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Title of Thesis: "The Role of Protein Synthesis and Monoamines
in the Production of Long-Term Potentiation in
the Rat Hippocampal Slice"

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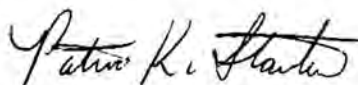
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ABSTRACT

Title of Dissertation: The Role of Protein Synthesis and Monoamines in
                          the Production of Long-Term Potentiation in the
                          Rat Hippocampal Slice.

Patric Kevin Stanton, Doctor of Philosophy, 1985

Dissertation directed by: John M. Sarvey, Associate Professor,
                             Department of Pharmacology

Long-term potentiation (LTP) in the hippocampus is a long-
lasting enhancement of synaptic efficacy produced by brief, high-
frequency stimulation of afferents. The long duration of LTP, and
implication of the hippocampus in learning, has generated interest in
LTP as an appropriate model, and perhaps a candidate mechanism, for
learning and memory. Thus, it is important to understand the mechanisms
of LTP, and also to investigate mechanisms already implicated in
learning and memory.

Three major questions were addressed in my study, based upon
what is known about the cellular mechanisms of learning and memory.
First, since inhibitors of protein synthesis are amnestic in a variety
of learning paradigms, can protein synthesis inhibitors also impair
production of LTP? Second, since the monoamine neurotransmitters
norepinephrine (NE) and serotonin (5-HT) have both been implicated
in performance of learning paradigms, what is the effect of depletion
of either of these monoamines on LTP? Finally, since NE has been
shown to produce a long-lasting potentiation in the dentate gyrus,
what are the mechanisms behind this potentiation, compared to LTP?

The <u>in vitro</u> hippocampal slice preparation was the system chosen to study LTP. Incubation for 30 min with the protein synthesis inhibitors emetine, cycloheximide, or puromycin decreased the frequency of occurrence of LTP in field CA1 and in the dentate gyrus. Blockade was dose-dependent, correlated with the ability of these inhibitors to inhibit incorporation of [^{3}H]-valine into proteins, and required a substantial preincubation period to block LTP. In contrast, the protein synthesis inhibitor anisomycin was unable to block LTP. These results suggest the necessity for a set of newly synthesized or rapidly turned over proteins for hippocampal LTP.

In studies investigating the effects of depletion of either NE or 5-HT on LTP, I have found that NE depletion markedly reduces the occurrence and amplitude of LTP in the dentate, but not in field CA1. In contrast, depletion of 5-HT does not impair LTP in either area. Furthermore, pharmacologic data indicates that β_1-receptor stimulation of adenylate cyclase is probably the mechanism of NE's action in the production of LTP in the dentate. These results suggest that endogenous hippocampal NE is more important to LTP in the dentate than is endogenous 5-HT. The effects of NE applied directly to hippocampal slices was also examined. NE produced a long-lasting potentiation of both the population spike and population EPSP in the dentate gyrus. NE-induced potentiation was confined to the dentate gyrus, where slices perfused for 30 min with concentrations of NE as low as 5 μM exhibited potentiation. Potentiation began within 15 min and lasted many hours after NE was washed out.

Pre-incubation of slices with emetine indicated that there are two distinct phases to NE-induced potentiation. The initial NE-induced potentiation during NE application was unaffected by a 30 min pre-incubation with emetine, whereas the long-lasting potentiation which persists for hours after NE washout was completely blocked by emetine at a concentration which I have shown is effective in blocking LTP, and in inhibiting [^{3}H]-valine incorporation into proteins. Furthermore, pharmacologic data support the conclusion that both phases of NE-induced potentiation are produced by β_1-receptor stimulation of adenylate cyclase. This result is consistent with the previous experiments indicating a role for β_1-receptor stimulation of adenylate cyclase in the production of LTP in the dentate.

Taken together, these studies indicate two important similarities between mechanisms of learning and memory, LTP, and NE-induced potentiation. First, protein synthesis seems to be necessary for their full expression. Second, NE seems to be an important neurotransmitter in their production. These conclusions support the continued study of LTP as a possible substrate for learning and memory, and suggest NE-induced long-lasting potentiation as another model of neuronal plasticity.

THE ROLE OF PROTEIN SYNTHESIS AND MONOAMINES IN THE PRODUCTION

OF LONG-TERM POTENTIATION IN RAT HIPPOCAMPAL SLICES

by

Patric Kevin Stanton

DEDICATION

To my parents, Lewis and Eleanor Stanton, for their love, belief and support throughout all the endeavors of my life, and to Diane Ellen Lindwarm, whose caring and friendship contributed greatly to my work, and more importantly, to the enjoyment of it.

ACKNOWLEDGEMENT

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INTRODUCTION

Background

A major function which has evolved in the mammalian brain is
the capability to learn from experience, and to recall what has been
learned at a later date. Beginning with D.O. Hebb (1949), it has been
assumed that learning and memory must involve long lasting changes in
neurons or their interconnections, but isolating specific cellular
changes produced by learning has so far not been possible. A clearer
understanding of mechanisms underlying modifications in neuronal excit-
ability may be a first step in understanding how learning takes place.

The hippocampus is a cortical structure which has fascinated
researchers for some time. It is a discrete and very organized part of
the limbic system, and is one of the earliest cortical structures to
evolve. One fact stands out from many experiments relating the hippo-
campus to behavior: it seems to be involved with learning, memory, and
behavioral state [Isaacson and Pribram, 1975; Olton et al., 1979].

In recent years, Bliss and Lømo (1973) have shown that truly
long lasting changes in synaptic strength, as a function of activity,
can occur in the hippocampus. Afferent stimulation of any one of a num-
ber of neuronal pathways to the hippocampus by application of a brief,
high-frequency train of repetitive stimuli can produce an increased
neuronal excitability lasting for weeks or months in the intact animal
[Bliss and Gardner-Medwin, 1973; Douglas and Goddard, 1975]. This pheno-
menon was labelled long-term potentiation (LTP). The persistence of this
alteration in excitability makes it an intriguing model of long-term
neuronal plasticity and for this reason much interest has developed in
the factors important in the generation of LTP in the hippocampus.

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Hippocampal electrophysiologic measures

Because of the lamellar organization of the hippocampal pyr-
amidal cells and their synaptic inputs, evoked population potentials
(population spikes) following a stimulus can be recorded extracellularly
from a number of cells producing synchronous action potentials. Intra-
cellular recordings have verified that population responses recorded
at various depths in the hippocampus accurately reflect cell body
action potential and dendritic excitatory post synaptic potentials
(EPSP) [Andersen et al., 1966]. It has been shown that extracellular
population spikes recorded in the cell body layers are reliable indi-
cators of summed single cell activity, based on intracellular recording
from dentate granule cells in response to perforant path stimulation
[Lømo, 1971; Andersen et al., 1971]. In addition, the amplitude and
latency of the population spike was found to be closely related to the
number and synchrony of activated cells [Andersen et al., 1971].

The major flow of information through the hippocampus is thought
to take place through a 'trisynaptic circuit' which passes via the
perforant path projection from the entorhinal cortex to the dentate
gyrus granule cells, is relayed to field CA3 of the hippocampus proper
by the mossy fiber system, and then proceeds to field CA1 by way of
the Schaffer collateral system [Ramon y Cajal, 1909; Andersen
et al., 1966]. At each of these three synaptic connections, repetitive
high frequency stimulation can give rise to LTP in the excited cell
area, which can be measured as a two- to ten-fold increase in amplitude
of the population spike produced by a single stimulus [Lømo, 1966;
Bliss and Lømo, 1973; Schwartzkroin and Wester, 1975; Alger and

Teyler, 1976]. Since extracellular recording of evoked potentials in the cell body layers and dendritic layers of the hippocampal subfields represents accurate measures of neuronal excitability and synaptic efficacy in a large number of neurons, and increases in amplitude of these responses are the major indication of the overall enhancement of synaptic efficacy seen in LTP, measurements of these evoked potentials in hippocampal subfields are an extremely useful index of summed activity of these populations of neurons.

The in vitro hippocampal slice

The study of LTP, as well as many other pharmacologic and electrophysiologic questions in the hippocampus, has been greatly facilitated by the development of the acute in vitro hippocampal slice technique [Yamamoto and McIlwain, 1966; Yamamoto, 1972]. Since the hippocampus is a lamellar structure, thin (300-500 μm) slices cut perpendicular to the axis of the hippocampus preserve the majority of the internal synaptic connections, making possible the examination of several monosynaptic and polysynaptic excitatory pathways to a cell field [Andersen et al., 1971; Yamamoto, 1972]. In addition, comparison of in vivo and in vitro intracellular recordings [Schwartzkroin, 1975; Dudek, et al., 1976] as well as morphologic [Yamamoto, et al., 1970] and metabolic [Yamamoto and Kurokawa, 1970; Okada, 1974] data, indicate that neurons in the slice are in good condition and behave much like those in vivo.

Brain slices allow greater control of the neuronal environment than in vivo preparations, while retaining the interneuronal organization absent in most tissue culture preparations. Advantages over other

recording systems include: (1) the relative ease of access to visually identifiable cell soma and dendritic layers in fields CA1, CA3, and the fascia dentata; (2) facility of application of a known drug concentration to the bathing medium or ejection of a drug from a micropipette placed in close proximity to the recording electrode; (3) absence of anesthetic effects, interactions with other drugs, or secondary systemic effects of drugs at remote sites; (4) control of the ionic content of the bathing solution; and (5) absence of respiratory and circulatory pulsation, which enhances the stability of extra- and intracellular recordings. Furthermore, LTP following application of repetitive stimulation is also seen in the hippocampal slice, where it lasts for up to 10 hours, its longevity being limited by that of the slice [Yamamoto and Chujo, 1978].

Properties of Long-Term Potentiation

As stated previously, LTP is a stable, persistent increase in the amplitude of the evoked population spike following repetitive, high-frequency stimulation of afferents. In addition, this increase usually extends to the dendritic EPSP. LTP has been demonstrated in all of the pathways of the hippocampal trisynaptic circuit, as well as in CA1 pyramidal cells following stimulation of the stratum oriens [Lømo, 1966; Bliss and Lømo, 1973; Schwartzkroin and Wester, 1975; Andersen et al., 1977].

Repetitive stimulation greater than or equal to 15 Hz with a total number of pulses of 100-200 represent usual stimulation parameters producing LTP, but a frequency range of 1-400 Hz has been effective in producing LTP [Swanson et al., 1982]. Following repetitive stimulation,

a short-term potentiation (STP) is often seen, on the order of a few minutes or less in duration [Stanton and Sarvey, 1984; Scharfman and Sarvey, 1985]. Presence or absence of this STP does not imply presence or absence of LTP to follow. This STP may be followed by a generalized heterosynaptic depression lasting from 1-10 minutes, and then by the development of LTP, which usually plateaus between 5 and 20 minutes post-stimulation. Once developed, LTP will last for many hours [Bliss and Lømo, 1973; Schwartzkroin and Wester, 1975; Andersen et al., 1977; Stanton and Sarvey, 1984].

The magnitude of increase in the amplitude of the population spike seen in LTP ranges from 0-1100% of baseline amplitude, and from 0-300% of baseline for the dendritic EPSP slope [Stanton and Sarvey, 1984; Stanton and Sarvey, unpublished results]. The 0% reflects the fact that repetitive stimulation sometimes fails to produce LTP in slices with otherwise healthy electrophysiologic responses. The observed frequencies in these studies for the occurrence of LTP in CA1 was 57%, and in the fascia dentata was 75%, in agreement with those seen in many other laboratories [Swanson et al., 1982].

Norepinephrine and serotonin in the hippocampus

Two of the major monoamine neurotransmitters in the hippocampus are norepinephrine (NE), and serotonin (5-HT). All norepinephrine-containing innervation of the hippocampus arises exclusively in the midbrain nucleus in the floor of the fourth ventricle known as the locus coeruleus (LC) [Ungerstedt, 1971; Lindvall and Bjørklund, 1974]. This structure is the major source of NE in the brain, with projections to most of the brain and spinal cord [Moore, 1973]. LC

fibers travel in the dorsal noradrenergic bundle, join the median forebrain bundle and then enter the hippocampus by three pathways: through the ventral amygdaloid bundle - ansa penducularis, the ipsilateral fasciculus cinguli, and the fornix [Ungerstedt, 1971; Lindvall and Björklund, 1974]. The densest innervation and highest NE content is in a layer interior to the blades of the dentate gyrus, followed by the stratum radiatum of CA3 and stratum lacunosum of CA1 and CA2 [Crutcher and Davis, 1980; Loy et al., 1980]. NE-containing fibers are less numerous in the rest of the hippocampus, but β receptors have been identified on soma and dendrites of pyramidal neurons in all areas [Crutcher and Davis, 1980].

Many conflicting hypotheses concerning the physiologic roles of the LC projections have arisen, including roles in learning [Crow and Wendlandt, 1976], arousal [Segal, 1978], emotion [Redmond, 1977], sleep [Hobsen et al., 1975], and cardiovascular function [Ward and Gunn, 1976]. In field CA1, extracellular studies have shown that NE has both β-receptor mediated excitatory, and α-receptor mediated inhibitory, effects on population spike amplitude [Mueller et al., 1981]. Intracellular studies have found NE to hyperpolarize, produce a moderate conductance increase, and decrease excitability, in both CA1 pyramidal neurons [Langmoen et al., 1981], and dentate granule cells [Haas, 1984]. Many investigators have suggested that NE preferentially reduces slow synaptic events, while leaving fast depolarizations like EPSP's unaffected, as a potential way of improving the signal-to-noise ratio of input to the hippocampus [Langmoen et al., 1981; Segal, 1982]. Interestingly, recent studies have shown that NE can decrease a Ca^{2+}-dependent K^+ current (I_c) in hippocampal pyramidal cells [Madison

and Nicoll, 1982; Haas and Konnerth, 1983]. NE may act via suppression of I_c to increase the repetitive firing of granule cells responding to high frequency stimulation.

In addition, NE superfusion of hippocampal slices produces metabolic changes in mitochondrial oxidative state, which are probably mediated by β receptor stimulation of cAMP production [Segal et al., 1980]. In the dentate gyrus, stimulation of the ascending noradrenergic pathway from the LC increases the synaptic efficacy of perforant path input to the dentate granule cells [Assaf et al., 1979]. This effect was blocked by prior injections of 6-OHDA into the dorsal noradrenergic bundle to deplete hippocampal NE. Finally, in vivo work has shown that NE depletion with 6-OHDA produces a reduction in the magnitude of LTP [Bliss et al., 1983] and also specific changes in perforant path excitability during slow wave sleep [Dahl et al., 1983]. It seems clear that NE effects in the hippocampus suggest great potential for a role for this neurotransmitter in modulating overall hippocampal excitability in response to external input.

The serotonergic innervation of the hippocampus originates from neurons within the midbrain raphé nuclei, almost exclusively in the median raphé, with the dorsal raphé contributing little, if any [Lorens and Guldberg, 1974]. These axons ascend in the median fore-brain bundle, passing via the fornix and cingulum to the hippocampus [Fuxe et al., 1970; Bjørklund et al., 1973]. Interestingly, auto-radiographic studies with ^{3}H-proline injected into the median raphé show that 5-HT terminal distribution significantly overlaps that of NE [Conrad et al., 1974]. The largest concentrations of terminals arise from axons entering via the cingulum and entorhinal cortex and are

found along the interior borders of the dentate granule cell layer and in a dense band in stratum lacunosum-moleculare of field CA1. There is sparser innervation of stratum oriens dendrites of field CA1, and stratum radiatum of fields CA2 and CA3 [Conrad et al., 1974; Azmitia and Segal, 1978].

Since the serotonergic input to the hippocampus originates in the raphé, speculation about its role in hippocampal function has centered around mechanisms of state of arousal and sleep. Prestimulation of the median raphé enhances the magnitude of the population spike produced by stimulation of the perforant path during slow wave sleep, but not during the alert state [Winson, 1980]. 5-HT applied in vivo to hippocampal neurons in the cat has proven more effective than NE in depressing firing, although in a small proportion of neurons it caused excitation, sometimes progressing to seizure discharge [Stefanis, 1964; Biscoe and Straughan, 1966]. In the rat in vivo, CA1 and CA3 pyramidal cell firing is depressed by 5-HT, and also by raphé stimulation, and both responses are antagonized by 5-HT antagonists [Segal, 1975]. Intracellular recordings from CA1 pyramidal cells show the depression is produced by a hyperpolarization accompanied by decreased spontaneous activity and increased membrane conductance [Jahnsen, 1980]. These responses are, in general, faster in onset but shorter in duration than NE responses. 5-HT might prove to be important in differentially modulating hippocampal function during sleep vs. awake states, in contrast to NE, where it seems more likely that levels of arousal in the awake animal are involved.

Cyclic nucleotides in the hippocampus

The cyclic nucleotides 3',5'-cyclic adenosine monophosphate
(cAMP) and 3'-5'-cyclic guanosine monophosphate (cGMP) have been
implicated as intracellular 'second messengers' mediating the actions
of a number of hormone and neurotransmitter systems. NE can stimulate
formation of cAMP in a variety of tissues including brain [Blumberg
et al., 1976; Dolphin et al., 1979; Segal et al., 1981]. However, the
physiologic relevance of increased cAMP levels in the brain, and
specifically in the hippocampus, has remained unclear. It has been
proposed that cAMP serves as a second messenger for NE actions and
that accumulation of cAMP promotes phosphorylation of certain membrane
proteins, leading in some way to hyperpolarization of the neurons
involved [Levitan and Adams, 1981; Forn et al., 1974]. In the cere-
bellum NE causes a hyperpolarization associated with increased
membrane resistance seeming to involve cAMP as intracellular mediator
[Hoffer et al., 1973].

Similar mechanisms may operate in rat hippocampus, since the
extracellular application of cAMP is depressant, and NE depression is
enhanced by concurrent application of a phosphodiesterase inhibitor
to inhibit the enzyme responsible for degradation of cAMP [Segal and
Bloom, 1974]. In the dentate gyrus in particular, stimulation of the
perforant path input from the LC or iontophoresis of NE can suppress
spontaneous activity of granule cells, and such stimulation also causes
elevation of cAMP levels in the freely moving rat [Segal et al., 1981].
NE, but not other putative neurotransmitters (including 5-HT), has
been shown to cause a 3-4 fold rise in cAMP levels in hippocampal

slices. This effect is probably mediated by both β1 neuronal receptors and β2 glial receptors, since there is an enhanced cAMP-generating capacity in kainic acid-treated rats, where, presumably, nearly complete destruction of hippocampal neurons and proliferation of glia has occurred. This cAMP generation was blocked by the β2 antagonist H35/25, but not by the β1 antagonist practolol, in the kainate-treated rats. In contrast, these two drugs were equipotent antagonists in untreated rats [Segal et al., 1981]. Segal and colleagues interpreted these results as indicating that β_1-receptors were primarily neuronal, and thus destroyed by kainic acid, while β2-receptors represented a population primarily localized on glial cells.

In abdominal ganglia of the marine mollusc Aplysia californica, a short term heterosynaptic facilitation along with an increase in cAMP levels is produced by prolonged stimulation, and also by 5-HT, dopamine, and octopamine [Klein et al., 1982; Klein and Kandel, 1978; Cedar and Schwartz, 1972; Pellmar, 1981; Brunelli et al., 1976].

The relation of cAMP mechanisms to the functioning of the LC input to the hippocampus was still unclear, and it remained to be tested whether cAMP mechanisms have any importance to LTP in the hippocampus. In investigating cAMP systems, the recently discovered diterpene, forskolin, has proved most useful. Forskolin has both direct stimulatory effect on adenylate cyclase activity [Seamon et al., 1981], and a low dose ($\leq 10 \mu M$) potentiation of the ability of agonists (such as NE) to stimulate cAMP production in a variety of systems. If cAMP mechanisms were indeed involved in LTP generation, forskolin promised to be a useful tool in studying these effects, especially the use of a low dose to enhance adenylate cyclase stimulation and attenuate

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or reverse the effects of monoamine depletion on LTP.

Evidence has also accumulated to support the view that cGMP is involved in hippocampal neural transmission, and perhaps in alterations following repetitive stimulation as well. cGMP has a net excitatory effect on hippocampal pyramidal neurons [DeFrance et al., 1978], and muscarinic cholinergic agonists produce a Ca^{2+}-dependent increase in cGMP in the rat cerebral cortex and hippocampus [Black et al., 1979]. Repetitive stimulation of the medial septal pathway, which supplies cholinergic innervation to the entire hippocampus, produces an increase in cGMP levels in field CA1 30-60 seconds after the stimulation [DeFrance et al., 1983]. However, longer term effects have not been reported. In view of the interaction between cAMP and cGMP levels in a variety of tissues, it is possible that cGMP plays a role in LTP in the hippocampus. If so, this role will probably prove to be as second messenger in septal cholinergic afferents.

In general, cyclic nucleotides seem to be intimately involved with certain afferent transmitter systems in the hippocampus. In the case of cAMP, NE seems to be the leading candidate, whereas for cGMP, acetylcholine is of probable importance. The role of cyclic nucleotides in LTP remains to be investigated, and, considering the many long-term modulatory functions they are postulated to serve in other systems, they seem likely candidates for involvement here.

Protein Synthesis and LTP

Although synaptic transmission and second messenger systems are probably involved in short-term neuronal plasticity, a variety of other mechanisms have been suggested as potentially important in main-

taining changes for long periods of time. Most changes in transmitter levels, or in cyclic nucleotide accumulation, produced by stimulation are not sufficiently persistent to explain such long lasting plasticity. However, high-frequency repetitive stimulation of the hippocampus produces numerous other biochemical effects, including changes in protein phosphorylation patterns [Bar et al., 1982], increases in specific protein fractions [Browning et al., 1979], and increased secretion of newly synthesized proteins [Duffy et al., 1981]. However, it was not known whether synthesis of proteins was required for, or merely a by-product of, LTP. Therefore, determination of the ability of the hippocampus to exhibit LTP in the presence of inhibitors of protein synthesis promised to help answer this question.

Norepinephrine, serotonin and LTP

In studying the mechanisms leading to the production of LTP, the presumption has been that synaptic transmission is required [Dunwiddie et al., 1978]. It is important to know which neurotransmitters are involved in generation of LTP in different hippocampal fields. It has been shown that glutamate antagonists are effective in blocking LTP produced in CA1 by repetitive stimulation of the Schaffer collaterals [Krug et al., 1982]. A recent in vivo study has shown that LTP in the dentate is reduced by prior depletion of norepinephrine (NE) by 6-hydroxydopamine (6-OHDA), or of serotonin (5-HT) by 5,7-dihydroxytryptamine (5,7-DHT) or p-chlorophenylalanine (PCPA) [Bliss et al., 1983]. However, it was impossible to tell in vivo whether this resulted from depletion in the hippocampus itself, or depletion of other brain

areas. Since stimulation of a variety of other brain areas has been shown to influence hippocampal neuronal excitability [Winson, 1980; Segal and Bloom, 1974; Swanson et al., 1982], it is possible that depletion of a noradrenergic or serotonergic input to one of these areas might secondarily modulate LTP in the hippocampus. In addition, generalized stimulation of the angular bundle in vivo may antidromic- ally stimulate brain areas supplying axons to this input [e.g. the median raphe ; Conrad et al., 1974; Azmitia and Segal, 1978], which may in turn project to the hippocampus either directly, or through other brain areas [e.g. the locus coeruleus; Conrad et al., 1974]. Therefore, I elected to examine the effects of depletion of NE or 5-HT on LTP in the in vitro hippocampal slice preparation. In this way, I could isolate the hippocampus from tonic extrahippocampal inputs, and apply compounds in the bath at known concentrations. I employed depletion methods identical to those in the in vivo study [Bliss et al., 1983], to facilitate data comparison.

Very recently, NE has been reported to produce a long-lasting potentiation of the population spike in the dentate of hippocampal slices, a potentiation which looked very much like LTP in duration [Neuman and Harley, 1983]. This result opened the way for a variety of pharmacologic studies to characterize the receptor subtype responsible, intracellular messengers involved, and the possible requirement for protein synthesis in NE-induced long-lasting hippo- campal plasticity. Preliminary data suggested β-receptors as a likely candidate for NE-induced long-lasting potentiation [Neuman and Harley, 1983]. Published data concerning the possible role of cAMP in noradrenergic transmission in the hippocampus and elsewhere

suggested this intracellular second messenger as a promising candidate for involvement. Therefore, I began by testing these hypotheses.

In better understanding the role of monoamine transmitters in LTP, we may learn more about the steps leading to LTP, the importance of two well-defined hippocampal afferents in its generation, and the potential role of these neurotransmitters in mechanisms of long-term neuronal plasticity.

Specific Aims

There has been a long-standing interest in the possible role of protein synthetic machinery in learning and memory [Barondes, 1970], and more recent interest in a possible role for protein synthesis in hippocampal LTP [Browning, et al., 1979; Duffy, et al., 1981]. However, it was not known whether protein synthesis was required for LTP production. Therefore, I investigated the ability of a variety of inhibitors of protein synthesis to block LTP in the hippocampus. In addition, experiments to measure inhibition of $[^3H]$-valine incorporation into proteins in hippocampal slices, experiments with reversible and irreversible inhibitors of protein synthesis, and time course characterizations of the effects of protein synthesis inhibitors, were performed. The interaction of inhibitors of protein synthesis with long-term neuronal plasticity produced by NE in the hippocampus was also examined.

