 4 min in other systems (Karrenbauer et al., 1990). Thus, a reasonable estimate for the minimal time required for somatically synthesized protein to reach the observed dendritic region is 5–10 min. These considerations make it extremely unlikely that the new protein observed in dendrites after presentation of the associated stimuli could have arrived via flow. The time from initial exposure to ^{3}H-leucine to fixation of the tissue was only 3 min.

The short exposure time means that diffusion of ^{3}H-leucine through the slice must be one of the rate-limiting factors for incorporation into the tissue. However, the possibility that the associated stimuli act by increasing the rate of diffusion through the slice (e.g., by increasing extracellular spaces) is eliminated by the lack of their effect on the 3 min uptake of ^{3}H precursor.

While there was significant synthesis of protein in the dendrites of carbachol-treated slices exposed to the standard stimulus paradigm, there was no measurable synthesis in the resting dendrites. This contrasts with cultured neurons described above, where dendritic synthesis occurs in the absence of exogenous stimulation (Torre and Steward, 1992). It is of interest that situations in which dendritic synthesis has been observed prior to the present study are ones in which dendrites were undergoing significant molecular reorganization. As described above, these include reestablishing synaptic connections *in situ*, or growth in cell culture (Steward et al., 1988). It may be that dendritic synthesis is coincident with remodeling of dendritic or synaptic structure and that the associated stimuli in the present study produce such conditions.

Mechanisms of the activated synthesis

The mechanisms by which dendrite synthesis is maintained at negligible basal levels in the resting slice, and by which the associated inputs activate it, were not determined. The activation is almost certainly at the level of translation rather than transcription because the flow of mRNA is several orders of magnitude too slow (11 μm/hr) to activate dendrite synthesis within 20 min of initiating the stimulus (Davis et al., 1990).

The most dramatic instance of rapid initiation of translation from a dormant state occurs during fertilization. Certain species of mRNA are activated, in many cases by apparent removal or modification of masking proteins (Richter, 1991). It is certainly possible that such mechanisms are involved here. Alternatively, several steps in mRNA translation are regulated by protein phosphorylation (Sarre, 1989; Morley et al., 1991) and both NMDA and muscarinic receptor activation affect protein kinases (El-Fakahany et al., 1988; Bading and Greenberg, 1991) and, via increases in Ca, phosphatases.

A very important aspect of the present findings is the necessity for combining activation of the Schaffer collaterals with addition of carbachol in order to activate synthesis. The effects of the transmitter antagonists suggest that this represents a requirement for simultaneous activation of muscarinic and NMDA receptors. In four out of six cases, D-APV caused the magnitude of the response to decrease dramatically after the first two to four 10 Hz trains within each minute (Fig. 8). Thus, at least in some cases, NMDA receptor activation appears necessary to allow maintenance of prolonged responses to high-frequency activity. This cannot account completely for its role in the activation of protein synthesis, because in the two cases where D-APV did not lead to a reduced response it did prevent activation of synthesis (data from individual experiments, not shown). Thus, another effect of NMDA receptor activation is likely to be involved in the initiation of protein synthesis.

There are several intracellular interactions that might explain the strong synergism between the NMDA and muscarinic receptor mechanisms. Two of these are mediated by Ca, activation of protein kinase C removes the depolarization block of NMDA receptor–mediated Ca channels (Chen and Huang, 1992), and muscarinic activation enhances protein kinase C activity in hippocampal slices (Bading and Greenberg, 1991). Also, muscarinic receptor activation greatly enhances the increase in intracellular free Ca caused by depolarization of cultured hippocampal pyramidal cells (Muller and Connor, 1991). Another potential interaction is not mediated by Ca. There are now cases where *both* tyrosine and serine/threonine phosphorylations have been shown to be necessary for activation of enzymes; this is true for the important regulatory protein MAP kinase (Anderson et al., 1990). This is of particular interest as a model because NMDA receptor occupancy activates a tyrosine kinase (Bading and Greenberg, 1991) while the muscarinic receptor activates protein kinase C (El-Fakahany et al., 1988), a serine/threonine kinase. If such a multiple phosphorylation were necessary for activation of proteins involved in mRNA translation in the dendrites, it would explain the requirement for coactivation of the NMDA and muscarinic receptors.

Significance of the effect

There are several features that suggest that the activation of protein synthesis described here is important in long-term memory. Formation of long-term memory in many cases appears to be dependent upon ongoing protein synthesis at the time of learning (Flood et al., 1975; Eichenbaum et al., 1976; Grecksch and Matthies, 1980; Matthies, 1989), upon ongoing activity of the muscarinic cholinergic system (Dunnett et al., 1982; Chrobak et al., 1989; Ridley et al., 1991), and upon NMDA receptors (Morris et al., 1986). These features reflect the properties of the associative protein synthesis described here. Also important is the fact that synthesis is activated from a negligible level, suggesting that the associated stimuli produce a qualitative, as distinct from quantitative, change in dendritic protein. The fact that low-frequency stimulation did not lead to protein synthesis while patterned stimulation, of the type likely to pertain during exploratory activity in the animal, did, strengthens the connection between the increased synthesis and plasticity. Low levels of activity will not activate protein synthesis. Clearly, such arguments are not conclusive. However, they are consistent with a conclusion that the activation of protein synthesis seen here makes a contribution to synaptic plasticity during learning. The nature and function of the proteins whose synthesis is activated are not known. The two species of mRNA that have so far been

identified in distal dendrites are those encoding the regulatory protein MAP-2 and the α-subunit of calmodulin dependent protein kinase II (Burgin et al., 1990; Kleiman et al., 1990).

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