Recent work _in vivo_ has shown that NE and 5-HT are also of importance to the generation of LTP in the dentate, since NE or 5-HT depletion led to decreased LTP in this area [Bliss, et al., 1983]. These results, however, were not compared with effects of depletion

on LTP in other hippocampal cell areas. Furthermore, it was impossible to tell whether the effect resulted from depletion in the hippocampus itself, or was secondary to depletion of other brain areas. Therefore, the second major set of experiments were performed to examine the effects of depletion of NE or 5-HT on LTP in the dentate vs. field CA1 of the isolated in vitro hippocampal slice.

The third major set of experiments centered around the recent discovery that NE by itself can produce potentiation in the dentate when microiontophoresed [Neuman and Harley, 1983], or bath applied [Stanton and Sarvey, 1985b]. Therefore, it was important to identify the receptor subtypes responsible, possible cyclic nucleotide involvement, and the effect of inhibitors of protein synthesis on this second example of long-term hippocampal plasticity.

Finally, hints have appeared concerning the possible involvement of cyclic nucleotides as second messengers of NE actions in the hippocampus [Segal et al., 1981], and even of changes in cyclic nucleotide levels during short-term potentiation in the hippocampus [DeFrance et al., 1983]. However, the long-term effects of repetitive stimulation or NE on hippocampal concentrations of cAMP were unknown. Therefore, experiments to directly assay cAMP concentrations in slices, and test the effects of repetitive stimulation and NE on cAMP levels, were performed.

The overall objectives of the studies presented here were to assemble a comprehensive picture of the neurotransmitters, intracellular messengers, and cellular synthetic machinery, involved in two intriguing examples of long-term neuronal plasticity, and to enumerate the similarities or differences between them. The larger implications

for hippocampal function and plasticity should help direct future investigations into the cellular mechanisms producing long-term changes and the significance of these models to learning and memory.

The specific aims of this study were:

1) Investigate the importance of protein synthesis in the production of long-lasting potentiation in the hippocampus, through the use of inhibitors of protein synthesis to impair ongoing synthesis.

2) Establish the extent of effects of NE or 5-HT depletion on LTP in the isolated in vitro hippocampal slice.

3) Investigate the distribution and pharmacology of NE-induced long-lasting potentiation in the hippocampus, and to identify cellular areas and receptors involved.

4) Investigate the possible importance of cyclic nucleotides as second messengers in noradrenergic mechanisms influencing LTP and NE-induced long-lasting potentiation in the hippocampus.

5) Relate the involvement of protein synthesis in LTP with NE's role in long-term hippocampal plasticity, by examining the necessity for ongoing protein synthesis in NE-induced long-lasting potentiation.

MATERIALS AND METHODS

Materials

Chemicals

Emetine hydrochloride (HCl), cycloheximide, puromycin, aniso-
mycin, α-methyl-p-tyrosine, 6-hydroxydopamine HCl, 5,7-dihydroxy-
tryptamine HCl, para-chlorophenylalanine HCl, 5-hydroxytryptamine HCl,
dl-norepinephrine HCl, propranolol HCl, Fast Green dye, O-phthaldehyde,
iodine, and l-valine, were obtained from Sigma Chemical Co. (St. Louis,
MO). n-Heptane, butanol, and methyl-tert-butyl-ether were obtained
from Fischer Scientific (Pittsburgh, PA). Puromycin aminonucleoside
was obtained from ICN Pharmaceuticals Division (Cleveland, OH). Chloro-
pent® anaesthetic, a mixture of 127 mg/kg chloral hydrate and 27 mg/kg
pentobarbital, was obtained from Fort Dodge Laboratories (Fort Dodge,
IA). EP liquid scintillation cocktail was purchased from Beckman
Instrument Co. (Fullerton, CA). Desmethylimipramine HCl was the gift
of Dr. T.P. Pruss, Revlon Health Care Group (Tuckahoe, NY). Metoprolol
HCl was the gift of Ciba-Geigy Pharmaceuticals (Summit, NJ). Forskolin
was the gift of Dr. John W. Daly, NIADD, NIH (Bethesda, MD).

All other chemicals were of certified A.C.S. reagent grade.

Radiolabelled Compounds

L-[3,4(n)-^{3}H]valine (36 Ci/mmol) in 2% EtOH was obtained from
Amersham Corp. (Arlington Heights, IL). RIANEN™ cAMP radioimmunoassay
kit was purchased from New England Nuclear Inc. (Division of DuPont,
North Billerica, MA). This kit supplies succinyl 3',5'-cyclic adenosine
monophosphate (cAMP) tyrosine methyl ester [^{125}I] as tracer (1.5

μCi/ ml in 1:1 n-propanol:H_2O), as well as the following radioimmuno-
assay reagents: $NaCH_3COOH$ buffer plus 0.1% NaN_3 as an antibacterial
(pH 6.2); lyophilized cAMP standard (5000 pmol/ml reconstituted), cali-
brated spectrophotometrically using the molar absorption coefficient
ε = 14.6 x 10^3 1· $mol^{-1}·cm^{-1}$ at 259 nm; lyophilized, pre-reacted first
and second antibody to cAMP; lyophilized normal rabbit serum; acetic
anhydride, triethylamine, and precipitation enhancer plus 0.1% NaN_3
in NaCH3COOH buffer.

Instrumentation

Extracellular evoked reponses were recorded from glass micro-
electrodes (2M NaCl, 2 to 5 megohms; Frederick Haer Co., Brunswick,ME),
signals passed through a high impedance preamplifier (either an NEX-1,
Biomedical Engineering Co., Thornwood, NY, or a WPI S-7071, World Pre-
cision Instruments Inc., New Haven, CT). Eight responses were averaged
on line with a Tektronix 7D20 digitizing plug-in in a Tektronix R7704
oscilloscope mainframe (Tektronix Inc., Beaverton, OR). Stimulus trigg-
ering pulses were generated with a WPI 1830 Interval Generator (World
Precision Instruments, New Haven, CT). Stimuli were supplied by a WPI
1850A DC stimulus isolation unit with model 870 rechargeable DC stimulus
isolators (World Precision Instruments, New Haven, CT). Calibration
pulses were supplied by a Stoelting calibrator (Stoelting Co., Chicago,
IL), placed in series with the chamber ground. The chamber ground
wire in the bathing solution was a Ag/AgCl half cell to prevent devel-
opment of DC junction potentials. Recording pipettes were pulled with
a vertical pipette puller (Model 700C, David Kopf Inc., Tujunga, CA).

The recording chamber was of the 'interface' style (Fig 1C;

Schwartzkroin, 1975], where the slice is placed on a nylon mesh at the interface between a modified Krebs-Ringer buffer perfused from below, and moist, warmed 95% O_2/5% CO_2 passed over the slice. Buffer was warmed to 37° C with a Thermomix heating water bath (Model 1420, Braun Inc.,Melsungen, West Germany) and pumped through the chamber by a Gilson Minipuls 2 peristaltic pump (Gilson Medical Electronics, Middleton, WI). Slices were illuminated from above with a fiber optics light (Math Associates, Great Neck, NY). Electrodes were positioned visually with the aid of a Zeiss operating microscope (Model OPM1, Zeiss Inc., Oberkochen, West Germany), and held in position with either Prior (England) or Leitz (Leitz Inc., Rockleigh, NJ) micromanipulators. Pressure ejection of nanoliter quantities of drugs was performed with a Picospritzer multichannel pressure unit (General Valve Corp., Fairfield, NJ).

The averaged evoked responses were transferred via an IEEE 488 Instrumentation Bus (1980 standard, International Society of Electrical Engineers) to a MINC 11/23 minicomputer (Digital Equipment Corp., Marlboro, MA) for disk storage and later analysis. Traces were plotted on a Hewlett-Packard 7225A plotter (Hewlett-Packard Co., Sunnyvale, CA).

The liquid scintillation counter used for ^{3}H-valine incorporation studies was a Beckman LS7800 (Beckman Instruments Co., Fullerton, CA). The gamma counter employed for cAMP measurements was a Micromedic Systems 4/600 (Micromedic Systems Inc., Horsham, PA). Spectroflourometric norepinephrine and serotonin assays employed an Aminco SPF-500 ratio spectrofluorometer (Travenol Lab Instrumentation Division, Deerfield, IL). Centrifuges used were either a Sorval RC-3B refrigerated centrifuge (Division of DuPont, Newtown, CT), an IEC CRU-5000

refrigerated centrifuge (Damon/IEC Division, Needham, MA), or a Beckman Microfuge B (Beckman Instrument Co., Fullerton, CA).

Animals

Adult, male, Sprague-Dawley rats (Hilltop Lab Animals, Scottsdale, PA) weighing 150-350 g were used in all studies. Animals had free access to tap water and standard rat chow (M/R/H 2000, Agway Inc., Ithica, NY), and were maintained 4 rats per cage at constant temperature on a 12 hour light/dark schedule.

Methods

Electrophysiology

Animals were decapitated, the brain removed and hippocampus gently dissected out within 5 min (Fig 1A), and transverse hippocampal slices (375-400 μm thick, Fig 1B) were prepared with a McIlwain tissue chopper (Brinkmann Instruments Inc., Westbury, NY). After a 30 min pre-incubation in modified Krebs-Ringer buffer (pH 7.2) at 30° C, slices were transferred to an interface recording chamber (Fig 1C; Schwartzkroin, 1975). They were placed on a nylon mesh at the interface between buffer and a humidified atmosphere of 95% O_2/5% CO_2, and continuously perfused at 35° C. The modified Krebs-Ringer buffer had the following ionic composition (mM): NaCl, 124; KCl, 5; $MgSO_4$, 1.3; KH_2PO_4, 1.25; $NaHCO_3$, 26; Glucose, 10; $CaCl_2$ was titrated to give a free $[Ca^{2+}]$ of 2.2 mM, as measured by a Ca^{2+}-sensitive electrode.

Slices were capable of producing stable orthodromic responses for at least 8 hours. Stimuli were delivered with bipolar electrodes placed on either the Schaffer collateral axon fibers in stratum radiatum,

Fig. 1. The hippocampal slice preparation and interface recording chamber

A: Schematic of the rat brain, showing a section of overlying neocortex cut away to reveal the hippocampus, and an outline of the transverse slice, cut parallel to the hippocampal lamellae.

B: Anatomy of the transverse hippocampal slice, from Ramón y Cajal (1909). The transverse orientation of the hippocampal lamellae permit preparation of a slice which retains the majority of its intrinsic synaptic connections intact, and the stereotypic orientation of the major cell types yield the characteristic extracellular evoked somatic and dendritic potentials recorded.

C: Schematic of the 'interface'-style recording chamber employed in all experiments. Slices were placed on a nylon mesh in the recording well, and physiologic buffer was perfused from below, while a warmed, moist 95% O_2/5% CO_2 gas mixture was passed over the upper surface of the slice. A, bathing medium input; B, O_2/CO_2 input; C, outflow for bathing medium; D, ground electrode; E, heating coil; F, constant temperature water bath; G, chamber cover; H, nylon mesh support for slice.
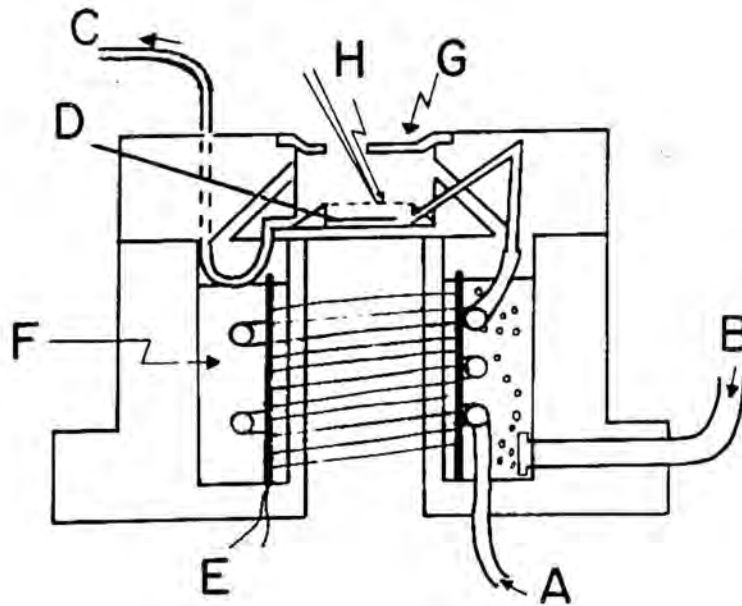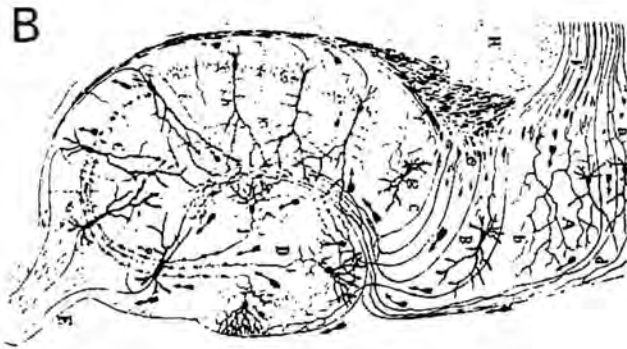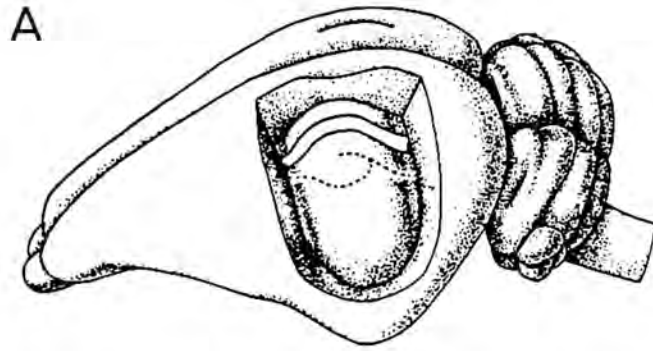
or the perforant path axons in stratum moleculare (Fig 2A). Ortho-dromic somatic field potentials were recorded with glass microelectrodes (2M NaCl, 2-5 megohms) placed in the CA1 pyramidal, or dentate granule, cell body layers, respectively. In some experiments, orthodromic den-dritic excitatory postsynaptic potentials (EPSP) were recorded in the dentate granule cell dendritic layer (stratum moleculare, Fig 10A).

Drugs were applied extracellularly at known concentrations by switching perfusion reservoirs. The perfusion flow rate was maintained at 3 ml/min with a peristaltic pump (Minipuls 2, Gilson Medical Elec-tronics Inc., Middleton, WI). All drug application times were corrected for transit time from the reservoirs to the slice (5 min). All drugs, except forskolin, were directly soluble in physiologic buffer at all concentrations employed. Forskolin was first dissolved in dimethyl-sulfoxide (DMSO) to a concentration of 100 mM, and then diluted in physiologic buffer. The largest final concentration of DMSO was 0.14 mM. One hundred times that concentration was found not to affect hippo-campal excitability, and has been shown not to alter adenylate cyclase activity [Huang et al., 1982].

Prior to repetitive stimulation, slices were incubated for 60 min, and 3 repetitions of the baseline input-output relation (I/O, stimulus intensity vs. population spike amplitude) were determined over a period of 10-15 mins. Baseline stimulus frequency for these I/O relations was 0.3 Hz, since this frequency did not alter the response amplitude or waveform over the course of the I/O determination. Slices were repetitively stimulated with 200 pulses, at a frequency of 20Hz for 10 sec via the stratum radiatum in CA1, or 100 Hz for 2 sec via the perforant path in the dentate, with an intensity that had evoked a

Fig. 2. Long-term potentiation (LTP) in the rat hippocampal slice

A: The hippocampal slice preparation, showing both recording sites (dentate and CA1), and both stimulus sites (perforant path and Schaffer collaterals). Only one set of recording and stimulus sites was employed in a given slice. Inset: Evoked population response in field CA1. The hatched area is the population spike, and spike amplitude equals $((X-Y)+(Z-Y))/2$. Asterisk denotes stimulus artifact.

B: Control LTP in field CA1. The population spike was recorded in CA1 just prior to (0'), and 30 min after (30'), repetitive stimulation of the Schaffer collaterals (20Hz/10sec). The potentiation after 30 min was 300% of control spike amplitude.

C: Control LTP in the dentate. The population spike was recorded in the dentate, and repetitive stimulation applied to the perforant path (100Hz/2sec). Potentiation here after 30 min was 225% of control amplitude. (Calibration for B and C: 1 mv, 5 msec)
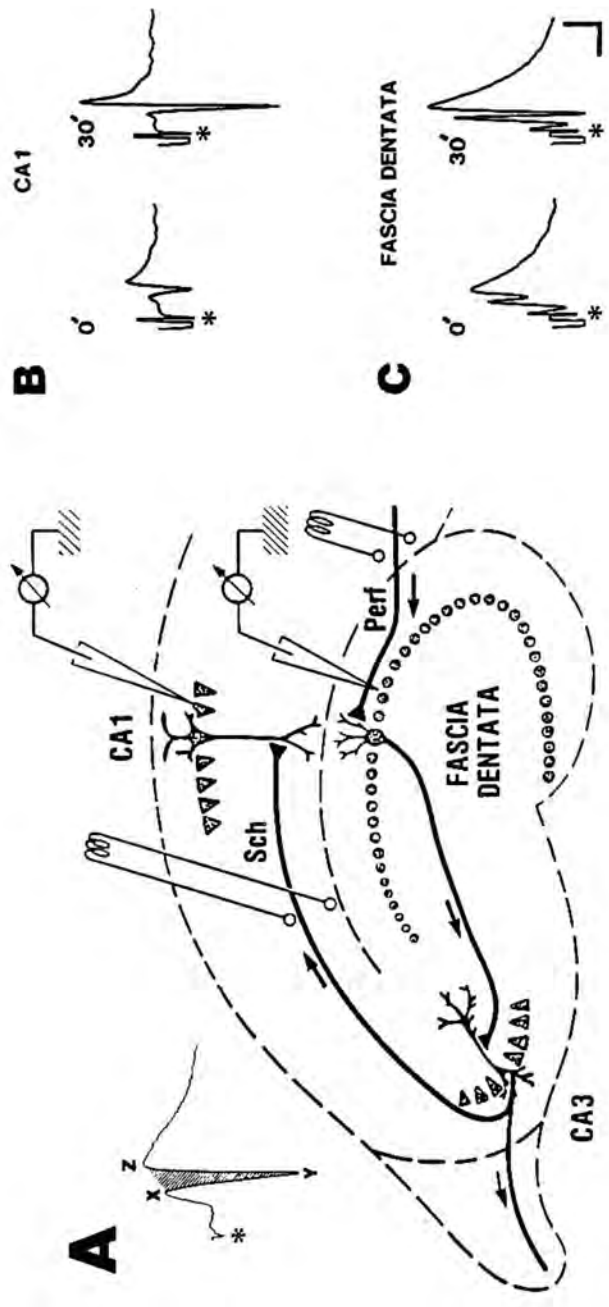
population spike of 20-40% of the maximal baseline response. These
stimulus parameters were chosen because they were maximally effective
in producing LTP in these areas. I/O curves were determined by
stimulating again at 0.3 Hz 1, 5, 15, and 30 min after repetitive
stimulation.

Measurement of [3H]-valine incorporation into proteins in slices

The level of inhibition of protein synthesis in slices was
measured by incorporation of [3H]valine into trichloroacetic acid
(TCA) precipitable macromolecules [Lipton and Heimbach, 1977]. Slices
were prepared identically with those used for electrophysiology, and
then placed in 1 ml of buffer (pH 7.2-7.4) in 24-well tissue culture
dishes (Costar). Slices were preincubated for 60 min at 37° C in a
humidified atmosphere of 95% air/5% CO_2. Inhibitors were then added to
the wells for a 30 min incubation, after which [3H]-valine (final
concentration 1 µCi/ml) was added. After an additional 60 min, incorp-
oration was terminated by dilution of the isotope in unlabelled valine
(final concentration 0.1 mg/ml) and homogenization in 10% TCA at 4° C,
followed by centrifugation at 12,000 g. Pellets were washed twice in
5% TCA, centrifuged at 12,000 g, and resuspended in 5% TCA. All super-
natants were discarded. The pellets were resolubilized in 1N NaOH over-
night at 37° C, then neutralized in 1N HCl. Aliquots were taken for
determination of radioactivity by liquid scintillation methods using a
Beckman LS 7800 scintillation counter. Protein was determined by the
method of Lowry et al. (1951), and results were expressed as dpm/mg
protein. Percent inhibition of [3H]-valine incorporation produced by
drug treatments was calculated by comparing incorporation in treated

slices with control slices in the same tissue culture plates. It has previously been shown that incorporation of [^{3}H]-valine into proteins with this methodology is linear for at least 60 min [Lipton and Heimbach, 1977], and we have verified this in our assay system.

Depletion of norepinephrine and serotonin

Animals to be depleted of NE with 6-OHDA were anesthetized with Chloropent® (3 mg/kg i.p.), and injected bilaterally over a period of 10 min in the dorsal noradrenergic bundle with a 10 µl microsyringe (Hamilton Inc., Reno, NV; 6 µg per side in 2 µl 0.9% NaCl containing 0.1-0.2 mg/ml l-ascorbic acid), using the following stereotactic coordinates (measured from zero at the midpoint of the interaural line): 2.4 mm anterior, 0.8 mm lateral, 2.9 mm dorsal, and the incisor bar was adjusted to achieve a 5° angle below the horizontal plane through the interaural line [König and Klippel, 1963]. The syringe was left in place at least 60 sec after injection and then removed. Sham operated controls were injected with an identical volume of 0.9% NaCl plus l-ascorbic acid. All animals were given 5 cc sterile 0.9% NaCl i.p. to counteract dehydration often seen in 6-OHDA-treated rats, lessen the effects of surgical trauma, and improve survival. In a few test animals, fast green dye (0.1 mg/ml) was injected into the dorsal noradrenergic bundle or lateral ventricle, to verify penetration of the dye into the desired sites.

Animals were depleted of 5-HT with 5,7-DHT by unilateral free-hand injections in the lateral ventricle (330 µg in 0.9% NaCl containing 0.2 mg/ml l-ascorbic acid), 1.5mm lateral to Bregma and 3.5mm below the skull [Noble et al., 1967]. These injections were performed by

exposing the skull, locating Bregma, and moving 1.5 mm lateral along
the coronal suture line. At this point, a small hole was bored in the
skull immediately caudal to the suture line, and a 10 μl microsyringe
(Hamilton Inc., Reno, NV) fitted with a sleeve 3.5 mm above the tip
was inserted down to this stop. Injections were performed slowly,
taking at least 60 seconds, and waiting an additional 60 seconds before
removing the syringe and closing the wound with wound clips. These
animals had been pretreated with desmethylimipramine (20 mg/kg i.p. in
sterile H_2O) 40-60 min before 5,7-DHT injection to protect noradrenergic
terminals. Sham operated controls were injected with an identical
volume of 0.9% NaCl plus l-ascorbic acid. Slices were prepared from
these animals 14-21 days after injection, when levels of depletion
were maximal [Bliss et al., 1983]. Animals were depleted of 5-HT with
PCPA by two injections, 48 and 24 hours prior to slice preparation
(400 mg/kg i.p. in sterile H_2O).

Spectrofluorometric measure of NE and 5-HT

Hippocampi of control and depleted animals were dissected out
within 5 min, frozen on powdered dry ice, and stored at -70° C until
NE or 5-HT was assayed by specific spectrofluorometric assay by the
method of Maickel, et al. (1968). In addition, at the beginning of
some experiments, one hippocampus was randomly selected for the prep-
aration of slices, while the remaining tissue from this side, and the
entire contralateral hippocampus, was frozen at -70° C for assay
later. These assays permitted resolution down to 2 ng of NE or 5-HT,
and standard curves were linear over a range of 5-100 ng. Internal
standards were employed to correct for percent recovery of the extrac-

tion procedure. There was <1% cross-reactivity in either assay for the other monoamine or dopamine. Protein was determined by the method of Bradford, et al. (1976). Transmitter levels were expressed as ng/mg protein.

After dissection and freezing, the hippocampus was later homogenized in 2.0 ml acid butanol, and centrifuged at 4° C at 22,000 g for 20 min. The maximum recoverable supernatant (1.7 ml) was then extracted with 0.1N HCl and n-heptane, shaken for 10 min, and centrifuged at 4° C at 1,000 g for 10 min. The organic phase was aspirated, and the aquaeous phase assayed for either NE or 5-HT.

For NE, 1.0 ml of the aqueous phase containing 5-100 ng NE was treated with 600 µl of 1M NaCH$_3$COOH containing 0.1M ethylenediamine tetra-acetic acid (EDTA). The tubes were mixed and 300 µl 1N iodine in absolute ethanol was added. After mixing, tubes were allowed to stand exactly two minutes at room temperature, then 600 ul 1N acetic acid was added, tubes heated in a 100° C water bath for two minutes, and the sample cooled and fluorescence read at excitation and emission wavelengths of 385 nm and 485 nm, respectively.

For 5-HT, 1.0 ml of the aqueous phase was placed in a test tube with 100 µl of freshly prepared 1% cysteine. Samples were treated with 2.0 ml of freshly prepared 0-pthaldehyde solution (4.0 mg/100 ml 10N HCl), vortexed, and heated in a 100° C water bath for 10 minutes. After cooling, samples were read at an excitation wavelength of 355 nm, and an emission wavelength of 470 nm.

cAMP radioimmunoassay

cAMP determinations in hippocampal slices were performed with the RIANEN™ radioimmunoassay kit (New England Nuclear, Division of DuPont Corp., North Billerica, MA). This assay employs the competition between radiolabelled and non-radiolabelled cAMP for a fixed, limiting number of antibody (Ab) binding sites. The radiolabelled tracer used was succinyl cAMP tyrosine methyl ester [125I], and the primary Ab was derived from rabbit. Unlabelled cAMP from standards or samples, and a fixed amount of labelled cAMP, were allowed to react with a limiting amount of Ab, and decreasing amounts of radiolabel bound to the Ab as sample [cAMP] increased. Separation of bound from free cAMP was achieved with a pre-reacted primary Ab/secondary Ab complex. After overnight incubation and centrifugation at 4,000 g for 30 min, the supernatant was decanted and discarded, and radiolabelled cAMP-Ab complex counted in the pellet to quantitate bound tracer.

Prior to assay, hippocampal slice cAMP was homogenized in 6% TCA at 4° C, and centrifuged at 4° C at 2,500 g for 15 min. The supernatant was collected and extracted 3 times with 5x volume of H_2O-saturated methyl-tert-butyl-ether, the ether phases discarded, and the aqueous phase evaporated to dryness. Samples were dissolved in 1.0 ml $NaCH_3COOH$ buffer (pH 6.2), and 100 or 200 µl aliquots used in the assay. These aliquots were reacted with triethylamine and acetic anhydride to acetylate sample cAMP, a modification which has previously been shown to enhance assay sensitivity 100-fold [Harper and Brooker, 1975]. Protein was determined by the method of Bradford, et al.(1976), and [cAMP] was expressed as pmol/mg protein. With this assay system, detection of

amounts of cAMP down to 0.10 pmol was possible, with a linear range of at least 0.10-4.0 pmol.

Data analysis

Population spike amplitude was defined as the average of the amplitude from the peak early positivity to the peak negativity, and the amplitude from the peak negativity to the peak late positivity [Alger and Teyler, 1976; Fig 2A inset, $((X-Y)+(Z-Y))/2$]. Population spike amplitude has been shown to reflect the number and synchrony of neurons firing in the vicinity of the recording electrode [Andersen et al., 1971], and to correlate with the slope of the excitatory post-synaptic potential as well as with spike latency [Bliss and Gardner-Medwin, 1973]. The standard deviation of control spike amplitudes was averaged from pooled control slice standard deviations, and LTP was defined as a spike amplitude increase of greater than two standard deviations from the mean of three control baseline responses 30 min after repetitive stimulation. Fig 2B (CA1) and Fig 2C (Dentate) show examples of population spikes exhibiting LTP 30 min after repetitive stimulation. In slices which showed potentiation at this time, potentiation lasted for hours (fig 3C). An earlier time was not chosen because large spike amplitude increases often occur immediately after repetitive stimulation and last up to 15 min in the absence of LTP [Stanton and Sarvey, 1984; Scharfman and Sarvey, 1985]. Therefore, we defined a spike amplitude increase of two standard deviations observed 1 min after repetitive stimulation as short-term potentiation (STP).

The maximum dendritic EPSP slope was determined on the initial negative-going phase of the population EPSP recorded in the dentate

granule cell dendritic layer (stratum moleculare). The computer calculated the moving averages of the negative-going slope for 8 time points spaced .1 msec apart around each digitized point, and returned the maximum slope value within the early portion of the EPSP uncontaminated by the somatic population spike. Table 1 summarizes the mean population spike and EPSP slope amplitudes and latencies to peak, both before and during LTP. These data show the increases in spike amplitude and EPSP slope produced by repetitive stimuli in the dentate and CA1.

All values in the text are expressed as mean ± standard error of the mean. Comparison of the frequency of occurrence of LTP was evaluated by χ^2 test, comparing all groups to the control frequency of occurrence of LTP in the appropriate recording area (75% for dentate, N=12; 57% for CA1, N=28). Comparison of average population spike amplitudes between different groups of slices was evaluated by two-tailed Student's t-test for unpaired observations. Comparison of pre- and post-stimulation responses in the same slices was evaluated by t-test for paired observations. The level of significance for all statistical tests was pre-selected to be $p<0.05$. Dose-response relations were transformed to double reciprocal (1/log[NE] vs. 1/response) relations, and the line of best fit calculated by Newton's method least squares linear regression. Curves were then plotted by re-transforming the double reciprocal equation of the fitted line. In the case of NEP (both with and without forskolin), the fit was significantly improved by employing the fractional threshold receptor occupancy assumption, which steepens the fitted curve by assuming that a minimum percentage of receptors (in this case 10%) must be occupied (the threshold) before any response is observed [Goldstein, et al., 1974].

Table 1. Extracellularly-recorded evoked population response parameters in the hippocampal slice, before (Control) and 30 min after (LTP) repetitive stimulation. A brief, high-frequency train of stimuli was applied to either the Schaffer collateral axons (20 Hz for 10 sec) or the perforant path axons (100 Hz for 2 sec), and evoked responses recorded in field CA1 or the dentate gyrus, respectively. Population spikes were recorded in the cell body layers (Fig 2A schematic), and population excitatory post-synaptic potentials (EPSP) recorded in the cell dendritic layers (Fig 10A schematic). Spike amplitude and EPSP slope were calculated as described in the text (pp.31-32), spike latency is the latency to the negative peak (Y in Fig 2 inset), and EPSP latency is the latency to the maximum slope. Dendritic EPSP's in field CA1 were not examined, since there was no effect of experimental manipulations on population spike LTP in this area (see results). All values are expressed as mean ± S.E.M., and N = number of slices in each group.

EVOKED POPULATION RESPONSE PARAMETERS

	CA1				DENTATE			
	Control	N	LTP	N	Control	N	LTP	N
Population Spike Amplitude (mV)	-2.67 ± 0.29	28	-4.93 ± 0.89	28	-2.00 ± 0.24	12	-3.31 ± 0.50	12
Population Spike Latency (msec)	4.45 ± 0.15	8	4.38 ± 0.21	8	3.73 ± 0.19	12	3.74 ± 0.18	12
EPSP Slope (V/sec)	_____		_____		-3.22 ± 0.34	10	-3.68 ± 0.22	10
EPSP Latency (msec)	_____		_____		3.06 ± 0.16	10	3.14 ± 0.28	10

RESULTS

I. <u>The role of protein synthesis in LTP</u>

<u>Blockade of LTP in hippocampal slices by
protein synthesis inhibitors</u>

Fig 3 illustrates the population spike in field CA1 before and
30 min after a single train of repetitive stimuli to the Schaffer
collaterals. An example of control LTP of approximately 200% increase
in amplitude is shown in Fig 3A. In Fig 3B, emetine (1.5 μM) was added
to another slice 30 min prior to repetitive stimulation, and was present
continuously until measurement of LTP 30 min after repetitive stimula-
tion. During this time, the baseline response remained stable. LTP
was completely blocked, and the population spike was unaltered.
Fig 3C illustrates the time course of typical changes in population
spike amplitude after repetitive stimulation for a control potentiated
slice, and for a slice treated with emetine (1.5 μM). Although LTP was
routinely assessed 30 min after repetitive stimulation, this time
course demonstrates that the response can remain potentiated for at
least 5 hours. It is important to note that emetine had no effect on
the population spike over the 5 hour period. Furthermore, intracellular
recordings from CA1 pyramidal neurons performed in our laboratory
indicated that the highest concentration of emetine used to block LTP
(1 μM) had no effect whatsoever on resting membrane potential, input
resistance, action potential amplitude or duration, EPSP or IPSP ampli-
tude, synaptic or direct threshold for evoked action potentials,
accomodation to a depolarizing current step, or the Ca^{2+}-dependent K^+
after-hyperpolarization [Stanton and Sarvey, 1984]. These data make it

Fig. 3. Effects of the protein synthesis emetine on LTP in field CA1

A: Control LTP in field CA1. The population spike was recorded
in CA1 just prior to (0'), and 30 min after (30'), repetitive stimulation
of the Schaffer collaterals (20Hz/10sec; arrow). Asterisk denotes
stimulus artifact.

B: Blockade of LTP in field CA1 by emetine. The slice was pre-
treated with the protein synthesis inhibitor emetine (1.5 μM) for 30
min prior to repetitive stimulation (20Hz/10sec), and the inhibitor
remained in the medium until measurement of LTP 30 min after repetitive
stimulation. LTP was completely blocked, and the population spike ampli-
tude was unchanged 30 min after repetitive stimulation. (Calibration
for A and B: 1 mV, 5 msec)

C: Time course of the increase in population spike amplitude
in control LTP is compared to the time course of blockade of LTP with
emetine (15 μM). These are different slices from A and B. The control
slice (closed circles) saw no inhibitor and was repetitively stimulated
at time zero (0', 20Hz/10sec; arrow). The treated slice (open circles)
received bath application of emetine (15 μM) throughout the experiment,
and was repetitively stimulated in identical manner. In the control
slice, LTP was clearly established at 30 min, peaked after 2 hours, and
lasted throughout the 5 hour recording period. In the treated slice, no
LTP occurred, and the response was stable throughout the 5 hour recording
period.

C

POPULATION SPIKE AMPLITUDE (mV)

TIME (min)

20Hz/10s

A CONTROL

0' 30'

*

B EMETINE 1.5μM

0' 30'

*
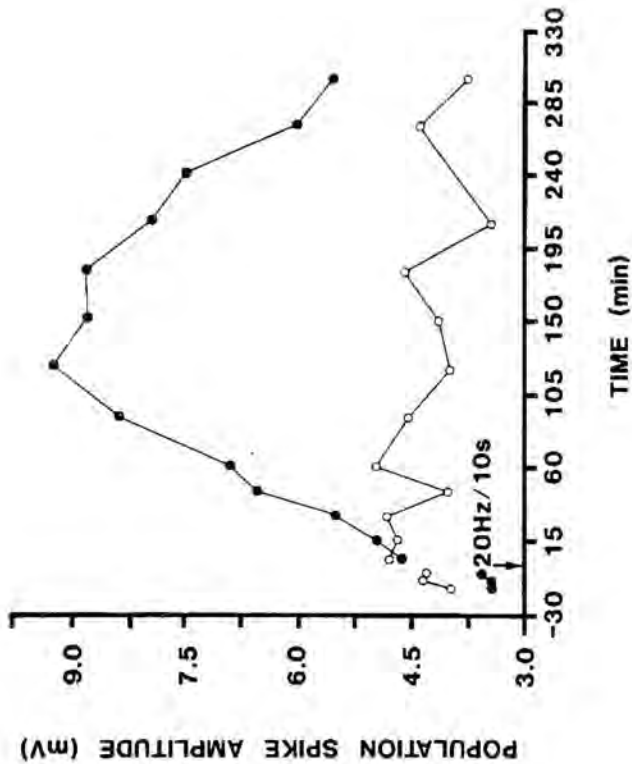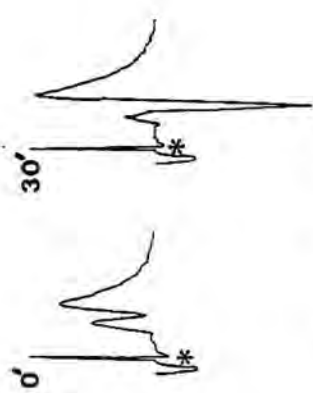
very unlikely that some generalized alteration in membrane properties
is responsible for emetine's blockade of LTP.

To test the generality of this result, the protein synthesis
inhibitor emetine (1.5 µM) was also tested for its ability to block
LTP in the dentate granule cell body layer produced by stimulation of
the perforant path axons. Emetine was also effective in preventing
LTP in this area, as measured by the frequency of occurrence of LTP
($0/4, x^2, p<0.05$ compared to a control frequency of 8/12), and the
average population spike amplitude increase after repetitive stimula-
tion (92.9 ± 9.3 % of baseline, Student's t-test, $p<0.05$ compared to
control amplitude of 169.6 ± 18.3 % of baseline).

Three protein synthesis inhibitors were effective in blocking
production of LTP in CA1 in a dose-dependent manner. Slices were pre-
incubated for 30 min with one of the protein synthesis inhibitors
emetine, cycloheximide, or puromycin. The Schaffer collaterals were
then repetitively stimulated, and the slices were maintained in the
drug solution for an additional 30 min. After this time, population
spike amplitude was measured, and the percent of slices exhibiting LTP
in control vs. drug conditions is shown in Fig 4A. Note that, in con-
trol conditions, 57% of the slices exhibited LTP.

The intermediate and high concentrations of emetine and cyclo-
heximide, and the high concentration of puromycin, were all able to
significantly decrease the frequency of LTP observed in CA1 ($x^2, p<0.05$).
In contrast, the low concentrations of all three inhibitors did not
significantly decrease the frequency of LTP. There were no alterations
in population spike waveform or amplitude produced by any of the three

inhibitors over the course of the experiments, with the exception of the highest concentration of cycloheximide (35 μM), which occasionally produced a transient increase in spike amplitude, followed by at most a 20% depression. The effect of the highest concentration of puromycin (100 μM) on the population spike was followed for longer time periods. Puromycin had no effect on population spike waveform or amplitude for periods up to 3 hours (N=4), but multiple population spikes were occasionally observed after this time.

The blockade of LTP by these inhibitors is specific to the long lasting enhancement of synaptic efficacy in LTP. Short-term potentiation (STP), often observed immediately after repetitive stimulation, was not affected by inhibition of protein synthesis. STP seen 2 min after repetitive stimulation (8 of 28 slices, 29%, mean amplitude increase 127.7 ± 20.4% of control), was not altered by the highest concentration of any of the three synthesis inhibitors (14 of 26 slices, 54%, mean amplitude increase 131.6 ± 8.51% of control), even though LTP did not occur.

Measurement of protein synthesis in slices by [^{3}H]-valine incorporation into TCA-precipitable macromolecules

Measurement of the level of inhibition of [3H]-valine incorporation into proteins achieved in slices was performed to determine if the percent inhibition correlated with the dose-dependence of blockade of LTP (Fig 4B). Slices were preincubated for 30 min with one of the three protein synthesis inhibitors prior to the addition of [3H]-valine (1 μCi/ml) to the medium. Uptake and incorporation of [^{3}H]-valine into proteins was allowed to proceed for an additional 60 min. Incorporation into TCA-precipitable proteins was measured as a percent of untreated slices.

Fig. 4. Effects of protein synthesis inhibitors on LTP and on incorporation of [^{3}H]-valine into Trichloroacetic acid (TCA)-precipitable macromolecules

A: Frequency of LTP in the presence of each of three protein synthesis inhibitors in the bath, compared to control incidence of LTP. Above each bar is the number of slices showing LTP per total number of slices tested. LTP was defined as having occurred when an increase in population spike amplitude greater than two standard deviations (>35%) over mean control baseline amplitudes was observed 30 min after repetitive stimulation.

Whereas the lowest concentration of each inhibitor was ineffective in blocking LTP, higher concentrations of each inhibitor significantly decreased the frequency of occurrence of LTP (*;χ^2,p<0.05).

B: Percentage inhibition of [^{3}H]valine incorporation into TCA-precipitable macromolecules by each protein synthesis inhibitor. Slices were incubated in tissue culture wells for 30 min in one of the protein synthesis inhibitors, and then [^{3}H]-valine (1 µCi/ml) was added and the slices were incubated for an additional 60 min. Incorporation into proteins was halted by homogenization in excess unlabeled valine (0.1 mg/ml) at 4° C, and TCA-precipitable macromolecules were separated and counted. Incorporation was expressed as disintegrations per minute per milligram of protein, and percentage inhibition was calculated compared to internal standard slices that received no inhibitor in the same plate.
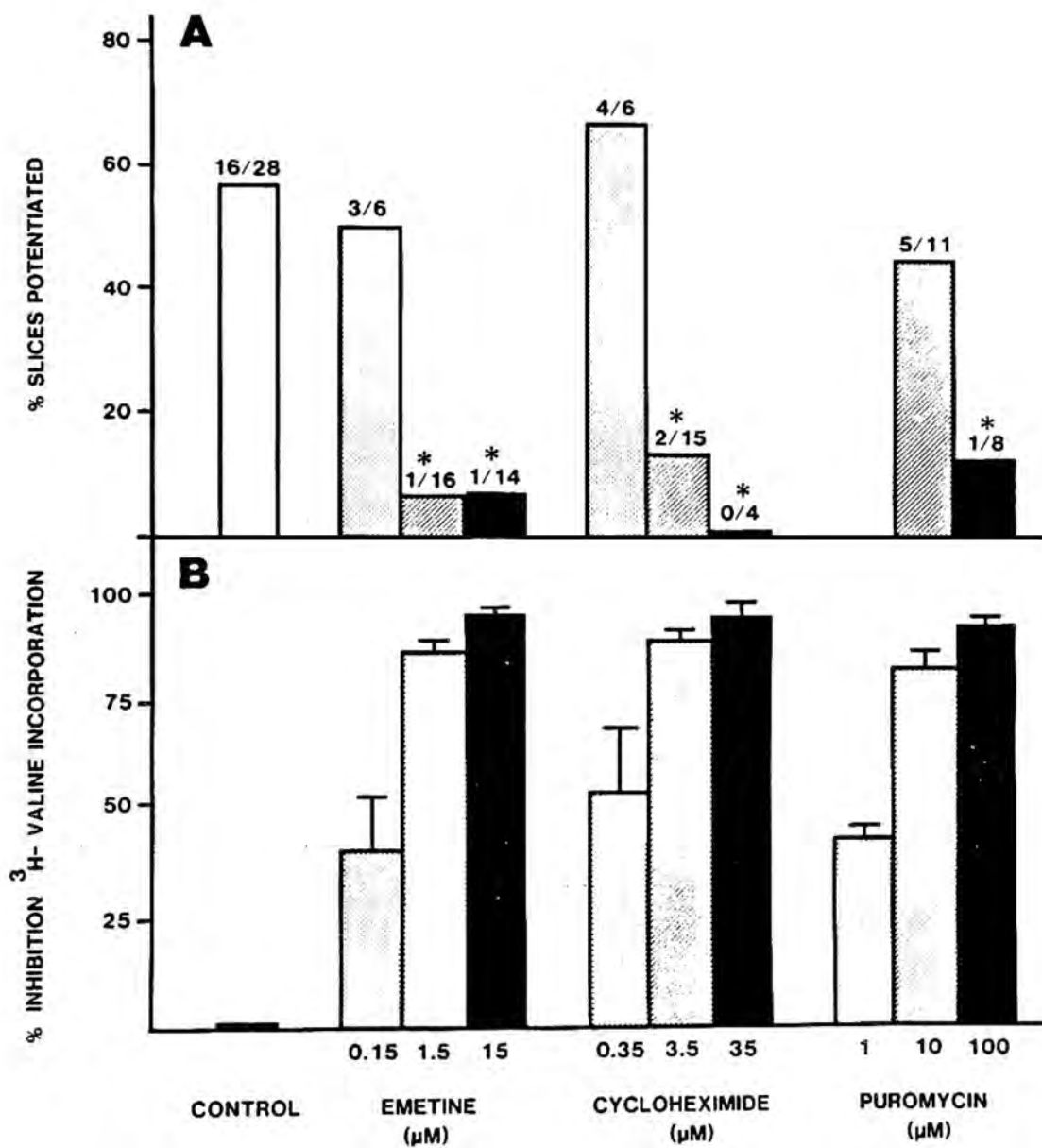
These inhibitors block protein synthesis in a dose-dependent manner. At concentrations which were effective in blocking LTP, the mean percent inhibition of protein synthesis was 93 ± 1.2% (N=26). At the low concentrations, which were ineffective in blocking LTP, the mean percent inhibition of protein synthesis was only 60 ± 7.7% (N=14). The degree of blockade of LTP produced by each of the three inhibitors was found to correlate (r=-0.843,p<0.05) with the attenuation of incorporation of [3H]-valine into proteins. Regression analysis of the amplitude of the population spike 30 min after repetitive stimulation as percent of prestimulation amplitude versus inhibition of incorporation of [3H]-valine also exhibited a significant correlation (r=-0.737,p<0.05). The mean percent inhibition of protein synthesis at concentrations that block LTP suggests that almost complete inhibition of protein synthesis is necessary to impair the production of LTP.

Blockade of LTP by emetine requires a preincubation period

In a variety of cellular systems, protein synthesis inhibitors are found to require several minutes to penetrate the cell, bind to ribosomes, and inhibit protein synthesis [Grollman, 1968]. If protein synthesis inhibition is the mechanism of LTP blockade, we would expect a preincubation period to be necessary for effective blockade. Conversely, if a faster acting property of these inhibitors is the mechanism of LTP blockade, we would expect presence of the inhibitor during and after the repetitive stimulation to be sufficient to block LTP.

As shown in Fig 5, blockade of LTP by emetine did require the 30 min preincubation period. If repetitive stimulation was delivered as emetine arrived in the bath, LTP was no longer blocked (N=11). In

Fig. 5. Blockade of LTP by emetine requires a preincubation period

Time lag in LTP blockade by emetine. Population spike amplitudes of responses recorded in CA1 before, and 2, 15, and 30 min after repetitive stimulation of the Schaffer collaterals (20 Hz/10 sec; large arrow). Emetine (1.5 μM; small arrow) was added to the bath 30 min before (circles, N=15) or simultaneous with (squares, N=11) the repetitive stimulation, and was present the entire time thereafter. Blockade of LTP by emetine did require the preincubation period, since simultaneous addition failed to block LTP (*; paired t-test, p<0.05).
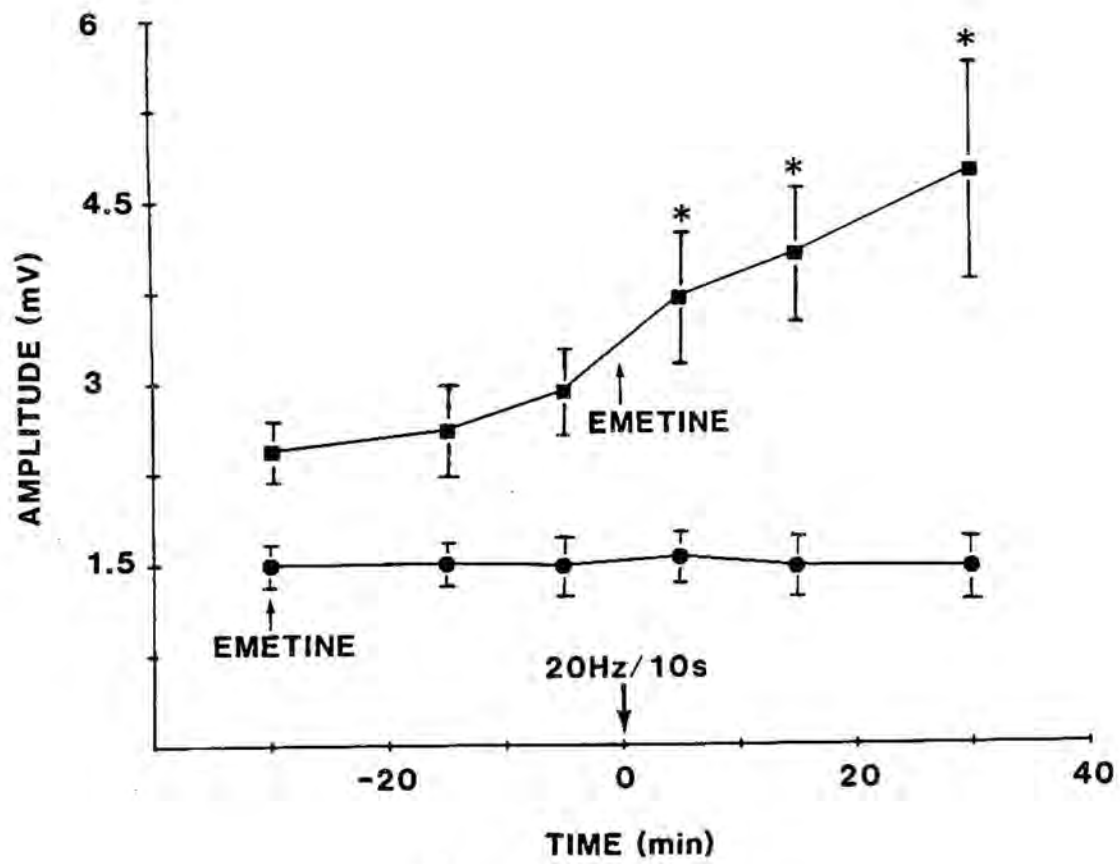
contrast, LTP did not occur when emetine was added 30 min prior to repetitive stimulation ($N=15$, χ^2, $p<0.05$). These results indicate that blockade of LTP by emetine is not simply a result of non-specific membrane effects, since when emetine was present in the bath only from the time of repetitive stimulation, up to 30 min after, LTP was no longer blocked.

Reversibility of protein synthesis inhibition and blockade of LTP

To further establish that these inhibitors block LTP by inhibiting protein synthesis, I determined whether differences in the reversibility of their inhibition of protein synthesis were matched by differences in reversibility of their blockade of LTP. Emetine is an irreversible inhibitor of protein synthesis, whereas cycloheximide is a reversible inhibitor of protein synthesis [Grollman, 1968]. Measurement of [^{3}H]-valine incorporation in slices verified this observation. Slices were treated with the same incubation and wash paradigm employed with the other inhibitors, shown in Fig 6A. Inhibition by emetine of [^{3}H]-valine incorporation in slices was not reversed by a 2 hour wash, while inhibition by cycloheximide was reversible.

Fig 6B illustrates that the differences in reversibility also apply to the blockade of LTP. As usual, preincubation for 30 min with either emetine (1.5 μM) or cycloheximide (3.5 μM) blocked the induction of LTP (χ^2, $p<0.05$). These same slices were then washed in drug-free buffer for 2 hours, after which a second repetitive stimulation was applied. Slices treated with the irreversible protein synthesis inhibitor emetine were still unable to potentiate ($N=4$, χ^2, $p<0.05$). However, slices treated with the reversible inhibitor cycloheximide were capable of

Fig. 6. Reversibility of protein synthesis inhibition and blockade of LTP

     Comparison of the degree of reversibility of inhibition of ^{3}H-valine incorporation (A), and blockade of LTP (B), by emetine (1.5 μM) and cycloheximide (3.5 μM).

A: TREATED: Slices incubated 30 min in emetine (open bars) or cyclohex-imide (hatched bars) exhibited inhibition of ^{3}H-valine incorporation greater than 80%.

  WASHED: Slices were incubated 30 min and then washed for 1 hour before ^{3}H-valine was added. Emetine's inhibition was irre-versible, while cycloheximide's was partially reversible (*, t-test, $p<0.05$ compared to treated slices).

B: TREATED: Slices bathed for 30 min in emetine or cycloheximide exhibited complete blockade of LTP (*, χ, $p<0.05$).

  WASHED: These same slices were washed for 2 hours and repetitively stimulated (20Hz/10sec) a second time. As in the case of ^{3}H-valine incorporation, emetine's blockade of LTP was irre-versible, while cycloheximide's (solid bar) was reversible.
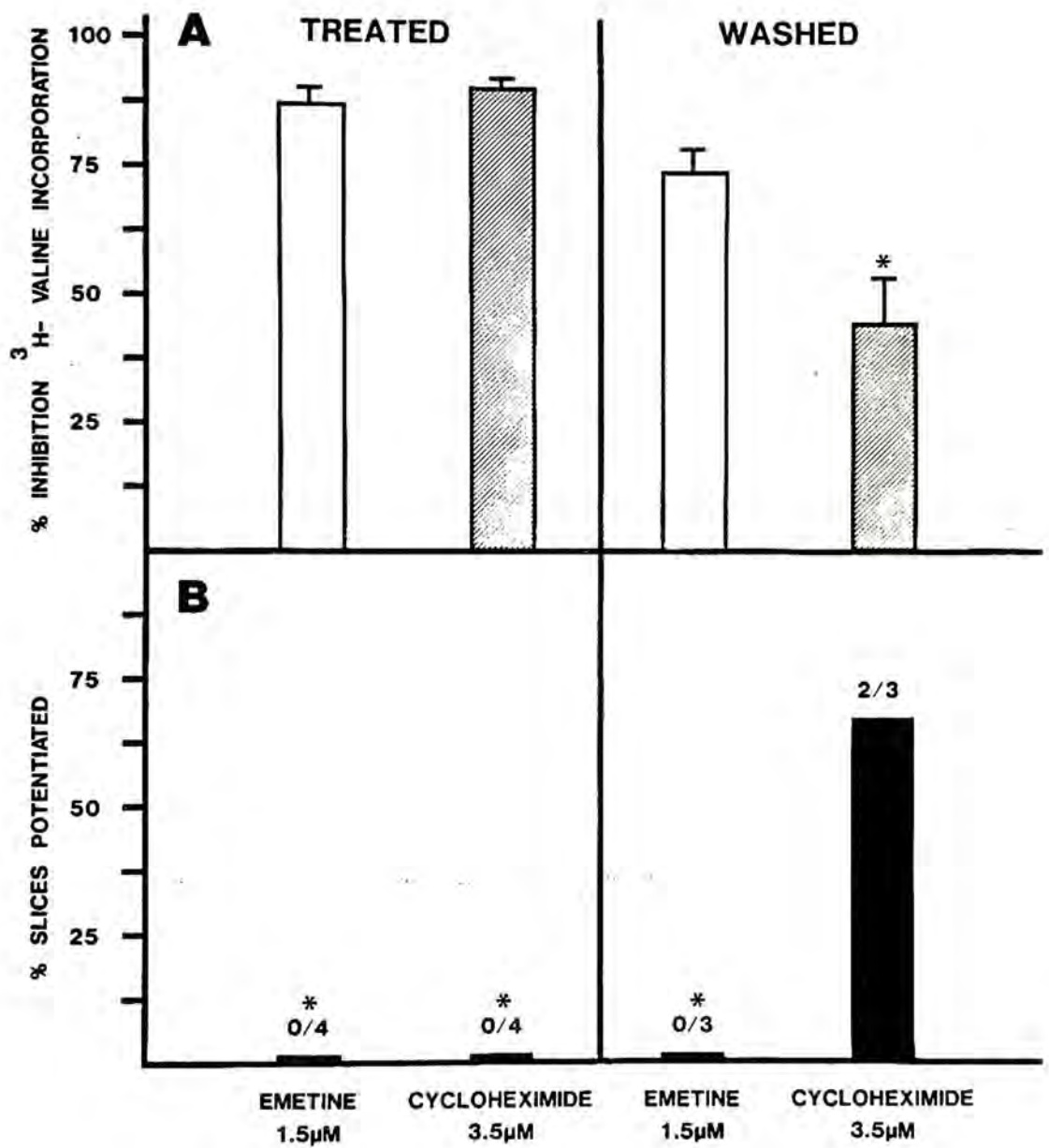
exhibiting LTP after wash (N=3). These results further support a link between LTP blockade and inhibition of protein synthesis. In addition, the ability of these slices to support LTP after cycloheximide treatment and wash convincingly demonstrates the viability of these slices, in spite of prolonged inhibition of protein synthesis.

Puromycin aminonucleoside does not block LTP or inhibit protein synthesis in slices

If inhibition of protein synthesis is essential for blockade of LTP, congeners of these compounds which are inactive in inhibiting protein synthesis should not be able to block LTP. Puromycin aminonucleoside (PA) is such a structural analog of puromycin that is relatively ineffective in inhibiting protein synthesis [Agranoff et al., 1966]. Experiments with PA are summarized in Fig 7. Measurement of [3H]-valine incorporation in slices treated with PA (100 µM) verified that it has very limited potency in inhibiting protein synthesis (17.3 ± 10.2%,N=2). When added to slices for 30 min prior to repetitive stimulation, PA (100 µM) was unable to prevent the induction of LTP (N=8). In contrast, the same concentration of puromycin was effective in blocking LTP (N=8, χ^2,p<0.05). These results support the conclusion that puromycin blocks LTP by inhibiting protein synthesis.

Tyrosine hydroxylase inhibition is not responsible for blockade of LTP by protein synthesis inhibitors

Inhibition of catecholamine synthesis is a side effect of cycloheximide, puromycin, and anisomycin, which has been suggested as a possible mechanism of their behavioral effects [Flexner and Goodman,

Fig. 7. Puromycin aminonucleoside (PA) does not block LTP or inhibit
         protein synthesis in slices

         Comparison of the percentage inhibition of $[^3H]$valine incorp-
oration by PA, with its ability to block LTP in slices. The left axis
and hatched bar show the inability of 100 μM PA to inhibit $[^3H]$valine
incorporation as measured by the same paradigm employed for the other
inhibitors. The right axis and hatched bar show that 100 μM PA was
also unable to block LTP in CA1 when tested with the same paradigm as
employed for the other inhibitors. The solid bars show the control (C)
values.
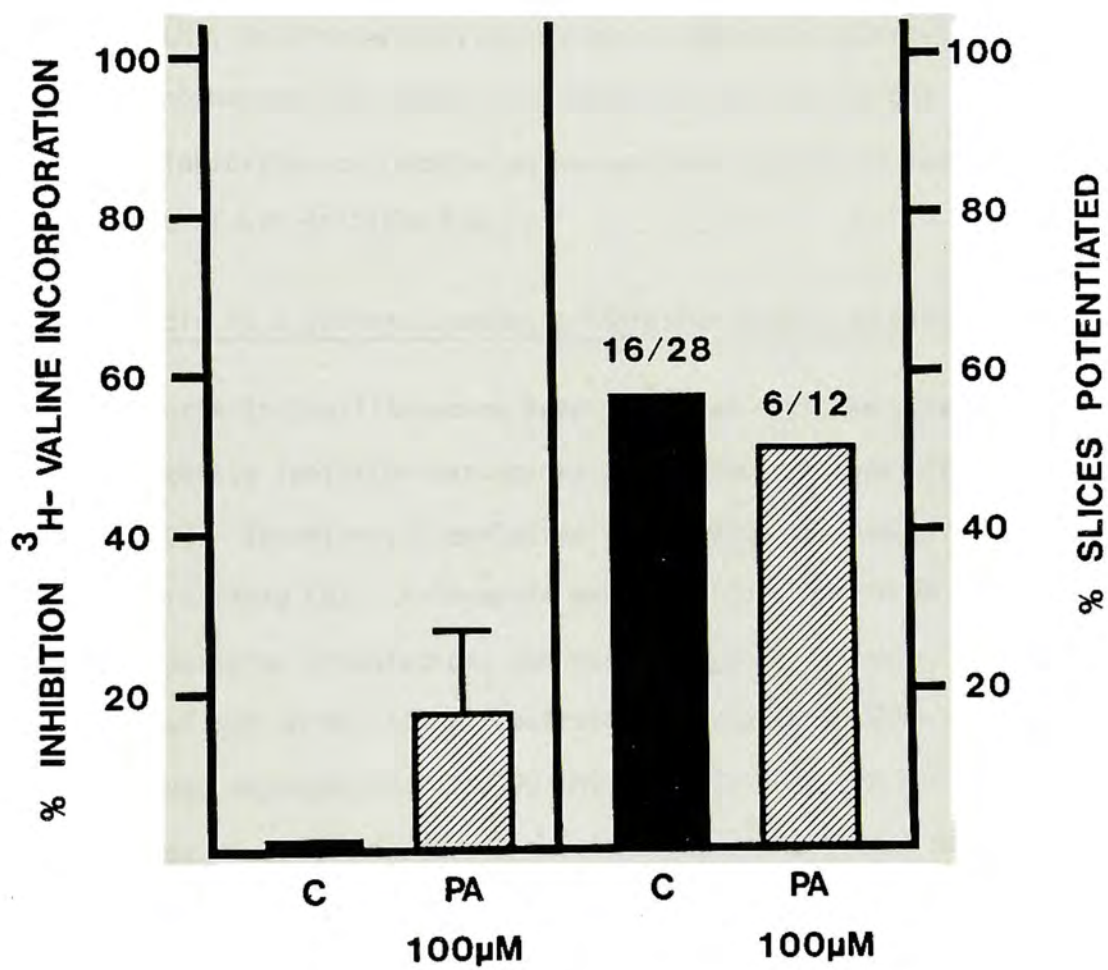
1975]. Other studies have indicated that inhibition of tyrosine hydroxylase, the rate limiting enzyme in catecholamine synthesis, is not responsible for the behavioral effects of protein synthesis inhibitors [Squire et al., 1974]. The possibility that inhibition of catecholamine synthesis by protein synthesis inhibitors might be responsible for blockade of LTP was evaluated by bath applying α-methyl-p-tyrosine (AMPT, 100 μM), an irreversible inhibitor of tyrosine hydroxylase. A 30 min preincubation with AMPT was unable to block LTP in CA1 (N=8). Therefore, inhibition of catecholamine synthesis cannot be responsible for blockade of LTP in field CA1.

Anisomycin is a protein synthesis inhibitor unable to block LTP

Reports in the literature have suggested that the pyrrolidine protein synthesis inhibitor anisomycin was unable to block LTP [Swanson, et al., 1982]. Therefore, I evaluated the ability of anisomycin to modify LTP in field CA1. Anisomycin was added to the bath 30 min prior to repetitive stimulation, and was present continuously until assessment of LTP 30 min after repetitive stimulation. None of the concentrations employed [3.8 to 190 μM] was able to alter the frequency of occurrence of LTP in field CA1 as tested by χ^2 analysis. However, when the population spike amplitude 30 min after repetitive stimulation was compared by a paired t-test with prestimulation amplitude in all slices, including those that failed to meet the criterion for LTP, an average increase in spike amplitude was not seen at any concentration of anisomycin tested. By this criterion, anisomycin blocked LTP. On those occasions when LTP did occur, the average amplitude of LTP in untreated slices was 311 ± 89.1% (N=16,% of pre-stimulated baselines),

while the amplitude of LTP in anisomycin treated slices was 199 ± 31.2% (N=9), and LTP amplitude in those treated with the other inhibitors, 196 ± 24.1% (N=6). Therefore, I conclude that emetine, cycloheximide, and puromycin may be able to decrease both occurrence and amplitude of LTP, while anisomycin appears only to decrease LTP amplitude, and not prevent its occurrence. This suggests a qualitative difference between anisomycin's actions and those of the other protein synthesis inhibitors.

Measurement of anisomycin's inhibition of [^{3}H]-valine incorporation into TCA-precipitable proteins indicated that all concentrations of anisomycin employed inhibited more than 90% (N=6).

Further experiments were performed to determine whether a greater time lag in the drug's effectiveness might be responsible for its failure to block LTP. Preincubation in anisomycin for up to 2 hours did not block LTP (N=4). Measurement of anisomycin's ability to inhibit [^{3}H]-valine incorporation after 2 hours in oxygenated buffer indicated that there was no significant loss of overall ability to inhibit [^{3}H]-valine incorporation over time (190 μM, N=2). Finally, I considered the hypothesis that anisomycin might be inhibiting [^{3}H]-valine incorporation in our assay system by inhibiting cellular uptake of [^{3}H]-valine, rather than by inhibiting protein synthesis. Therefore, I separated the intracellular and extracellular [^{3}H]-valine from that incorporated into TCA-precipitable proteins, and found that cellular uptake of [^{3}H]-valine was not inhibited by anisomycin (190 μM, N=5).

II. The role of norepinephrine and serotonin in LTP

Transmitter levels in depleted animals

Norepinephrine. The mean level of hippocampal NE in control animals
(N=8) was 210 ± 30 pg/mg tissue wet weight. In animals injected bilat-
erally with 6-OHDA in the dorsal noradrenergic bundle, and assayed
14-21 days later (N=12), the NE concentration was 35.5 ± 7.8 pg/mg
tissue. Thus, mean depletion of NE was 83.1 ± 5.4%. This value is comp-
arable to depletion produced with these methods in in vivo experiments
[Bliss, et al., 1983]. In animals pre-treated with the noradrenergic
uptake blocker desmethylimipramine (DMI) to block uptake of 6-OHDA
into noradrenergic terminals, mean depletion of NE was only 44.9% (N=2).

Serotonin. The mean level of hippocampal 5-HT in control animals
(N=8) was 328 ± 15 pg/mg tissue wet weight. The mean depletion in
animals treated with 5,7-DHT plus DMI (N=5) was 69.0 ± 6.0%, and in
animals treated with PCPA (N=3), 76.2 ± 1.4%. These values are also
comparable to those produced with these methods in vivo [Bliss, et
al., 1983].

Depletion of NE specifically reduced LTP in the dentate

The frequency of occurrence, and the percent change in the
amplitude of the evoked population spike, during LTP were compared for
normal slices and those depleted of NE or 5-HT. After establishing a
stable baseline I/O relation for 15-30 min, the slices were repeti-
tively stimulated via the perforant path for dentate recordings,

or via the Schaffer collaterals for CA1 recordings. A schematic of the slice with both stimulating and recording paradigms is shown in fig 2A.

Dentate.

Depletion of NE with 6-OHDA (fig 8A) virtually eliminated both the frequency of occurrence of LTP (χ^2,p<0.05) and the increase in average population spike amplitude (Student's t-test, p<0.05) produced after repetitive stimulation. Slices cut from NE-depleted animals were no different in population spike or dendritic excitatory postsynaptic potential (EPSP) waveform or amplitude, but were markedly impaired in their ability to exhibit LTP in the dentate.

Experiments to control for depletion of dopamine or other non-specific effects of the toxin were performed by injecting animals with desmethylimipramine (DMI) 40-60 min prior to injection of 6-OHDA, to block uptake of the toxin into noradrenergic nerve terminals. In slices cut from these animals 14-21 days later, neither the frequency of occurrence nor the amplitude of LTP differed from those seen in control slices (fig 8A).

CA1.

In contrast to dentate, depletion of NE by 6-OHDA did not impair either the frequency of occurrence or amplitude of LTP in field CA1, when repetitive stimulation was applied to the Schaffer collaterals (fig 8B).

Fig. 8. Effects of norepinephrine (NE) depletion on LTP in the dentate
and field CA1

A: Percent occurrence of LTP (clear bars), and percent increase
in population spike amplitude (Mean ± S.E.M., hatched bars), in the
dentate 30 min after repetitive stimulation of the perforant path. Above
each clear bar is the number of slices showing LTP/ Total number of
slices tested. LTP was defined as an increase in population spike ampli-
tude greater than two standard deviations over mean control amplitudes
30 min after repetitive stimulation.

Depletion of NE with 6-hydroxydopamine (6-OHDA) virtually
eliminated both the frequency of occurrence (*, χ^2, p<0.05), and the
increased amplitude (*, Student's t-test, p<0.05), of LTP. When animals
were pretreated 40-60 min before 6-OHDA injection with desmethylimipramine
(DMI, 20 mg/kg i.p.) to prevent uptake of the toxin into noradrenergic
terminals, there was no effect on LTP.

B: In contrast, depletion of NE by 6-OHDA did not impair LTP
in field CA1, when repetitive stimulation was applied to the Schaffer
collaterals.
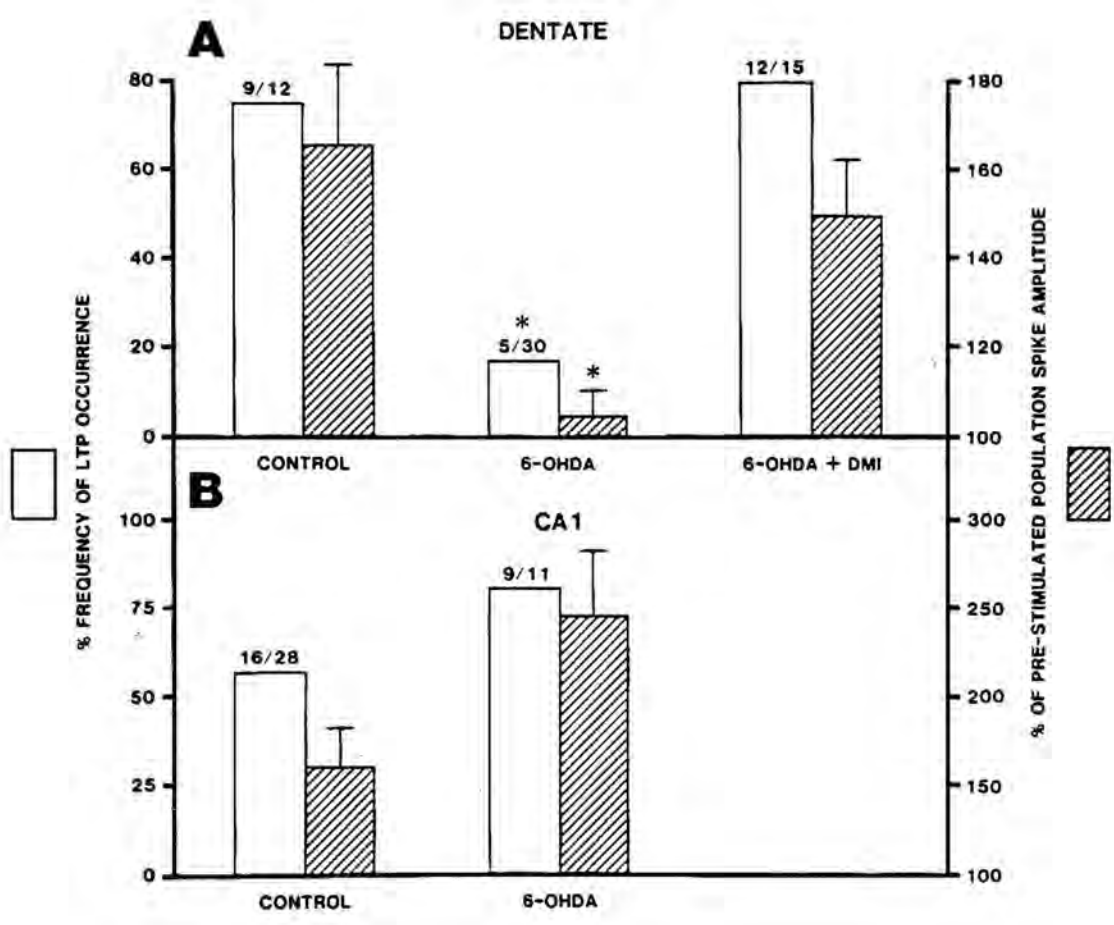
Fig. 9.   Effects of serotonin (5-HT) depletion on LTP in the dentate
          and field CA1

        A: Percent occurrence of LTP (clear bars), and percent increase
in population spike amplitude (Mean ± S.E.M., hatched bars), in the
dentate 30 min after repetitive stimulation of the perforant path. Above
each clear bar is the number of slices showing LTP/ Total number of
slices tested.
        Depletion of serotonin (5-HT) with either 5,7-dihydroxy-
tryptamine (5,7-DHT) or para-chlorophenylalanine (PCPA) produced no
decrease in the frequency of occurrence of LTP. However, depletion of
5-HT with PCPA did produce some decrease in the population spike amplitude
increase seen in LTP (*, Student's t-test, p<0.05), although there was no
significant decrease with 5,7-DHT treatment.

        B: In field CA1, depletion of 5-HT with either 5,7-DHT or PCPA
had no effect on either frequency of occurrence of LTP, or population
spike amplitude increase during LTP.
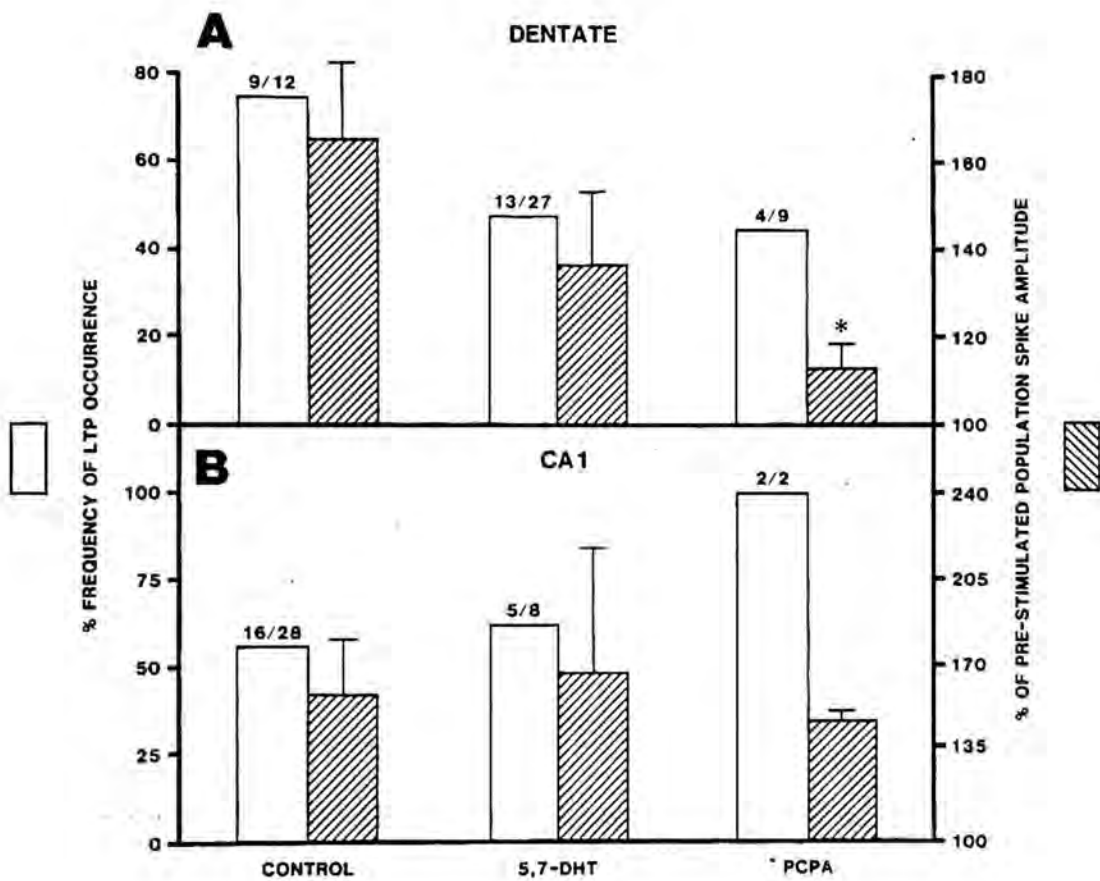
Depletion of 5-HT did not affect LTP in either dentate or CA1

Dentate.

Depletion of 5-HT with either 5,7-DHT or PCPA was unable to affect the frequency of occurrence of LTP produced in the dentate by stimulating the perforant path (fig 9A). In the case of 5,7-DHT, there was no significant difference in the average population spike amplitude increase seen during LTP. However, PCPA was able to reduce somewhat the average population spike amplitude increase after repetitive stimulation (Student's t-test, $p < 0.05$).

CA1.

In field CA1, depletion of 5-HT had no effect on either the frequency of occurrence of LTP or the average population spike amplitude after repetitive stimulation of the Schaffer collaterals (fig 9B).

Depletion of NE, but not 5-HT, also reduced LTP
of the dendritic EPSP in the dentate

LTP has been measured in a number of ways, including increased population spike amplitude (LTP) [Bliss and Lømo, 1973; Douglas and Goddard, 1975; Alger and Teyler, 1976; Stanton and Sarvey, 1984], and increased maximum dV/dt of the early, negative-going dendritic EPSP (EPSP-LTP) [Bliss and Gardner-Medwin, 1973]. However, studies have suggested some dissociation in the time course of population spike LTP from that of the dendritic layer EPSP [Douglas and Goddard, 1975; Abraham, 1984]. Therefore, I examined the effect of depletion of NE on the maximum dV/dt recorded in the stratum moleculare dendritic layer

Fig. 10. Dendritic excitatory postsynaptic potential (EPSP)-LTP and LTP
in the hippocampal slice

A: The hippocampal slice preparation, showing both the cell body
layer (B), and the dendritic layer (C), recording sites in the dentate,
and the perforant path axon stimulus site.

B: Control population spike LTP in the dentate. The population
spike was recorded in the dentate just prior to (0'), and 30 min after
(30'), repetitive stimulation of the perforant path (100 Hz/2 sec). The
potentiation after 30 min was 200% of control population spike amplitude.
Asterisk denotes stimulus artifact.

C: Simultaneously recorded dendritic EPSP-LTP in the dentate in
the same slice as in B. The dendritic EPSP was recorded just prior to
(0'), and 30 min after (30'), repetitive stimulation. Potentiation here
was measured by the increase in the maximum early, negative-going dV/dt,
which in this experiment was 175% of pre-stimulated amplitude.
(Calibration for B and C: 1 mV, 5 msec)
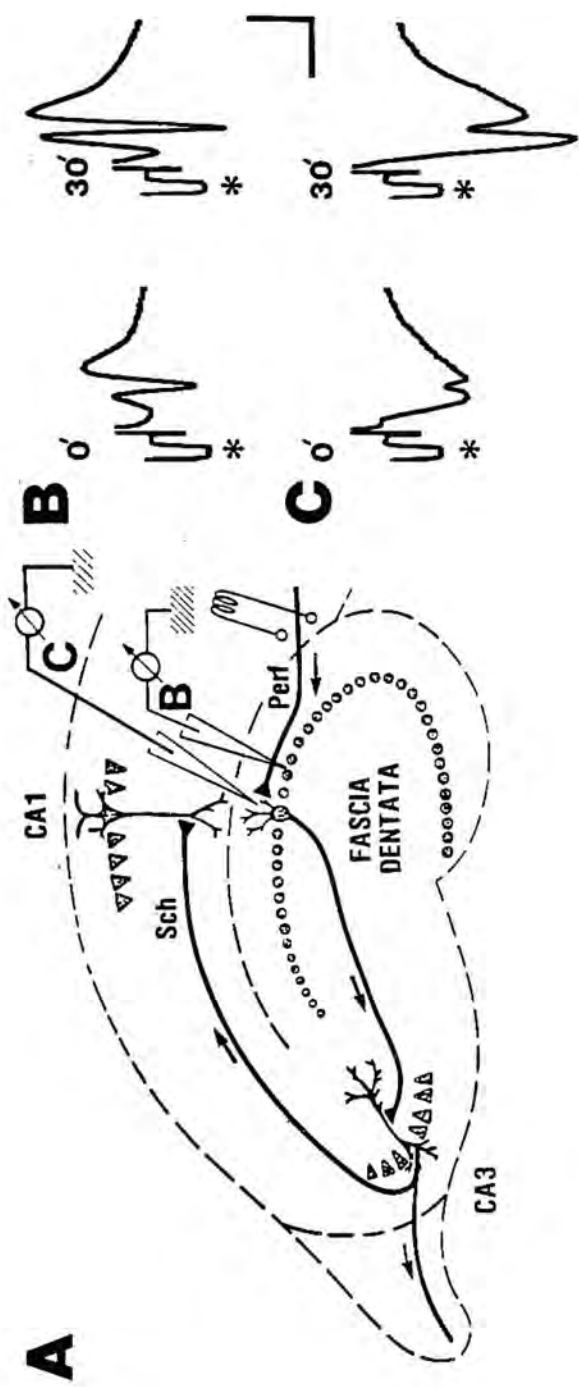
Fig. 11. Effects of NE and 5-HT depletion on dendritic EPSP-LTP
        in the dentate

The effect of depletion of NE or 5-HT on percent increase in
population spike amplitude (LTP, Mean ± S.E.M., clear bars), and percent
increase in maximum dendritic dV/dt (EPSP-LTP, Mean ± S.E.M., hatched
bars), in the dentate 30 min after repetitive stimulation of the perforant
path (100 Hz/2 sec). (N) = Number of slices.

As in previous experiments, depletion of NE with 6-OHDA markedly
reduced population spike LTP (*, Student's t-test, $p < 0.05$), while deple-
tion of 5-HT with 5,7-DHT did not. Furthermore, depletion of NE also
markedly reduced the maximum dendritic slope EPSP-LTP recorded in these
slices simultaneously (*, Student's t-test, $p < 0.05$), while depletion of
5-HT had no significant effect on dendritic slope EPSP-LTP.
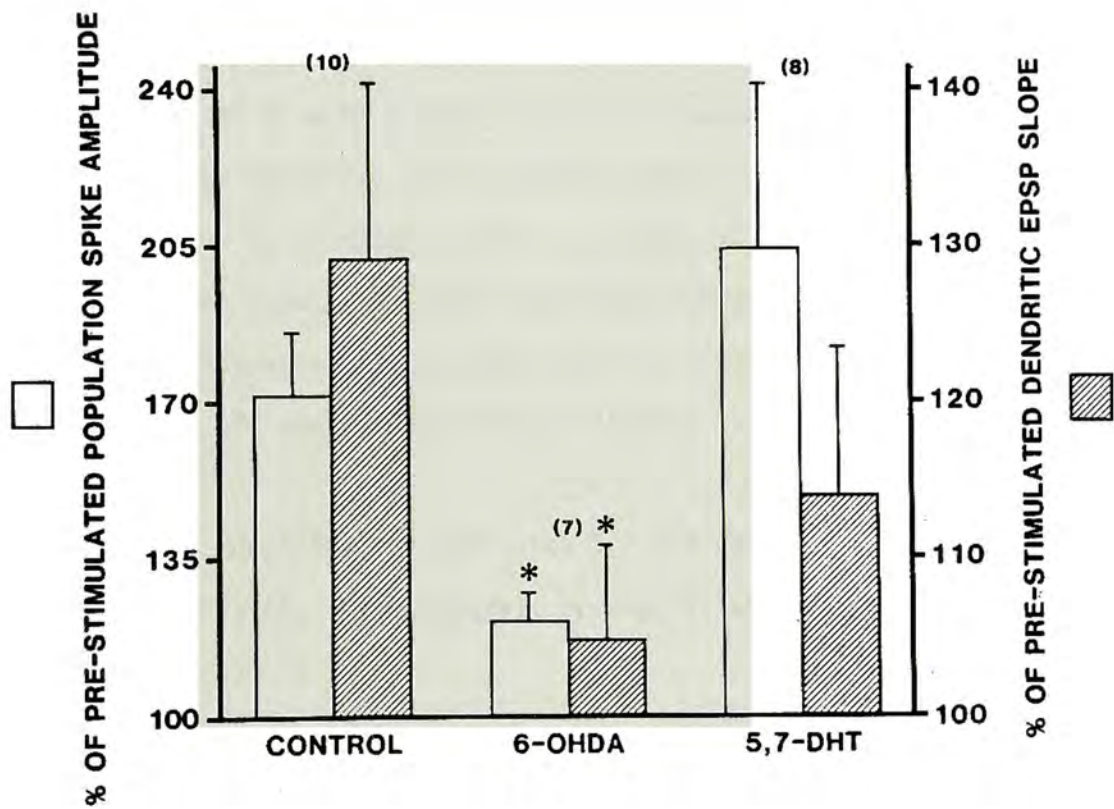
in the dentate (Fig 10A). A typical example of control LTP of the
population spike is shown in figure 10B, where the response just prior
to (0'), and 30 min after (30'), repetitive stimulation is shown in
the dentate granule cell body layer (stratum granulosum). In this same
slice, potentiation of the dendritic EPSP recorded in stratum moleculare
is shown prior to (0'), and 30 min after (30'), stimulation (Fig 10C).

Depletion of NE with 6-OHDA (Fig 11) virtually eliminated the
increase in maximum dendritic dV/dt produced after repetitive stim-
ulation (hatched bars, Student's t-test, $p<0.05$), as well as the
increase in average population spike amplitude (clear bars, Student's
t-test, $p<0.05$). Therefore, I conclude that the necessity for NE in
the expression of LTP applies to EPSP-LTP as well as population spike
LTP.

Similarly, depletion of 5-HT with 5,7-DHT was unable to affect
either EPSP-LTP (Fig 11, hatched bars), or population spike LTP
(Fig 11, clear bars).

The time course of the recovery of hippocampal NE levels
was paralleled by the recovery of LTP in the dentate

Another group of animals was treated with 6-OHDA to deplete
hippocampal NE, and then allowed longer periods (21-42 days) for hippo-
campal NE levels to recover. The time course of recovery of hippocampal
NE levels was closely paralleled by the recovery of LTP produced in
the dentate by repetitive stimulation of the perforant path. Increasing
percent depletion in the hippocampus was well correlated with a greater
reduction in the mean population spike amplitude increase after
repetitive stimulation (fig 12, $r=-.926$, Student's t-test, $p<0.05$ that

Fig. 12.   The time course of recovery of hippocampal NE and LTP

        Average percent increase in population spike amplitude
(Mean ± S.E.M.) recorded in the dentate vs. percent depletion of hippo-
campal NE in rats treated with 6-OHDA and allowed varying lengths of
time (2-5 weeks, indicated next to points) for hippocampal NE levels to
recover.
        The time course of recovery of NE levels was closely paral-
leled by the return of the ability to produce LTP in the dentate. In-
creasing percent depletion was correlated with smaller increases in aver-
age population spike amplitude after repetitive stimulation ($r = -.926$,
$p < 0.05$ that $r = 0$, control N=12 slices, each depleted animal N=4 slices).
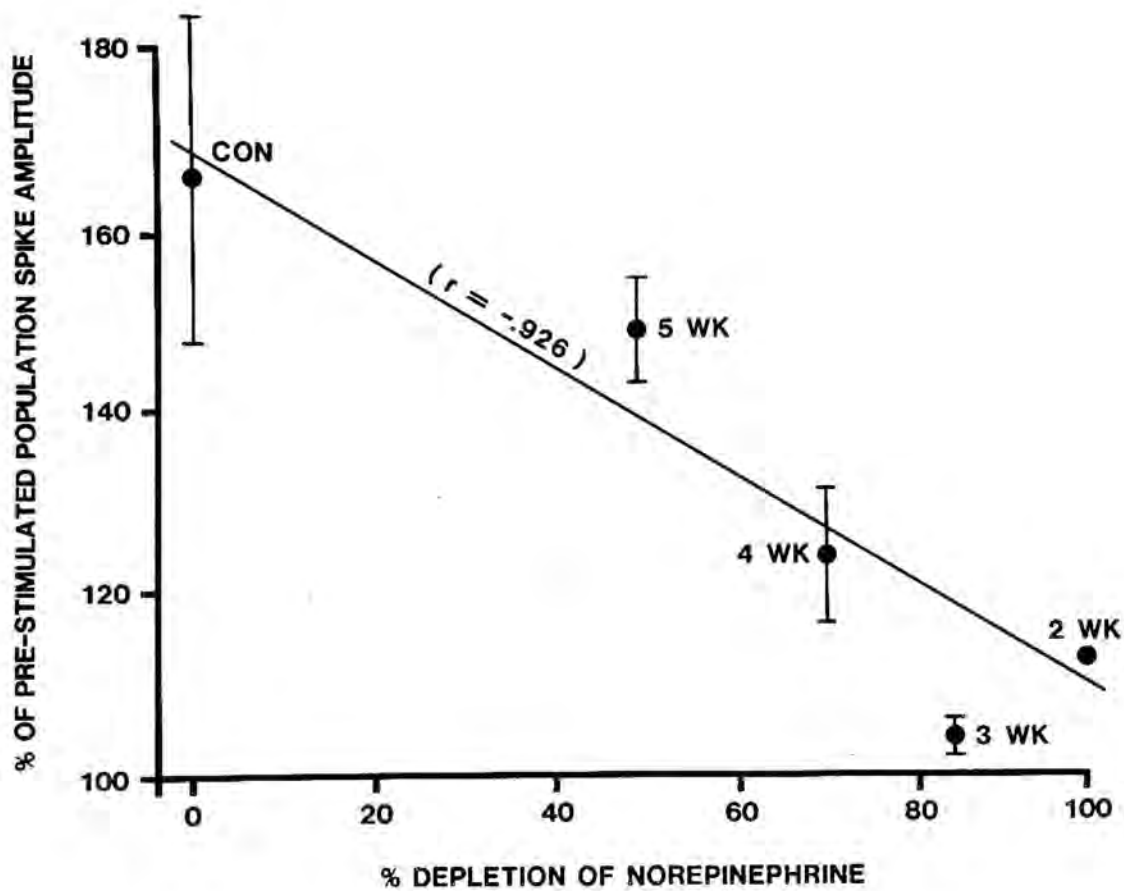
r=0). This supports the hypothesis that the reduction in LTP seen with 6-OHDA treatment is due to depletion of hippocampal NE.

The β-antagonist propranolol and β_1-antagonist metoprolol decrease LTP in the dentate

Since involvement of NE in production of LTP in the dentate was strongly supported by my data, the next question became the identification of the noradrenergic receptor subtype mediating the effect. Work in our and other laboratories has shown that NE alone, under a variety of application procedures, can produce a long-lasting potentiation of the population spike in the dentate, and that this effect is antagonized by β-receptor antagonists [Neumann and Harley, 1983; Stanton and Sarvey, 1985b]. Therefore, I began by testing the ability of the β-antagonist propranolol [Aurbach, et al., 1974] and the β_1-antagonist metoprolol [Minneman, et al., 1979a,b] to affect LTP produced in the dentate by repetitive stimulation of the perforant path.

Slices from non-depleted rats were pre-incubated for 30 min with bath applied propranolol (20 μM) or metoprolol (20 μM), then repetitively stimulated via the perforant path. β-receptor blockade markedly reduced both the frequency of occurrence (x^2, $p<0.05$), and the increased population spike amplitude (Student's t-test, $p<0.05$) of LTP (fig 13). Neither antagonist alone affected the population spike amplitude or waveform. These results further support the conclusion that the action of NE in supporting potentiation of the population spike in the dentate is mediated by β-receptor activation, and probably β_1-receptors specifically.

Fig. 13. Effect of β-antagonists on LTP in the dentate

Frequency of occurrence of LTP (clear bars), and percent increase in population spike amplitude (Mean ± S.E.M., hatched bars), in the dentate 30 min after repetitive stimulation of the perforant path. Above each clear bar is the number of slices showing LTP/ Total number of slices tested.

The β-antagonist propranolol (20 μM), and the β_1-antagonist metoprolol (20 μM), were effective in reducing both the frequency of occurrence (*, χ , p<0.05), and the amplitude increase (*, Student's t-test, p<0.05), of LTP in the dentate.
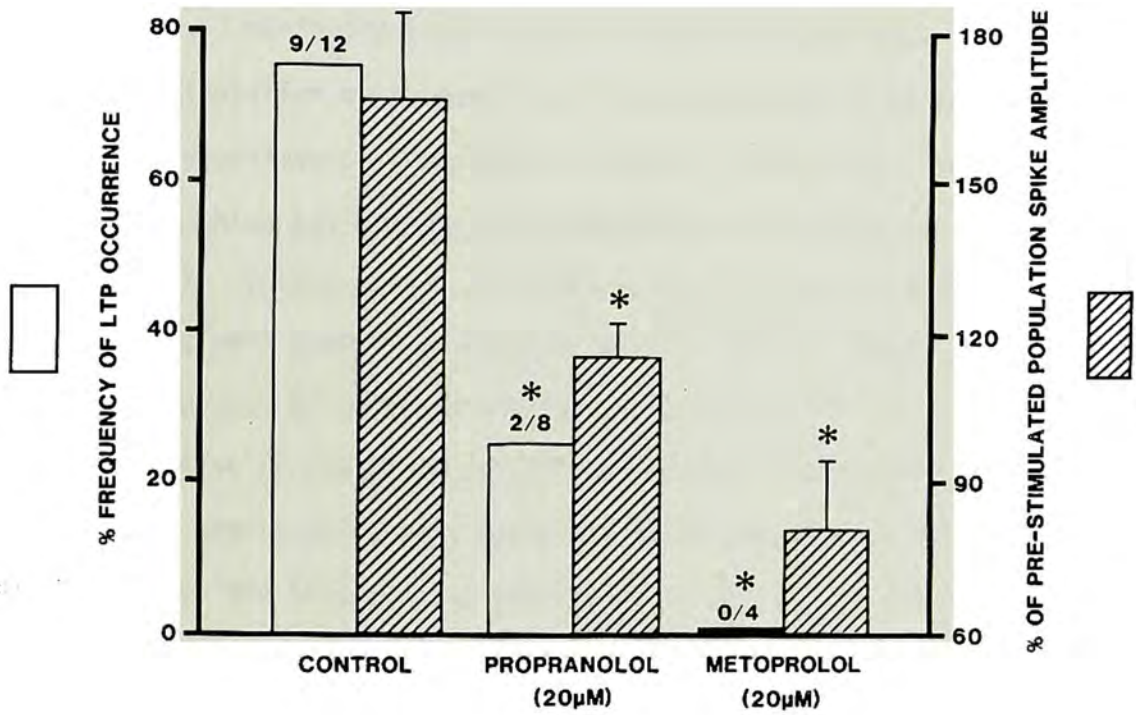
In contrast, LTP in field CA1 did not seem to be reduced by pre-incubation in propranolol (20 μM, Mean=202%, N=2), indicating much less importance for β-receptors in LTP in this area.

The adenylate cyclase stimulant forskolin restores LTP in the dentate of slices depleted of NE

Since β-receptor activation in a wide variety of neuronal systems has been shown to employ cAMP as an intracellular 'second messenger', I performed experiments to test the importance of adenylate cyclase stimulation by β-receptors in the action of NE in the dentate. For these experiments, I employed a recently discovered diterpene, forskolin, which has been found to potentiate agonist stimulation of adenylate cyclase at a 1 μM concentration, without directly stimulating cAMP production [Seamon, et al., 1981]. These experiments demonstrate that NE-depleted slices retain the ability to exhibit LTP in the dentate if adenylate cyclase activation is enhanced.

As previously shown, depletion of NE with 6-OHDA markedly reduced both the frequency of occurrence of LTP and increased population spike amplitude during LTP in the dentate (figs 8 & 14). However, when 6-OHDA depleted slices were preincubated for 30 min with the direct acting adenylate cyclase stimulant forskolin (1 μM, in 0.14 mM DMSO) prior to repetitive stimulation, LTP measured 30 min after repetitive stimulation of the perforant path was restored to normal (fig 14). This concentration of forskolin alone had no effect on population spike amplitude in the dentate of either depleted (N=8) or control (N=3) slices.

Fig. 14.   The adenylate cyclase stimulant forskolin restores LTP
           in the dentate of NE-depleted slices

           Frequency of occurrence of LTP (clear bars), and percent in-
crease in population spike amplitude (Mean ± S.E.M., hatched bars), in
the dentate 30 min after repetitive stimulation of the perforant path.
Above each clear bar is the number of slices showing LTP/ Total number
of slices tested.
           As previously shown, depletion of NE_2 with 6-OHDA markedly
reduces both the frequency of occurrence (*, χ^2, $p < 0.05$), and the
increased population spike amplitude (*, Student's t-test, $p < 0.05$), of
LTP in the dentate. However, when NE-depleted slices were preincubated
for 30 min with the adenylate cyclase stimulant forskolin (1 μM) prior
to repetitive stimulation, LTP was restored to control values. This
concentration of forskolin had no effect alone on the population spike
amplitude (not shown).
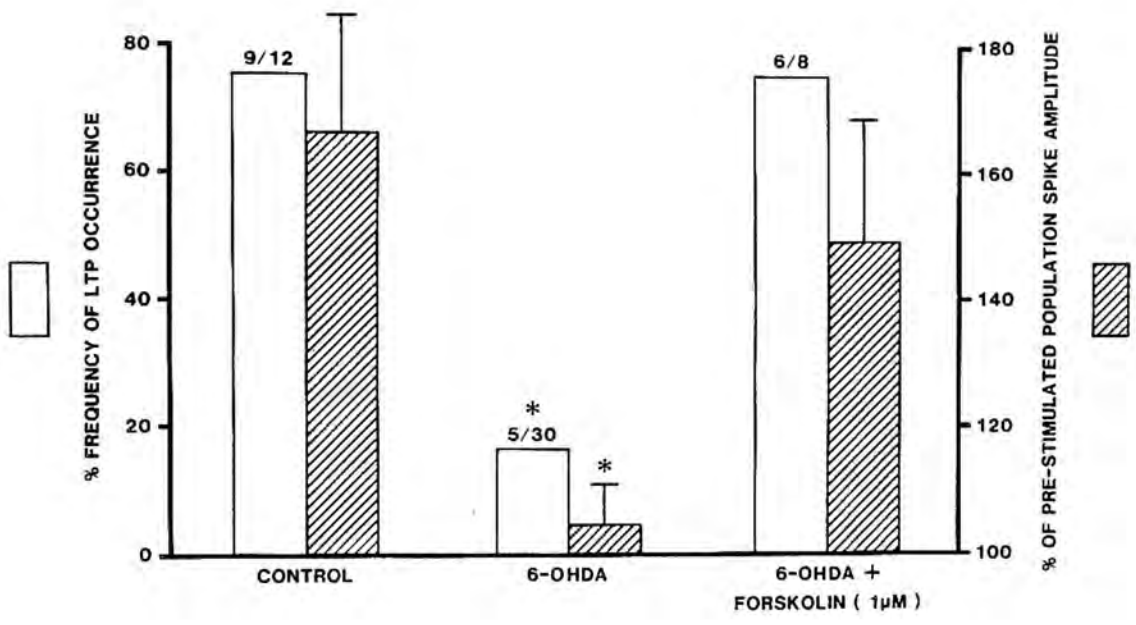
III. The pharmacology of, and role of protein synthesis in, norepinephrine-induced long-lasting potentiation

Norepinephrine produces a long-lasting potentiation specific to the dentate

Bath application of NE (50 µM, fig 15A) for 30 min potentiated the granule cell population spike evoked by perforant path stimulation. The experimental paradigm is shown in fig 2 in the dentate gyrus of the slice schematic. Potentiation appeared 5-10 min after NE reached the perfusion chamber, and increased steadily throughout the 30 min bath application. Population spike amplitude remained increased for many hours after NE was washed out. In figure 15A, the evoked population spike in the dentate is shown before (0'), after a 30 min NE application (30' NE), and after a subsequent 30 min wash period (30' WASH). An illustration of the long lasting nature of this potentiation is shown in figure 15C, where a 30 min application of NE (10 µM) produced a 200% potentiation in the dentate granule cell population spike amplitude which lasted throughout the 5 hour recording period.

In contrast, NE (50 µM, fig 15B) bath applied for 30 min produced only a slight, reversible depression of the evoked population spike in field CA1, when stimulating the Schaffer collaterals in stratum radiatum. The depression appeared 5-10 min after NE reached the perfusion chamber, and reversed in the same amount of time after the start of the wash (N=6).

Fig. 15. Effects of NE on evoked population spikes in the dentate
and field CA1

A: NE produces a long-lasting potentiation of the perforant
path evoked population spike recorded in the dentate. 0' is the response
immediately prior to bath application of NE (50 µM). 30' NE is the
response after 30 min of continuous NE application. 30' WASH is the
response after a subsequent 30 min drug-free wash. Asterisk denotes
stimulus artifact.

B: In contrast, NE (50 µM) produces only a slight reversible
depression of the Schaffer collateral evoked population spike in field
CA1. This depression appeared within 5-10 min of the start of NE appli-
cation, and the response had returned to pre-NE baseline amplitude within
5-10 min of the start of the wash. (Calibration for A and B: 1 mV, 5 msec)

C: The time course of NE-induced long-lasting potentiation of
the evoked population spike in the dentate. Potentiation was clearly
established at 30 min, continued to rise for 3 hours, and lasted through-
out the 5 hour recording period.

A FASCIA DENTATA

0'   30' NE   30' WASH

B CA1

0'   30' NE   30' WASH

C

POPULATION SPIKE AMPLITUDE (mV)

NE 10µM   WASH

TIME (min)
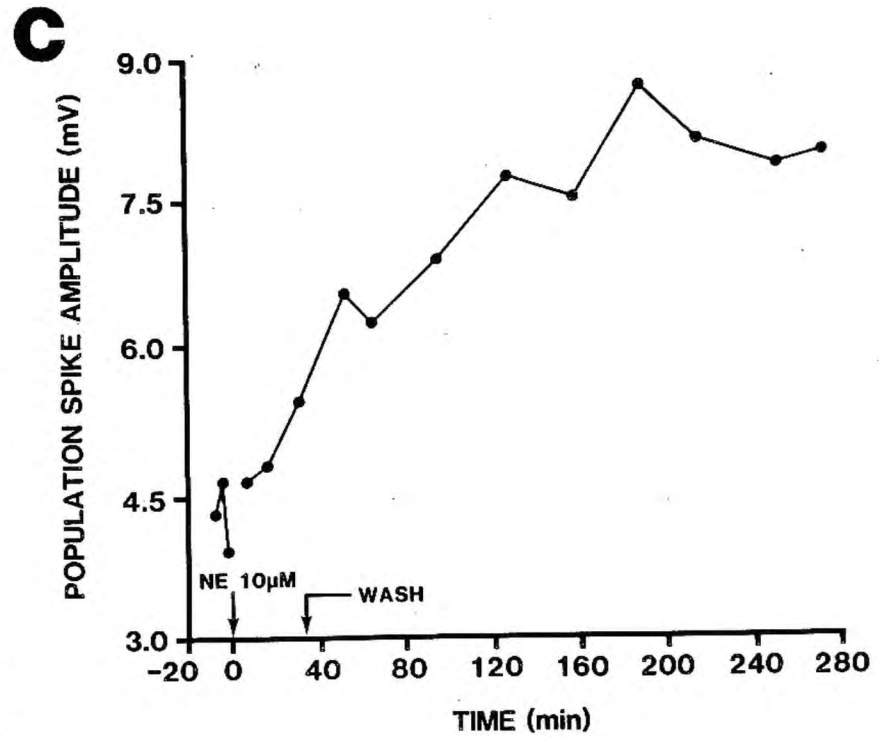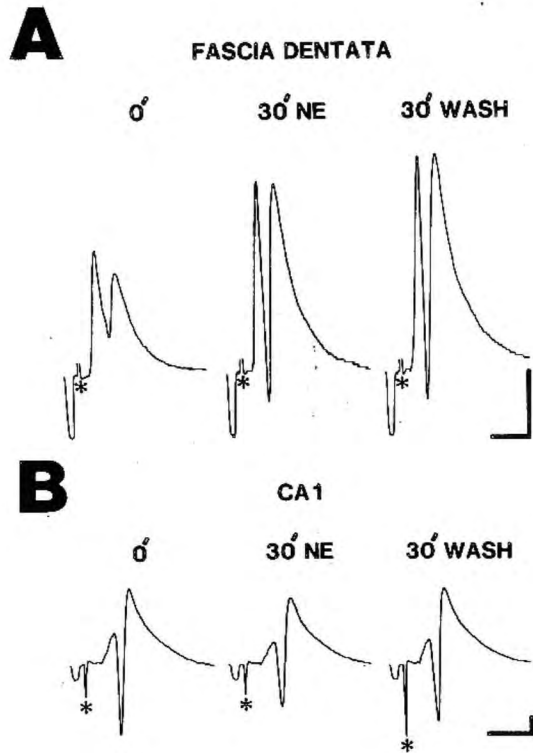
Norepinephrine also produces a long-lasting potentiation of the dendritic EPSP in the dentate

By analogy with experiments concerning the effects of deple-
tion of NE on population spike LTP vs. dendritic EPSP-LTP (pp.46-47),
experiments were performed to examine the effects of NE on the maximum
dendritic dV/dt of the EPSP recorded in stratum moleculare of the
dentate (experimental arrangement, Fig 10A). As was the case for the
population spike, bath application of NE for 30 min also potentiated
the maximum dendritic EPSP. Shown in figure 16 A and B are recordings
from a typical slice where NE (50 µM) application produced potentia-
tion of both the population spike (30'NE, Fig 16A) and the dendritic
EPSP dV/dt (30'NE, Fig 16B). In both the cell body and dendritic layers,
the potentiation was long-lasting, remaining increased after NE was
washed out (30'WASH). These experiments are summarized in figure 16C,
where the increase in population spike amplitude (clear bars), and
maximum dendritic EPSP slope (hatched bars), is shown after 30 min of
NE application (50 µM, 30'NE), and after a subsequent 30 min drug-
free wash (30'WASH). Consistent with the effects of NE depletion on
EPSP-LTP, these data do not differentiate the role of NE in somatic
population spike, or dendritic EPSP, long-term plasticity.

The protein synthesis inhibitor emetine specifically blocks NE-induced long-lasting potentiation in the dentate

In light of the data presented demonstrating that a variety
of inhibitors of protein synthesis are able to block the induction
of hippocampal LTP when protein synthesis in the slice is markedly
inhibited at the time of high-frequency repetitive stimulation (Fig 4),

Fig. 16. Effects of NE on evoked population EPSP in the dentate

A: NE-induced long-lasting potentiation in the granule cell body layer of a different slice from figure 15. 0' is the response before NE application. 30' NE is the response after 30 min of NE application (50 µM). 30' WASH is the response after the subsequent 30 min drug-free wash. Asterisk denotes stimulus artifact.

B: Simultaneous recordings show that NE also produced a long-lasting potentiation of the maximum dendritic EPSP dV/dt, both during application (30' NE), and in the wash (30' WASH). (Calibration for A and B: 1 mV, 5 msec)

C: Average percent increase in population spike amplitude (Mean ± S.E.M., clear bars), and in the maximum dendritic EPSP dV/dt (Mean ± S.E.M., hatched bars), recorded after a 30 min NE application (30' NE, 50 µM), and after a subsequent 30 min drug-free wash (30' WASH).
NE produced potentiation of the dendritic EPSP slope during NE application, and long-lasting dendritic slope potentiation, which paralleled the population spike NEP and NELLP.
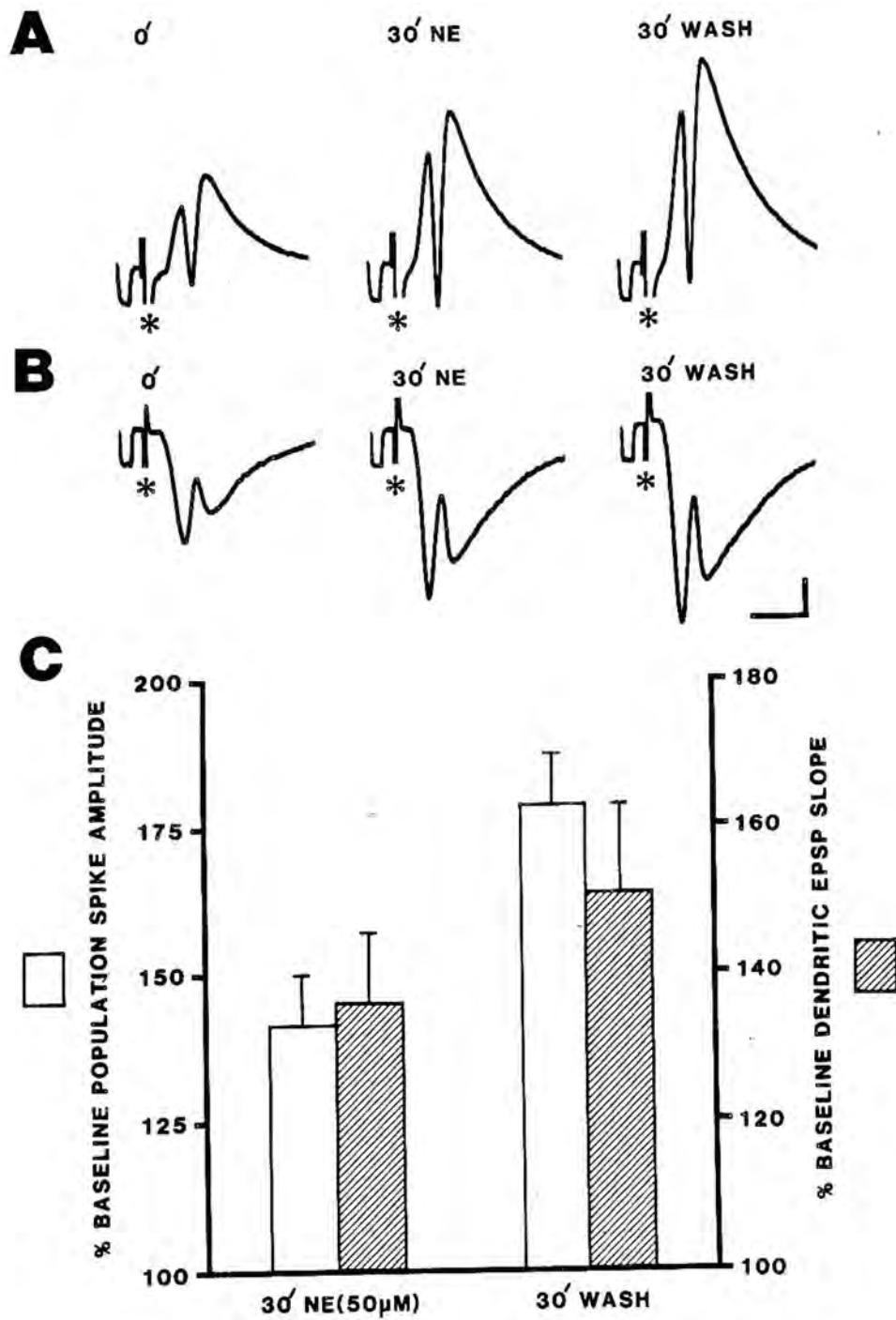
Fig. 17. Effects of the protein synthesis inhibitor emetine on
NE-induced potentiation in the dentate

Average percent increase in population spike amplitude (mean ±
S.E.M.) in the dentate after bath application of NE (50 μM) for 30 min
(NEP, clear bars), and after a subsequent 30 min drug-free wash (NELLP,
hatched bars). (N)= number of slices.

In slices where the protein synthesis inhibitor emetine (EME,
15 μM) was added to the bath 30 min before addition of NE (50 μM), and
was continuously present, two phases of NE-induced potentiation were diff-
erentiated. Only the long-lasting potentiation which persists after NE
washout was blocked by emetine (*, Student's t-test, p<0.05 compared to
control NELLP), while NEP during the application of NE was unaffected.

In contrast, in slices where EME (15 μM) was added to the bath
15 min after the start of NE (50 μM) perfusion, EME was unable to block
NELLP.
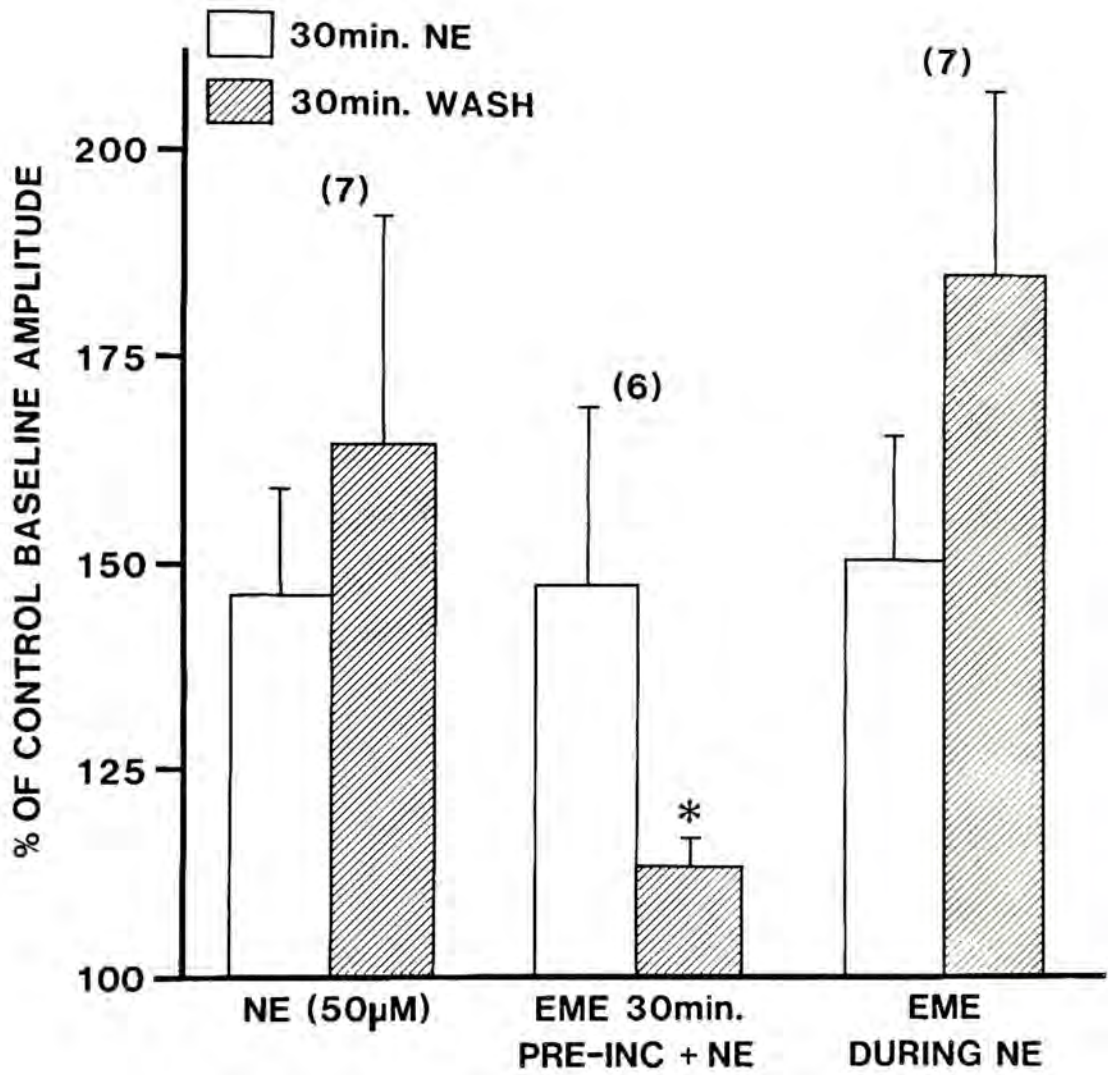
I investigated the hypothesis that protein synthesis might also be required for NE-induced potentiation in the dentate. Shown in figure 17 is data from experiments where the protein synthesis inhibitor emetine (15 μM) was bath applied to slices either 30 min before, or 15 min after the start of, application of NE (50 μM). In each case, the inhibitor was present throughout the remaining NE application, and then slices were washed in NE-free buffer containing emetine for an additional 30 min. The potentiation seen _during_ the NE application was not affected by either emetine treatment paradigm. However, NELLP measured _after_ the subsequent 30 min wash was specifically blocked by the 30 min emetine pre-incubation ($p < 0.05$, Student's t-test compared to control NE potentiation). In contrast, when emetine was added 15 min after the start of NE application, neither NEP nor NELLP was affected. Thus, pre-incubation with emetine differentiated two phases of NE-induced potentiation, an initial phase during NE application which did not require ongoing protein synthesis, and a later phase after NE washout which did. Note that this concentration of emetine virtually eliminated hippocampal LTP (Fig 4A), and inhibited [^{3}H]-valine incorporation into TCA-precipitable macromolecules in slices by >95% (Fig 4B).

The protein synthesis inhibitor anisomycin is also unable to block NELLP

Since the only protein synthesis inhibitor that was paradoxically unable to block LTP was anisomycin, it was important to test this inhibitor for its ability to impair NELLP. Anisomycin (3.8 μM) was bath applied 30 min before the application of NE (50 μM), and was present in the medium throughout NE application, after which slices were washed in NE-free buffer containing anisomycin for an additional

30 min. I have already determined that this concentration of aniso-mycin produces greater than 98% inhibition of [3H]-valine incorpora-tion into TCA-precipitable macromolecules in slices. In contrast to emetine, anisomycin did not effect either the potentiation seen during NE application (NEP), or the NE-induced long-lasting potentiation (NELLP) measured after the 30 min wash period. Taken together, the predominance of data suggests that ongoing protein synthesis is re-quired for full expression of LTP, and also for NELLP. However, both of these examples of long-lasting neuronal plasticity seem to be resis-tant to the inhibition of protein synthesis produced by anisomycin. Potentially important studies may be possible to determine what proteins are still synthesized in the presence of anisomycin, and then to focus on their interaction with LTP and NELLP.

Dose-response relations for NE-induced potentiation in the dentate

I conducted a number of experiments to characterize more fully the dose-response relation for the NE-induced potentiation (NEP) in the dentate during a 30 min application, and for the NE-induced long-lasting potentiation (NELLP) present after a 30 min drug-free wash. Figure 18 (NEP) shows the dose-response relation for NEP in the dentate, where each point is the average of 4 to 8 slices. In these experiments, the approximate NE concentration for half-maximal potentiation was 14 μM, and the calculated maximal response was approximately 160% of the control baseline spike amplitude.

Figure 18 (NELLP) shows the dose-response relation for NELLP after a 30 min drug-free wash. Again, each point is the average of the same 4 to 8 slices as in figure 18 (NEP). Here, the approximate NE con-

Fig. 18. Dose-response relations for NE-induced potentiation
          in the dentate

        NEP: The dose-response relation for NE-induced short-term
potentiation in the dentate, measured after a 30 min bath application of
NE. Each point is the mean ± S.E.M. of 4 to 8 slices.

        NELLP: The dose-response relation for NE-induced long-lasting
potentiation in the dentate, in the same slices as A, after a subsequent
30 min drug-free wash.
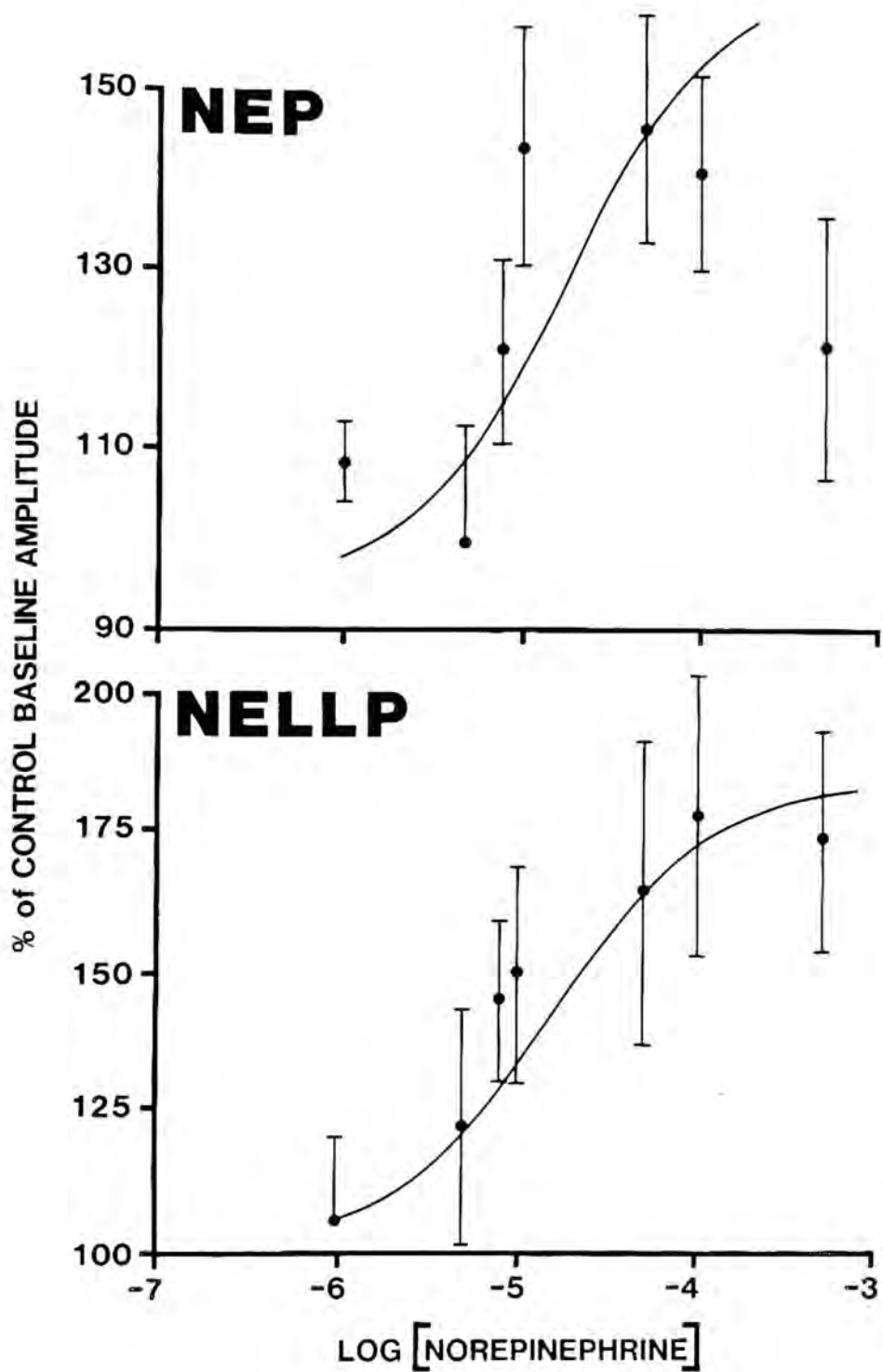
centration for half-maximal potentiation was 20 μM, and the calculated maximal response approximately 185% of control amplitude.

The adenylate cyclase stimulant forskolin shifts the NE dose-response curves to the left

Now that I had some idea of the dose-dependence of NEP and NELLP, I attempted to identify the mechanism of action of NE-induced potentiation in the dentate. Evidence has suggested that NE is able to stimulate adenylate cyclase production of 3',5'-cyclic adenosine mono-phosphate (cAMP) in hippocampal slices [Segal and Bloom, 1974], and that enhanced cAMP levels can produce long-lasting neuronal plasticity in invertebrate systems [Castellucci, et al., 1980; Camardo, et al., 1983]. Furthermore, my previous studies has strongly suggested a functional role for β_1-receptors in the production of LTP in the dentate. Recently, a unique diterpene, forskolin, has been found to directly stimulate adenylate cyclase activity at concentrations of 10-100 μM, and to potentiate the ability of other agonists to stimulate cAMP production without stimulating the cyclase itself, at a 1 μM concentration [Seamon, et al., 1981]. Therefore, I tested the ability of 1 μM forskolin, which had no effect on population spike amplitude or waveform alone, to enhance NE-induced potentiation in the dentate.

Slices were pre-incubated in forskolin for 30 min, then exposed to forskolin plus NE for 30 min, and finally washed with drug-free buffer. Figure 19 (NEP) shows the dose-response relation for NEP in the presence (open circles, dotted line), or absence (closed circles, solid line), of forskolin (N=4 to 8 slices per point). Forskolin shifted the dose-response curve to the left (half-maximal [NE]=1.6 μM,

Fig. 19. The adenylate cyclase stimulant forskolin shifts the
         NE dose-response curves to the left

NEP: The direct-acting adenylate cyclase stimulant forskolin
shifts the dose-response curve for NEP in the dentate to the left. The
solid line represents the NEP dose-response relation for bath application
of NE for 30 min (closed circles, mean ± S.E.M. of 4 to 8 slices). The
dashed line represents the NEP dose-response relation for slices that were
pre-incubated for 30 min with forskolin (1 μM), and then exposed to
forskolin + NE for 30 min (open circles, mean ± S.E.M. of 4 to 8 slices).

NELLP: Forskolin also shifts the dose-response curve to the
left for NELLP, in the same slices as in NEP, after a subsequent 30 min
wash. The solid line represents the NELLP dose-response relation for NE
alone (close circles). The dashed line represents the NELLP dose-response
relation when forskolin (open circles, 1 μM) was applied 30 min before,
and during, NE application.
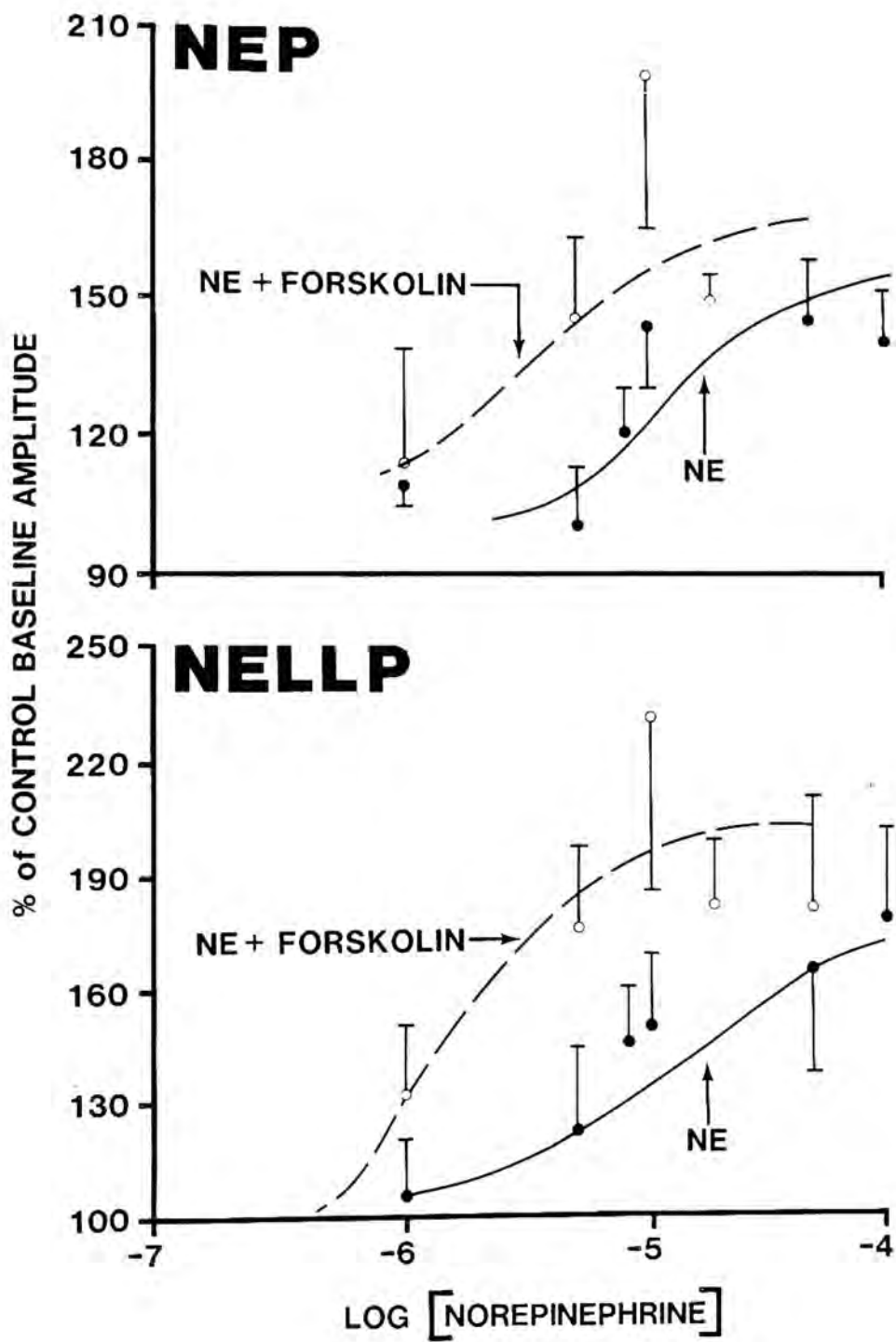
Duncan's multiple range test, p<0.05 compared to NE alone), suggesting that the adenylate cyclase stimulation produced by NE was enhanced, and that such stimulation appears to be important for NEP. Similarly, figure 19 (NELLP) shows that forskolin also shifted the dose-response curve for NELLP in these slices to the left (half-maximal [NE]=2.6 μM, Duncan's multiple range test, p<0.05 compared to NE alone), indicating that adenylate cyclase stimulation is important to both phases of NE-induced potentiation in the dentate.

The β-antagonist propranolol and β_1-antagonist metoprolol block all phases of NE-induced potentiation in the dentate

Since cAMP seemed to be involved in NE-induced potentiation, experiments were performed to test the ability of the β-antagonist propranolol, and the β_1-antagonist metoprolol, to block NE-induced potentiation in the dentate. As shown in figure 20, a 20 μM concentration of each of these antagonists was able to block completely both phases of NE-induced potentiation produced by a 50 μM concentration of NE (p<0.05 compared to control NE potentiation). Furthermore, there was no consistent effect of either propranolol or metoprolol at this concentration on the evoked spike amplitude or waveform, prior to the addition of NE. Therefore, I conclude that the long-lasting potentiation of population spike amplitude produced by NE is probably mediated by β_1-receptor stimulation of adenylate cyclase activity.

Fig. 20.   Effects of β-antagonists on NE-induced potentiation
           in the dentate

        Average percent increase in population spike amplitude (mean ±
S.E.M.) in the dentate after bath application of NE (50 μM) for 30 min
(NEP, clear bars), and after a subsequent 30 min drug-free wash (NELLP,
hatched bars). In these experiments, either the β-antagonist propranolol
(20 μM), or the β_1-antagonist metoprolol (20 μM), was bath applied
for 30 min before the application of NE, and was continuously present in
the bath for the rest of the experiment. (N)= number of slices.
        Each antagonist was effective in blocking both phases of NE-
induced potentiation (*, Student's t-test, p<0.05 compared to control NE
potentiation).
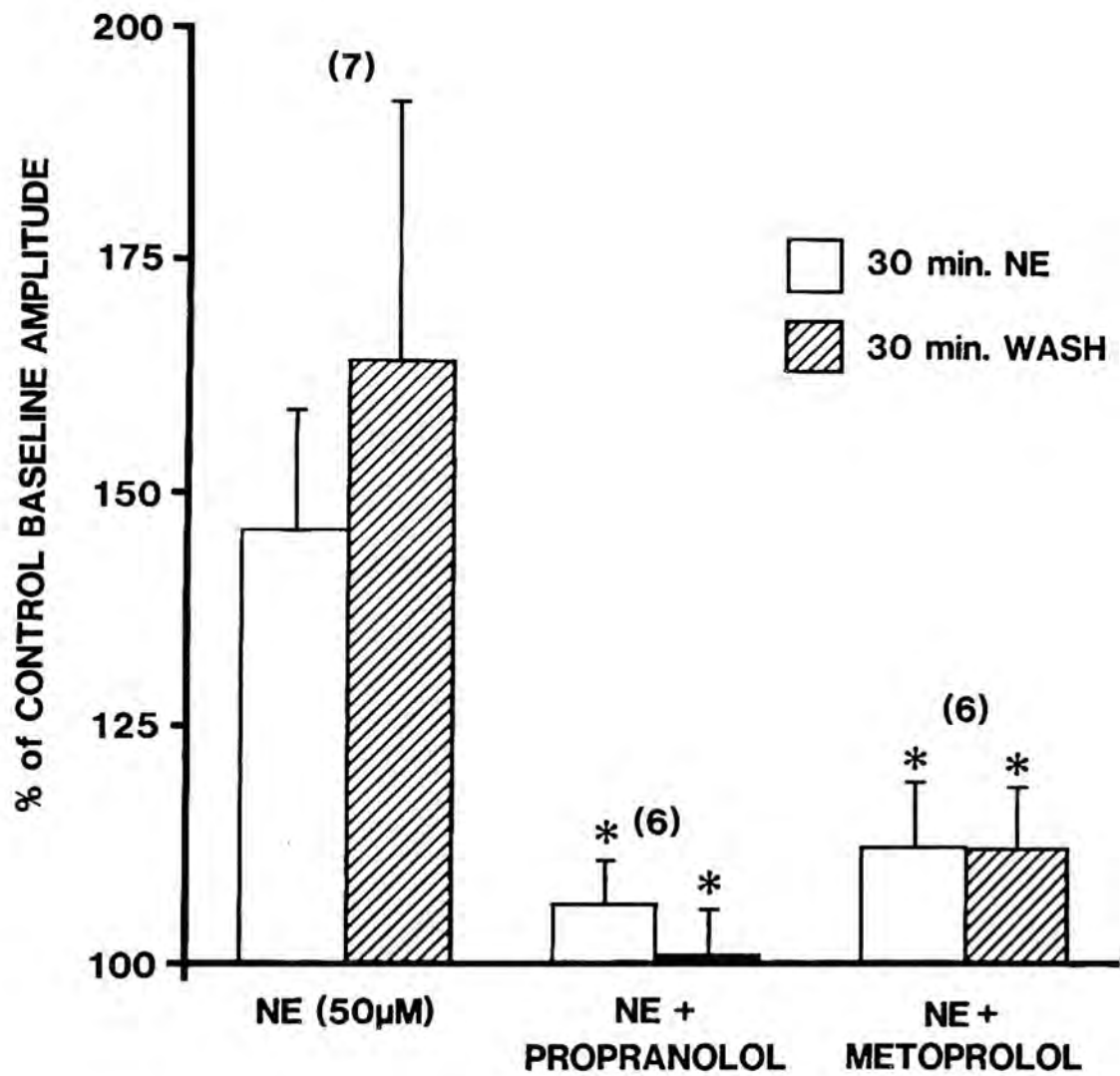
IV. <u>The effects of repetitive stimulation and NE on hippocampal</u>
    <u>concentrations of cyclic 3',5' adenosine monophosphate (cAMP)</u>

<u>Repetitive stimulation produces brief, but not long-lasting,</u>
<u>increases in cAMP levels in the dentate</u>

In view of the consistency of the previous experiments in

implicating adenylate cyclase stimulation in production of both LTP

and NELLP, I next chose to investigate the magnitude and time course

of stimulation of hippocampal cAMP concentrations produced by repeti-

tive stimulation, and by NE. Previous work in another laboratory has

shown that NE can produce 3-4 fold increases in [cAMP], in a 5-10 min

application period [Segal et al., 1981]. However, nothing was known

about the effects of prolonged application of NE, the effects of repeti-

tive stimulation on [cAMP], or the effects of depletion on NE's ability

to stimulate cAMP concentrations. These are the questions I chose to

address, by directly measuring [cAMP] in hippocampal slices.

The first set of experiments involved the effect of repetitive

stimulation of the perforant path on [cAMP] in the dentate. Slices were

prepared in the usual way, and the recording arrangement was the same

as for previous dentate experiments (Fig 2A, fascia dentata). However,

slices were placed in the chamber on a circle of filter paper (Mellita

Corp., No.1), on top of the nylon mesh. This was done to facilitate

the removal of slices for the cAMP assay, and did not affect hippo-

campal evoked potentials in any way. At either 1 min or 30 min after

repetitive stimulation of the perforant path (100Hz/2sec), the evoked

population spikes were recorded, slices were removed, and the dentate

was microdissected away from the rest of the slice on ice within 1 min.

Dentates were then frozen at -70° C with liquid nitrogen, and stored

at that temperature until assayed. The assay employed was a radio-immunoassay kit for cAMP, in which samples were first homogenized, extracted with tert-methyl butyl ether, lyophilized, and reconstituted in sodium acetate buffer. After overnight incubation with primary antibody to cAMP, separation of bound from free cAMP was achieved with a second antibody precipitation of bound antibody-cAMP complex, decanting the supernatant, and counting the pellet (for complete details, see pp 29-30).

The results of these experiments are shown in figure 21. Basal levels of cAMP in the dentate were found to be 19.0 ± 2.4 pmol/mg protein (N=6), a value which is in excellent agreement with the study of Segal and co-workers (1981). In addition, the cAMP concentrations in the dentate were 2-4 times those in the hippocampus proper (4.06 pmol/mg protein; N=2), also in agreement with these investigators.

In these experiments, the concentration of cAMP in the dentate was increased 2.5 fold 1 min after repetitive stimulation (Student's t-test, $p<0.05$ compared to basal levels). However, at 30 min post-stimulation, cAMP concentrations had returned to basal values, at a time when the average population spike LTP recorded electrophysio-logically was $183.2 \pm 26.9\%$ of pre-stimulated baseline amplitude. From these results, I conclude that, although stimulation of adenylate cyclase and production of cAMP are very likely to be necessary for full expression of LTP in the dentate, there is no sustained increase in [cAMP] required to maintain LTP. Therefore, studies into which 'second messenger' activities of cAMP are influencing LTP production should focus on effects of cAMP rises triggered within the first few minutes after repetitive stimulation.

Fig. 21. Effects of high-frequency repetitive stimulation on cyclic
3',5'-adenosine monophosphate (cAMP) levels in the dentate

cAMP concentrations (Mean ± S.E.M., pmoles/mg protein) in the
dentate of hippocampal slices in the absence of repetitive stimulation
(Basal), 1 min (1' Post-stim), and 30 min (30' Post-stim) after repetitive
stimulation of the perforant path (100 Hz/ 2 sec). [cAMP] in the dentate
was increased by 2.5 fold over basal levels at 1 min post-stimulation
(*, Student's t-test, p<0.05), but had returned to basal levels 30 min
post-stimulation, at a time when the population spike was 183.6 ± 26.9%
of pre-stimulated baseline amplitude. cAMP was measured by radioimmuno-
assay in isolated dentates microdissected away after electrophysiologic
recordings, and frozen at -70° C in liquid nitrogen.
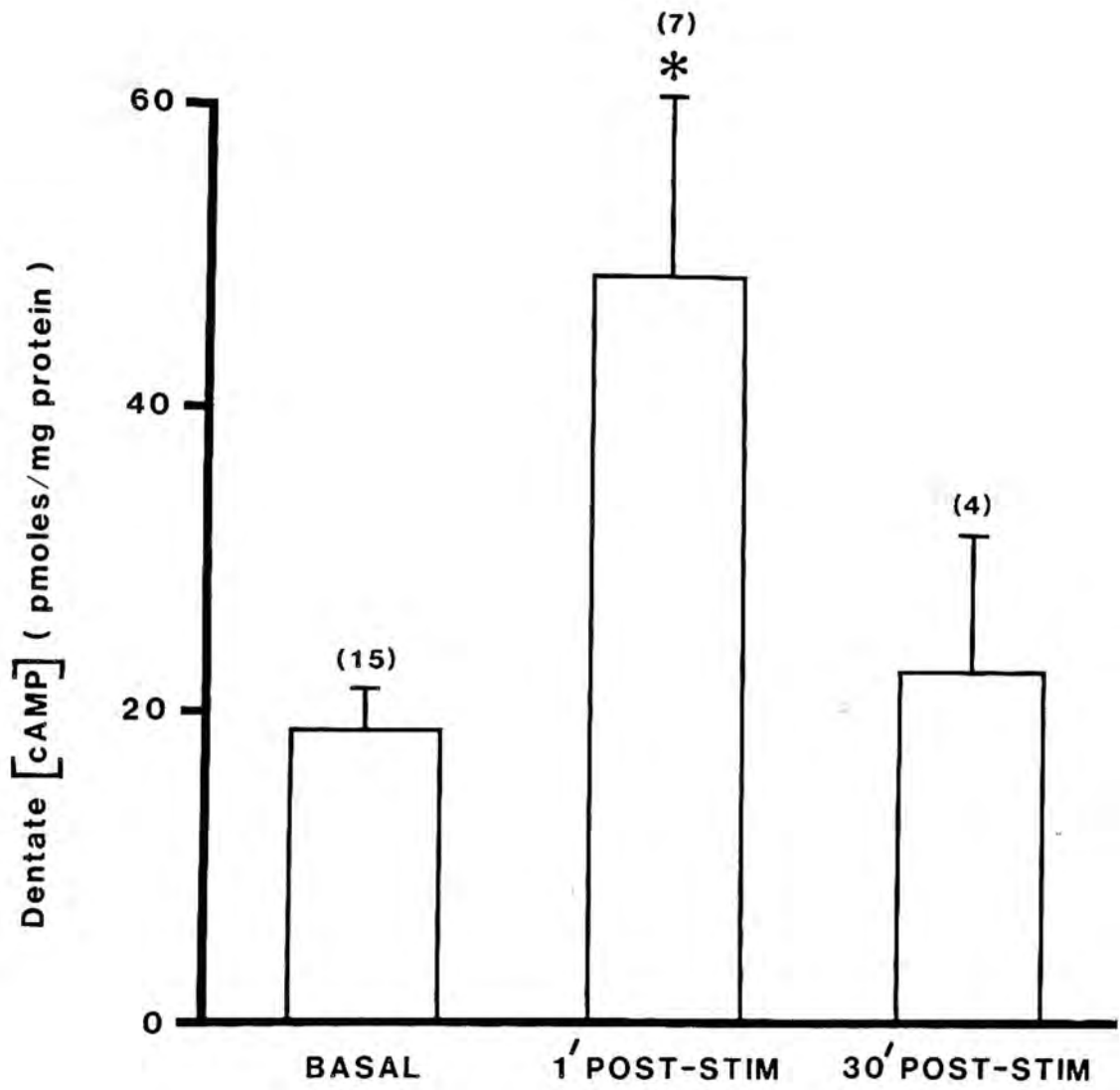
Norepinephrine stimulates cAMP production, and depletion of NE does not alter this stimulation

Norepinephrine was tested for its ability to stimulate cAMP production in hippocampal slices from control and NE-depleted animals, and the time course of stimulation characterized. Slices were prepared in the usual manner, and then incubated in beakers containing physiologic buffer for at least 1 hour before starting the experiments. This delay was similar to the usual delay before placing the slices in the chamber for electrophysiologic recording. Additionally, recent work by Wittingham and co-workers (1984) has shown that there is a large early rise in [cAMP] in hippocampal slices immediately after cutting, and that levels have returned to low basal concentrations by 1 hour after cutting. Therefore, slices were pre-incubated for 1 hour after cutting, and then transferred to beakers containing physiologic buffer plus NE (10 μM, this concentration produced the same amount of cAMP stimulation as 50 μM). After 30 min NE treatment, slices were transferred to another beaker containing drug-free buffer, and washed for 30 min. Although the longest treatment of slices was for this period, whole slices were removed at random after 1, 5, and 30 min of NE, and after the 30 min wash, and frozen at -70° C with liquid nitrogen for assay later.

Shown in figure 22 are data from experiments comparing NE-stimulated cAMP production in control (closed circles) and NE-depleted (open circles) slices treated with NE (10 μM). The basal concentration of cAMP in the control slices was 13.1 ± 1.35 pmoles/mg protein, and in the NE-depleted slices was only 8.59 ± 1.33 pmoles/mg protein (Student's t-test, $p < 0.05$ compared to control basal levels), indicating

Fig. 22. The time course of NE stimulation of cAMP accumulation in NE-depleted vs. control slices

The time course of cAMP accumulation produced by NE in control (closed circles) and NE-depleted (open circles) hippocampal slices. Each point represents the Mean ± S.E.M. (N=5, except for the 60' NE-depleted point, where N=3) of the ratio of cAMP accumulated (stimulated/basal). There was no difference in the ability of NE to stimulate cAMP production in NE-depleted vs. control slices (2-way ANOVA, p>0.05).
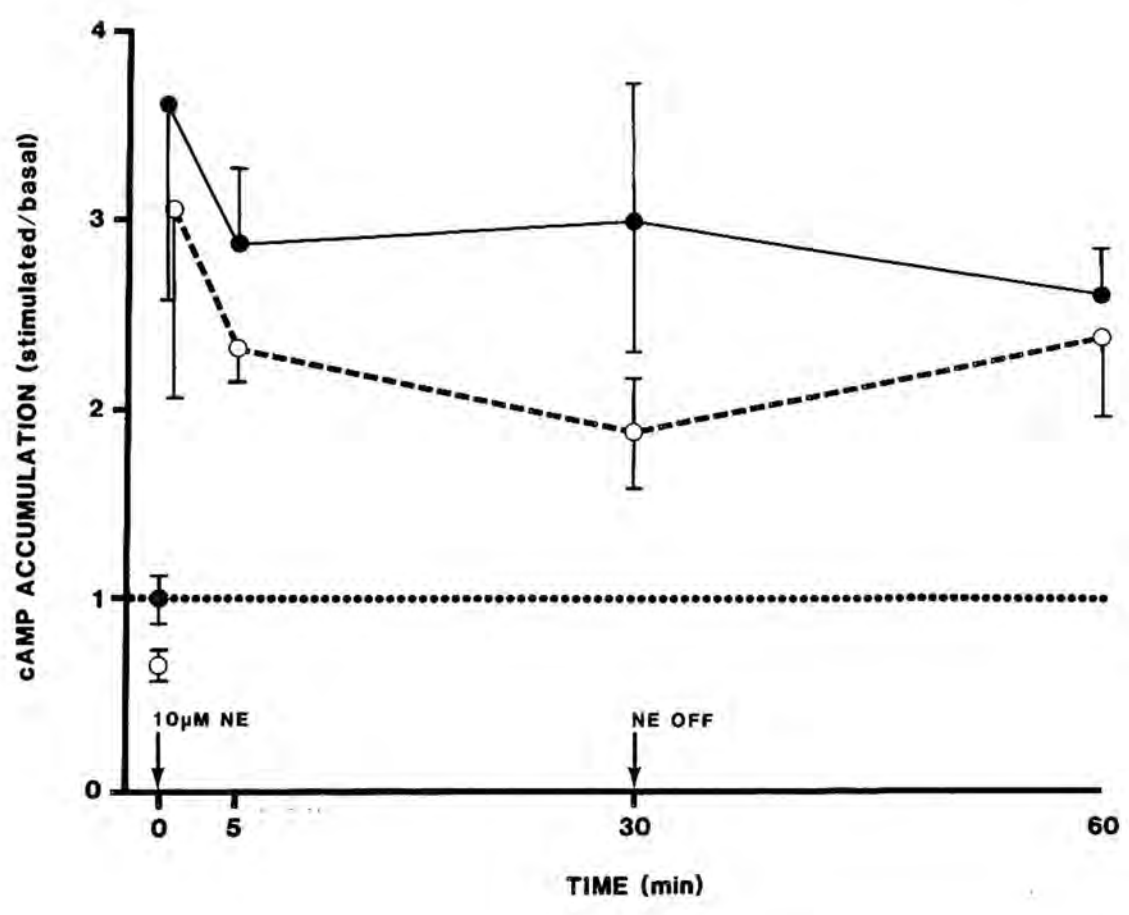
that depletion of NE did reduce the basal [cAMP] in hippocampal slices (mean depletion of NE in these slices was 75%). However, the ability of NE to stimulate cAMP production was not altered by prior NE depletion (2-way ANOVA, $p > 0.05$ compared to control stimulation). This supports the conclusion that, although depletion of NE does impair LTP and reduce basal cAMP concentrations, the ability to exhibit LTP in the presence of forskolin (Fig 14), and cAMP accumulation in response to NE (Fig 22), are unimpaired.

DISCUSSION

The preceding sections of this thesis have delineated a series of studies aimed at explaining some of the cellular mechanisms underlying two extremely long-lasting examples of plastic changes in hippocampal neuronal excitability. Implicit in the rationale for these studies has been a history of attempts to explain brain function in terms of neuronal properties, a history beginning with D.O. Hebb's Organization of Behavior (1949). Hebb postulated a set of physiologic assumptions as the basis for a theory of psychological function, and these assumptions still set the framework today for much of the research into the ways in which neuronal firing patterns may affect behavior.

In order to provide a substrate for learning, Hebb assumed that the strength of a synapse could be modified in some way by the pattern of neuronal activity supplying input to that synapse. Furthermore, he realized that the rules by which these changes took place would help define the way networks of such elements operated. Over twenty years later, Bliss and Lømo (1973) first showed that truly long-lasting changes in the strength of a synapse, as a function of activity, can occur in the adult mammalian brain. In response to brief, high-frequency bursts of repetitive stimulation of afferent fibers, the hippocampus could exhibit enhanced excitability to stimuli lasting weeks or months [Lømo, 1966; Bliss and Lømo, 1973]. This phenomenon has been labelled long-term potentiation (LTP).

The discovery of LTP, and its occurrence in the hippocampus, a brain structure implicated for many years in learning and memory [Milner, 1972], has led to continued speculation about the importance of these mechanisms to learning, and the usefulness of LTP as a model

for learning-based plasticity [Swanson, 1982].

Today, we still remain much in the dark about the answer to two extremely important and intriguing questions: 1) What, if any, relevance to the real accomplishment of learning does LTP have? and 2) What are the neurophysiologic and cellular mechanisms underlying LTP, and how do they relate to brain function? The studies presented in this thesis address only the first part of question (2), but their motivation stems from the hope that the information gleaned may have wider implications for understanding how neurons change the way they respond. Therefore, this discussion will have two goals, to critically evaluate the conclusions to which they lead us, and to attempt to integrate these conclusions in a way that will suggest future directions towards reaching Hebb's goal of understanding how neuronal mechanisms translate into brain function.

Protein Synthesis Inhibitors and LTP

Several conclusions can be drawn from the experiments performed with inhibitors of protein synthesis. (1) Emetine, cycloheximide, and puromycin, protein synthesis inhibitors with dissimilar chemical structures and sites of action, are all effective in preventing the production of LTP in CA1. (2) The dose-response relation for blockade of LTP parallels that for inhibition of incorporation of $[^3H]$-valine into TCA-precipitable proteins by these compounds. (3) There is a time lag for blockade of LTP which is similar to that necessary for inhibition of protein synthesis. (4) Blockade of LTP and inhibition of protein synthesis are either both essentially irreversible, as with emetine, or both reversible, as with cycloheximide. (5) Puromycin aminonucleoside,

an inactive analog of puromycin, is ineffective in blocking LTP.

(6) Inhibition of catecholamine synthesis is not responsible for blockade of LTP in CA1. (7) Anisomycin is a protein synthesis inhibitor which is ineffective in blocking the production of LTP.

While evidence has accumulated linking LTP with increased protein synthesis [Browning et al., 1979; Duffy et al., 1981], it was not previously known whether synthesis of proteins is necessary for LTP. The data presented suggest that functioning protein synthesis is required for LTP. However, I cannot say whether that role is in the activity-related synthesis of a set of proteins not previously expressed, an increase in the rate of production of a set of proteins already being synthesized, a requirement for a set of rapidly turned over proteins, or some combination of all three.

The dose-response relations for blockade of LTP and inhibition of incorporation of $[^3H]$-valine into proteins are correlated. Furthermore, there appears to be a requirement for very substantial inhibition of protein synthesis before production of LTP is blocked. This is interesting, considering that there is a similar requirement for the amnestic effects of these inhibitors [Barondes and Cohen, 1967; Barondes, 1970].

In addition, the requirement of a preincubation period for blockade of LTP by emetine suggests that inhibition of protein synthesis needs to be effective at or fairly soon after the repetitive stimulation. Further experiments to determine the precise time course of the blockade of LTP will be useful in identifying the kinds of proteins involved, what posttranslational processing may be occurring, and their sites of synthesis and action in supporting LTP.

Although the primary effect of these compounds is inhibition of protein synthesis, neuronal side effects other than inhibition of protein synthesis have been reported for some of these compounds. Cycloheximide and puromycin have been shown to have a direct local anesthetic effect, but at concentrations significantly higher than those used in my studies [Paggi and Toschi, 1971]. In the slice preparation, the low and intermediate concentrations of cycloheximide had no effect on population spike amplitude or waveform when present up to 2 hours. However, the highest dose of cycloheximide exhibited an ability to transiently increase, and then depress population spike amplitude. Puromycin injected intracerebrally in mice produces striking epileptiform abnormalities in hippocampal theta rhythm, but these effects occur 5 hours after injection [Cohen et al., 1966]. In the slice preparation, there is no effect of puromycin on population spike amplitude or waveform for up to 3 hours. However, in some slices followed for longer time periods, multiple evoked population spikes were sometimes observed. Given the delayed onset of these effects, I conclude that they are not important in LTP blockade.

One of the side effects exhibited by some of these protein synthesis inhibitors which has been proposed as a possible explanation for their behavioral actions is inhibition of catecholamine synthesis [Flexner and Goodman, 1975]. However, since preincubation with the irreversible tyrosine hydroxylase inhibitor α-methyl-p-tyrosine indicated that inhibition of catecholamine synthesis does not block LTP in field CA1, I eliminate this possibility.

The general consensus has been that anisomycin and emetine are relatively specific protein synthesis inhibitors lacking many of the

side effects attributed to other inhibitors [Barondes, 1970; Flood et al., 1973; Dunn, 1976]. In fact, extracellular population spike amplitudes and waveforms show no significant changes with bath application of either emetine or anisomycin, at any of the concentrations tested, for several hours.

The specificity of the blockade of LTP was demonstrated in two ways. First, even the highest concentration of emetine employed to block LTP does not alter the amplitude or waveform of the extracellular field potential. Second, the short-term example of activity-related plasticity which has been termed STP cannot be blocked by inhibition of protein synthesis. Previous studies have shown that STP is not a necessary condition for production of LTP [Misgeld et al., 1979]. It has also recently been shown that specific blockade of postsynaptic population spike firing with TTX, γ-aminobutyric acid, or pentobarbital, blocks production of LTP, without affecting the production of STP [Scharfman and Sarvey, 1985]. My results further support a sharp differentiation between STP and LTP.

The ability of three protein synthesis inhibitors with differing mechanisms of action to block LTP, the close parallel in dose-response relations for blockade and inhibition of [^{3}H]-valine incorporation, and the close parallels in time course and reversibility, all support the conclusion that these inhibitors block LTP by inhibiting protein synthesis. However, these results conflict with the inability of anisomycin to prevent LTP, although my studies indicate that anisomycin may be able to reduce the amplitude of LTP when it does occur. While I have ruled out a longer time lag in the drug's effectiveness, inactivation of the drug over time, or inhibition of valine uptake as possible

explanations for this difference, other possible explanations remain to be tested. It is important to remember that my use of $[^3H]$-valine incorporation to measure inhibition of protein synthesis is an overall measure of such synthetic rates, and may not reflect a relative resistance of a subclass of protein synthesis to anisomycin. Indeed, Steward and Levy (1982) have visualized a subclass of ribosomes localized to the dendritic spines of hippocampal neurons. Studies of the pharmacology of these uniquely placed ribosomes may prove very fruitful, and aniso-mycin resistance may prove to be a useful tool in determining what components of protein synthesis are important in the production of LTP.

A large amount of data has accumulated correlating the appearance of newly synthesized proteins in the hippocampus with acquisition of a learned behavior [Hydén and Lange, 1970; Hydén and Lange, 1983]. Also, it has been shown that the production of LTP in the hippocampus is associated with a preferential increase in the synthesis of proteins destined for secretion into the extracellular space [Duffy et al.,1981]. However, my present results supply the first evidence suggesting a necessity for protein synthesis in the production of LTP, since the inhibition of protein synthesis is correlated with inhibition of LTP. Future lines of investigation should include isolation and characterization of proteins whose synthesis is anisomycin-resistant, production of antibodies to some of the specific protein fractions affected by repetitive stimulation or training, and examination of their ability to modify the production of LTP in the hippocampus. Such data might serve both to identify the specific proteins necessary for the production of LTP, and also to strengthen the linkage between proteins important to LTP and those important in mechanisms of behavioral plasticity.

Effects of depletion of norepinephrine or serotonin on LTP

Several conclusions can be drawn from the monoamine depletion studies. (1) Depletion of NE with 6-OHDA reduces LTP frequency of occurrence, population spike amplitude increase, and increase in slope of dendritic EPSP-LTP, in the dentate, but not in field CA1. (2) In contrast, depletion of 5-HT with either 5,7-DHT or PCPA does not reduce occurrence of LTP in either area, although the average increase in population spike amplitude in the dentate is somewhat reduced by PCPA. (3) In 6-OHDA treated animals that are allowed longer lengths of time for hippocampal NE levels to recover, there is a reversal of the reduction in population spike amplitude which correlates well with the recovery of NE levels. (4) The β-receptor antagonist propranolol and β_1-antagonist metoprolol are effective blockers of LTP in the dentate, but not in field CA1. (5) The adenylate cyclase stimulant forskolin, at a concentration (1 μM) which primes the cyclase for enhanced agonist stimulation without directly stimulating cAMP production [Seamon et al., 1981], reverses the effects of depletion of NE on LTP in the dentate.

In the recent _in vivo_ study examining the effect of depletion of NE or 5-HT on LTP, it was found that depletion of either monoamine yielded significant reductions in LTP produced in the dentate by stimulation of the perforant path [Bliss et al., 1983]. In contrast, I have found that depletion of 5-HT produces little or no reduction of LTP in the dentate of hippocampal slices, but that depletion of NE virtually eliminates LTP in this area. It seems unlikely that I was simply unable to deplete hippocampal 5-HT sufficiently to observe effects on LTP, since the levels of depletion achieved were virtually identical

to those produced in the _in vivo_ study of Bliss and colleagues. The most likely explanation for this difference is that NE endogenous to the hippocampus is extremely important to the production of LTP in the dentate, but that the importance of 5-HT to LTP is at sites elsewhere in the brain. Indeed, there is a significant innervation of the locus coeruleus by neurons of the raphé nuclei [Conrad et al., 1974], and it is possible that deficits in serotonergic transmission in this area might secondarily produce alterations in noradrenergic transmission to the hippocampus _in vivo_ that cannot be operative _in vitro_. Alternatively, hippocampal 5-HT may pre-synaptically modulate tonic transmission levels of another hippocampal input, which is altered or no longer effectively modulated by 5-HT in the explanted slice.

An apparent inconsistency within my findings is the result that PCPA depletion of 5-HT significantly reduced the average increase in population spike amplitude, but did not prevent the occurrence, of LTP in the dentate, while 5,7-DHT depletion of 5-HT had no effect on either. This may be simply due to the slightly larger amount of depletion produced by PCPA. However, it may also be explained by the recent finding that PCPA-treatment reduces the affinity of NE for β-receptors, and reduces the amount of cAMP production stimulated by NE in cerebral cortex membrane preparations [Manier et al., 1984]. Any effect of 5-HT depletion is certainly much less dramatic than the effect of NE depletion on LTP in the dentate.

Although the depletion data presented here suggest an important role for NE localized in the dentate in the production of LTP, there is no clue to its mechanism of action. There is data indicating that locus coeruleus stimulation can modulate learned task performance

[Crow and Wendlandt, 1976, Mason and Iversen, 1977], and increase the synaptic efficacy of the perforant path input to dentate granule cells [Assaf et al., 1979]. In field CA1, extracellular studies have found that NE has both β-receptor mediated excitatory, and α-receptor mediated inhibitory, effects on population spike amplitude [Mueller et al., 1981]. Intracellular studies have found NE to hyperpolarize, produce a moderate conductance increase, and decrease excitability, in both CA1 pyramidal neurons [Langmøen et al., 1981], and dentate granule cells [Haas, 1984]. Many investigators have suggested that NE preferentially reduces slow synaptic events, while leaving fast depolarizations like EPSP's unaffected, as a potential way of improving the signal-to-noise ratio of input to the hippocampus [Langmøen et al., 1981; Segal, 1982]. Interestingly, recent studies have shown that NE can decrease a Ca^{2+}-dependent K^+ current (I_c) in hippocampal pyramidal cells [Madison and Nicoll, 1982; Haas and Konnerth, 1983]. This effect remains to be tested in dentate granule cells, but NE may act via such a suppression of I_c to increase the repetitive firing of granule cells responding to high frequency stimulation. The mechanism of action of NE in LTP expression in the dentate still remains to be extracted from the variety of noradrenergic receptors and effects reported in the hippocampus.

The most likely site for NE's actions is in the dense terminal layer in the dentate hilus, immediately interior to the dentate granule cell body layer. Consistent with this hypothesis is the observation that a train of stimuli, delivered to either the contra- or ipsilateral hilus just before or during a conditioning train to the perforant path, can prevent the induction of LTP [Douglas et al., 1982]. This is also

supported by our data showing that NE alone can produce a long-lasting potentiation of the evoked population spike in the dentate [Neuman and Harley, 1983; Stanton and Sarvey, 1985b], but not in field CA1 [Stanton and Sarvey, 1985b]. Since both the depletion data and NE-induced potentiation data indicate a role for NE in dendritic EPSP-LTP, as well as population spike LTP, the site or sites of changes in cellular properties produced by NE within the dentate are still unclear.

The present studies have begun to elucidate the receptor mechanisms behind NE's action in the dentate. The ability of the β-antagonist propranolol and β_1-antagonist metoprolol to reduce LTP in the dentate indicates that $\beta1$-receptor activation is probably important in LTP, and also strengthens the conclusion that NE endogenous to the hippocampus normally plays a role in the expression of LTP. This conclusion is further supported by the experiments where the ability to exhibit LTP was restored in NE-depleted animals with a low concentration of forskolin, probably acting by enhanced β-receptor mediated stimulation of adenylate cyclase. This result assures us that the neuronal mechanisms necessary for the production of LTP are intact in the 6-OHDA treated animals, but that there simply is not a sufficient concentration of NE for expression of LTP.

Forskolin has been shown to have both a direct stimulating effect on adenylate cyclase activity at high doses (100 μM), and a low dose (1-10 μM) potentiation of the ability of other agonists (such as NE) to stimulate cAMP production in a variety of systems [Seamon et al., 1981]. The concentration of forskolin used in my experiments is probably too low to directly stimulate adenylate cyclase activity, but is sufficient to prime the cyclase for enhanced stim-

ulation by the residual NE present in the depleted slices, and so to restore the ability of these slices to exhibit LTP.

The results of these depletion studies suggest separate sites of action for NE and 5-HT in the production of LTP first elucidated by Bliss and colleagues in vivo [Bliss et al., 1983]. My results support the hypothesis that NE endogenous to the hippocampus is important to expression of LTP, whereas the sites of action of 5-HT are probably remote to this structure. Furthermore, I have identified the probable involvement of β_1-receptor activation of adenylate cyclase in NE's actions. An interesting question remaining to be answered concerns what temporal contingency is required between noradrenergic activity and the conditioning train. Co-stimulation experiments with electrodes placed in the locus coeruleus or the median raphé and in the angular bundle should answer this question. Hippocampal monoaminergic systems should provide a useful tool for the study of potentiative mechanisms, and their relation to long-term neuronal plasticity.

NE-induced long-lasting potentiation and inhibitors of protein synthesis

Several conclusions can be drawn from the NE-induced long-lasting potentiation studies: (1) Norepinephrine produces a long-lasting potentiation of the perforant path evoked population spike, and the dendritic EPSP, in the dentate gyrus, but not of the Schaffer collateral evoked response in field CA1. (2) My data suggest this potentiation is produced by β_1-receptor stimulation of adenylate cyclase activity. (3) The protein synthesis inhibitor emetine blocks NELLP but not NEP, at a concentration that I have previously shown also blocks LTP and produces greater than 95% inhibition of protein synthesis in slices. (4) These results suggest that a set of newly synthesized, or rapidly turned over, proteins is necessary for persistence of NE-induced potentiation in the dentate, a result which closely parallels the blockade of LTP by protein synthesis inhibitors.

Anatomic data on NE innervation of the hippocampus fits well with the hypothesis that the site of action in NELLP is the dense terminal layer of the dentate hilus [Crutcher and Davis, 1980]. It has been shown that a train of stimuli, delivered to either the contra- or ipsilateral hilus just before or during a conditioning train to the perforant path, can prevent the induction of LTP [Douglas, et al., 1982] . Furthermore, depletion of forebrain NE specifically reduces LTP in the dentate both in vivo [Bliss, et al., 1983] and in vitro [Stanton and Sarvey, 1985a], while not affecting LTP in field CA1 [Stanton and Sarvey, 1985a]. Therefore, my data indicating that NELLP is confined to the dentate gyrus are quite consistent with effects of NE depletion and its likely site of action in modulating LTP.

I have previously shown that β_1-receptor antagonists can

block production of LTP in the dentate gyrus and that forskolin re-stored LTP in slices from NE-depleted animals [Stanton and Sarvey, 1985a]. The parallel experiments indicate that a β_1-antagonist can also block NE-induced potentiation, and that forskolin can also enhance the potency of exogenously applied NE in potentiating population responses in the dentate. These results strengthen the conclusion that β_1-receptor stimulation of adenylate cyclase plays an important role in long-term neuronal plasticity in the dentate gyrus.

Forskolin has been shown both to produce a direct stimulation of adenylate cyclase activity at high doses (100 µM) and to potentiate the ability of other agonists to stimulate cAMP production at low doses (1-10 µM) [Seamon, et al., 1981]. Since the concentration of forskolin used in my experiments (1 µM) had no effect alone on population spike amplitude, I conclude that this concentration of forskolin is probably too low to directly stimulate adenylate cyclase activity. However, forskolin's ability to shift the NE dose-response curves to the left suggests that this concentration is sufficient to prime the cyclase for enhanced NE stimulation.

Perhaps the most interesting feature of these results is the area specificity they exhibit. Experiments examining the long-term effects of NE stimulation of adenylate cyclase on biochemical parameters and membrane properties may be more likely to yield changes in the dentate, than in field CA1, since the effects of depletion and NE-induced long-lasting potentiation are localized to the dentate [Stanton and Sarvey 1985b]. In contrast, very recent work has suggested that there is a long-lasting potentiation produced by dopamine in field CA1 which may be mediated by intracellular rises in cAMP [Gribkoff and

Ashe, 1984; Gribkoff, et al., 1984; Lin-Liu, et al., 1984], while dopamine does not produce potentiation in the dentate [Stanton and Sarvey, unpublished results]. NE and dopamine may prove to be area-specific potentiators with final common mechanisms of action.

Since my experiments with LTP and NELLP in the dentate suggested similar receptor mechanisms, I felt that a logical and intriguing hypothesis was that similar kinds of protein synthesis mechanisms might be involved as well. I had already shown that protein synthesis inhibitors were effective in blocking hippocampal LTP [Stanton and Sarvey,1984], and the data presented here suggest a similar requirement for protein synthesis in the persistence of NE-induced potentiation for long periods beyond the drug application. Furthermore, since protein synthesis must be inhibited during the application of NE for blockade of NELLP, expression of newly synthesized proteins must be occurring during NE application to maintain the potentiation throughout the wash period. This is very similar to the pre-incubation period required for inhibitors of protein synthesis to block LTP. In this way, NELLP and LTP seem to have very similar time courses of requirement for functional protein synthesis.

In considering possible links between noradrenergic receptor systems and protein synthesis, there is some data from another system suggesting that NE-stimulated production of cAMP may be affected by protein synthesis inhibitors. It has been shown that the protein synthesis inhibitors cycloheximide and acetocycloheximide will prevent desensitization of cAMP production normally seen with repeated NE application in cultured glioma cells [Terasaki, et al., 1978]. It may be that locally high NE concentrations achieved in the synapse with

high-frequency repetitive stimulation can produce desensitization which may be part of the chain of events leading to long-lasting potentiation in the dentate. If so, NE-depleted slices should be less able to achieve such locally high synaptic NE concentrations. Nevertheless, it remains to be tested whether such hypothetical NE-receptor desensitization has any normal physiologic role in hippocampal potentiation.

These results should stimulate some specific investigations of common mechanisms which may underlie hippocampal LTP and NELLP. Since I have now shown a requirement for a newly synthesized, or rapidly turned over, set of proteins in both these forms of long-lasting neuronal plasticity, labelling experiments designed to isolate specific protein fractions exhibiting increased incorporation during LTP and NELLP should prove useful. In addition, previous studies concerning cAMP-dependent protein kinase-induced plasticity and possible phosphorylation susbstrates [Routtenberg, 1979; Routtenberg and Benson, 1980; Gribkoff, et al., 1984] have already suggested protein kinase-mediated phosphorylation of phosphoprotein F_1 as one possible target for hippocampal NE which should be examined during NELLP. There is a precedent in the invertebrate literature for cAMP-mediated neuronal plasticity [Castellucci, et al., 1980; Camardo, et al., 1983], and it will be interesting to see what mechanisms are preserved, and how they have been altered in vertebrate evolution.

In parallel, intracellular studies should begin to focus on long-term alterations in dentate granule cell membrane properties which might underlie NELLP, especially those that prove to require ongoing protein synthesis to occur. The question of localization of NE action within the slice, and a pre- vs. postsynaptic site of action, remain open.

Effects of high-frequency repetitive stimulation and NE on cAMP levels

Although a number of experiments remain to fully characterize the role of cAMP in long-term hippocampal plasticity, some conclusions can be drawn from my experiments measuring slice cAMP concentrations: (1) the concentration of cAMP in the dentate is increased 2.5-fold 1 min, but not 30 min, after high-frequency repetitive stimulation; (2) basal cAMP concentrations in the dentate are reduced 35% by depletion of NE; (3) NE (10 μM) stimulates slice cAMP concentrations throughout a 30-min application, and levels remain elevated after a 30 min post-NE wash; and (4) slices from NE-depleted rats show unaltered stimulation of cAMP levels by NE (10 μM).

My earlier studies have all pointed toward cAMP as a likely second messenger involved in both LTP and NELLP in the dentate. However, the most direct evidence of an effect on cAMP systems comes from actually measuring the cAMP concentration in slices, as I have done. In response to high-frequency repetitive stimulation of the perforant path, there indeed were increased cAMP levels in slices frozen 1 min after stimulation. However, these increases are more short-lived than LTP, since cAMP concentrations are returned to basal levels 30 min post-stimulation. Therefore, I conclude that a relatively short-lived cAMP spike during and/or shortly after repetitive stimulation is sufficient to support cAMP's role in LTP. This is not to say that there may not be a requirement for a number of concurrent neuronal events as well. This is clear, since one of these other requirements is for ongoing protein synthesis. Nevertheless, it should prove helpful to know that any actions of cAMP necessary for LTP are probably active shortly after repetitive stimulation.

Also of interest are the measurements of basal cAMP concentrations in the dentate of slices from NE depleted rats. Prior to these experiments, it was not clear that NE in the dentate exerted a tonic influence on cAMP concentrations. However, the significant drop in basal dentate cAMP levels with noradrenergic denervation of the hippocampus directly supports this conclusion.

NE is also able to increase cAMP concentrations in hippocampal slices. This result was first shown by Segal and co-workers (1981). These investigators and I both found 2-4 fold stimulation. However, Segal used a 100 μM concentration of NE, while I was able to produce equivalent stimulation with 10 μM or 50 μM NE. Interestingly, cAMP levels were found to remain elevated during a 30 min exposure to NE, and also through a subsequent 30 min drug-free wash. However, cAMP concentrations had plateaued at the elevated levels within 1-5 min of NE application. This suggests that β-receptors may well be desensitized by prolonged NE application.

The final set of experiments tested the ability of NE-depleted slices to exhibit NE stimulation of cAMP levels. I found that these slices were stimulated by NE just as well as control slices. This result might be considered somewhat surprising, since β-receptor up-regulation and enhanced cAMP stimulation might be expected with noradrenergic denervation. However, recent work by Dunwiddie and co-workers (1983) in hippocampal slices showed no changes in β-receptor number or affinity when NE was depleted by 73% with the neurotoxin DSP4. More recently, these investigators have achieved up-regulation of sensitivity to β-agonists when NE depletion greater than 90% was produced [Zahniser, et al., 1984]. Average depletion in my cAMP studies was 76%,

which may not have been enough to up-regulate β-receptors or alter cAMP stimulation. Nevertheless, this result supports the conclusion that the forskolin experiments with depleted slices led us to, namely, that the NE-depleted hippocampus retains intact the mechanisms for cAMP stimulation at depletion levels which reduce LTP, but that the amount of NE available is simply too low to allow expression of LTP.

Summary

The body of data presented here all serve to support some specific conclusions about the cellular mechanisms underlying long-term neuronal plasticity in the hippocampus. There is good reason to believe that protein synthetic machinery is required for one or more of the steps producing such plastic changes in excitability. However, it remains unknown which proteins are involved, whether these proteins are synthesized from pre-existing messenger RNA or new message is expressed as a result of stimulation, or whether a set of rapidly-turned over proteins cannot be replaced. This issue can be approached from two different directions. We need to know the molecular nature of the set of proteins enhanced by stimulation and NE, and we also need to know how these proteins function in the neuron to alter its responsiveness to hippocampal inputs.

Recently, I have begun pursuing an exciting and potentially useful approach to both of these problems. Preparation of monoclonal antibodies raised to cell-surface antigens on dissociated neonatal rat dentate gyrus cells has produced an antibody which is effective in blocking LTP, and eliminating LTP after it has been produced, in the dentate and field CA1 [Stanton, Sarvey, and Moskal, in review]. In

field CA1, this antibody is only effective in blocking LTP when applied to the dendritic layer [Stanton, Sarvey, and Moskal, unpublished results]. This represents the most direct proof to date for a dendritic locus for LTP production. Furthermore, it represents the promise of identifying the cell-surface antigen involved by affinity purification, and the electrophysiologic effects of this antibody on specific intracellularly-recorded membrane properties.

We also now have good reason to believe that NE will be intimately involved in long-term neuronal plasticity in the dentate gyrus. The pharmacologic similarities between NELLP and LTP are striking, and suggest a role for $\beta 1$-receptor simulation of cAMP, as well as for protein synthesis, in long-term hippocampal plasticity. This transmitter model of long-term plasticity should supply a pharmacologic way to stimulate neuronal changes, a likely modulatory input to study (the locus coeruleus), and possibly a number of behavioral effects as well.

The conclusions presented here also serve to clarify an ongoing debate in the study of hippocampal LTP. Previously, studies have indirectly suggested roles for newly synthesized proteins associated with LTP [Duffy et al., 1981], and also for changes in phosphorylation-dephosphorylation of existing neuronal phosphoproteins [Browning et al., 1979; Routtenberg, 1979]. Therefore, the literature has led to controversy over the new proteins versus phosphorylation mechanisms of LTP production. In fact, my data support the hypothesis that both types of macromolecular changes may be important to LTP production. cAMP-dependent protein kinase may well prove to modulate the function of important macromolecules by phosphorylation, and levels of some of

the phosphorylation substrates may be affected by inhibitors of protein synthesis. As suggested before, it seems likely that a number of parallel neuronal events are required, with close temporal association, for the full expression of long-term plastic changes in the hippocampus.

We are brought to an exciting threshold in the pursuit of D.O. Hebb's activity-modifiable synapse. We have some idea of cellular mechanisms which mediate a long-lasting enhancement in excitability produced by high-frequency neuronal stimulation, and of a transmitter (NE) likely to modulate it. It should prove fruitful to examine proteins being synthesized or rapidly turned turned over, cell-surface antigenic determinates, and to focus on the locations of dendritic synaptic connections. The vistas for isolating the pivotal changes in neural activity produced are good, and from there these characteristics may help to define the properties of the active elements in the larger neuronal aggregate. Pharmacologic intervention to ameliorate learning defects, and to prevent pathologic neuronal plasticity such as epilepsy, lies in the further future. Beyond that, knowledge of the cellular mechanisms of long-term neuronal plasticity may, as Hebb envisioned, help to define the ways in which information processing occurs in the brain.

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