Title
Synaptic transmission and long-term potentiation at the mossy fiber synapse of the hippocampus

Author
Weisskopf, Marc Gabriel

Publication Date
1995

Peer reviewed|Thesis/dissertation

Synaptic Transmission and Long-Term Potentiation at the Mossy Fiber Synapse of the Hippocampus

by

Marc Gabriel Weisskopf

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

To my grandfather, Viki

Who, from the beginning, instilled in me a wonder for
the universe and all the things in it

Acknowledgments

There are many people to whom I owe a great debt of gratitude for their support and help during my graduate training at UCSF. First of all, my graduate advisor, Roger Nicoll, whose friendliness, intelligence and eagerness to discuss scientific questions have been instrumental in my training. His accessibility at all stages of my work has been absolutely invaluable. In many ways Roger has created a wonderful working atmosphere within the lab, not the least of which is the people he has brought in to work together. These people have all contributed greatly to my scientific development with not only their help, but also their insights during innumerable discussions. They have made both the scientific and personal atmosphere first rate and for this I wish to thank each one of them: Massimo Scanziani, Pablo Castillo, Paul Salin, Pierre-Marie Lledo, Gang Tong, Takao Hensch, Albert Hsia, Steven Gomperts, Olivier Manzoni, David Wyllie, Toshiya Manabe, Jeff Isaacson, David Perkel, Dimitri Kullmann, and last, but most certainly not least, Bob Zalutsky, whose selfless generosity and keen wit made my transition into the lab as smooth as could be and laid the foundation for all of my doctoral work.

I would also like to thank the members of my thesis committee, Professors Peter Sargent, Lily Jan, Rob Malenka and Robert Zucker for their support during my training and helpful comments on drafts of my thesis. The other students in the Neuroscience program have also helped not only create a great learning atmosphere, but a fun one. In particular, my classmates Erik Ullian, Sophia Colamarino, Deda Gillespie and our honorary classmate, Jeff Diamond. The staff in both physiology and pharmacology have also been helpful in keeping things running smoothly, in particular Patricia Arrandale and Chris Olsen whose friendship has been a constant bright spot during my time at UCSF.

Finally, and most importantly, none of this would even have begun to be possible without my family. Their love and support through the ups and downs, the times of certainty and questioning has been a constant grounding influence. Finally, I wish especially to thank my grandmother, Ellen. Although she did not live to see me through my Ph.D., she has been with me the whole time.

Synaptic Transmission and Long-Term Potentiation at the Mossy Fiber Synapse of the Hippocampus

Marc Gabriel Weisskopf

Understanding synaptic transmission is one of the primary goals of neurophysiology. Within the central nervous system (CNS), the accessibility and simple synaptic connectivity of the Schaffer-collateral to CA1 pyramidal cell synapse of the hippocampus has made it one of the most favored sites for examining synaptic physiology. The physiology of the equally accessible hippocampal synapse between the dentate granule cell and CA3 pyramidal cell, the mossy fiber (MF) synapse, has received less attention. Since the anatomy and neurochemistry of these synapses differ dramatically from those in area CA1, these synapses offer the possibility of understanding synaptic events that may be very different. Indeed, some of the physiological properties of the MF synapse are already known to be different, notably the induction mechanism for long-term potentiation (LTP). In this study, we have used extracellular and whole cell recording techniques in the *in vitro* hippocampal slice preparation to pursue the physiology of this synapse further.

We have found that the opioid peptide dynorphin is released upon high frequency stimulation of MFs and acts with a slow time-course to depress the release of glutamate from surrounding MF terminals. This is the first evidence for a synaptic role for a peptide within the CNS and suggests that its actions in the CNS are similar to those described at some invertebrate and peripheral synapses.

We have also found that MF LTP, while independent of postsynaptic depolarization and any resultant calcium entry, is critically dependent on

presynaptic calcium entry. We provide evidence that normal glutamatergic transmission at MF synapses depends more heavily on P-type calcium channels than N-type, although the induction of LTP can occur in the absence of either subtype. Finally, we have found that MF LTP is critically dependent on the cAMP second messenger system. Enhancement of cAMP levels causes an increase in MF synaptic transmission that occludes LTP, and blockers of the cAMP dependent protein kinase (PKA) inhibit LTP. We propose that presynaptic calcium entry during a tetanus activates a calcium dependent adenylyl cyclase to elevate intraterminal cAMP levels, which acts via PKA to enhance glutamate release.

Table of Contents

Note: Abbreviations for the drugs used in this study are spelled out on pp. 35, 36

List of Figures

CHAPTER ONE

INTRODUCTION

A. General Introduction

In the late 1800's, as neurobiologists began to probe the fine structure of the brain, there was much debate over the nature of this structure: was it a syncytium or a collection of individual nerve cells? By the early 1900's, the latter view, the "neuron theory", had gained considerable acceptance although the opposing, "reticular theory" was perhaps not completely put to rest until the application of electron microscopy to the brain. The improved resolving power of the electron microscope over the light microscope was able to demonstrate individual neurons, each with their own membrane, separated at junction points by gaps tens of nanometers wide. We now refer to these gaps as "synapses", a term first coined by Charles Sherrington.

A clear implication of the neuron theory, which was recognized early on, was that neurons must communicate across the synapses. This issue engendered much debate about whether this synaptic transmission was electrical or chemical. While we now know that both are possible, the first definitive demonstration of chemical transmission was done by Otto Loewi and colleagues in the 1920's studying the depression of heart rate by stimulation of the vagus nerve. They found that the heart rate of an unstimulated preparation was reduced when bathed with Ringer's taken from a second, stimulated preparation. The active compound transferred in this procedure, the chemical neurotransmitter, was identified as acetylcholine (ACh). It was not long before ACh was determined to be a neurotransmitter at other synapses such as ganglia of the autonomic nervous system and at the neuromuscular junction (NMJ). These initial steps led to extensive examination of chemical synaptic transmission by many researchers using the cholinergic NMJ preparation in particular. By the 1960's the explosion of research into synaptic transmission had already elucidated many of the fundamental principles involved in this process (Eccles, 1964).

Much of the appeal of the NMJ and other peripheral and invertebrate synapses was, and still is, the relative ease they afford experimental recording and manipulation. The complexity and sheer number of connections within the central nervous system (CNS), and the size of the elements giving rise to them, contributed to the initial difficulty in studying its synaptic physiology. Nonetheless, the potential for understanding the functioning of the brain, as well as associated disorders, made CNS study a prime goal.

As techniques have progressed, researchers have been able to turn their attention to CNS synapses and much knowledge has been gained about their neurotransmission. One of the primary preparations used in this pursuit has been the *in vitro* hippocampal slice since it offers easy accessibility and relatively simple synaptic architecture. These aspects allow for easy manipulation of the experimental conditions, and thus the details of transmission can be probed with high resolution. In particular, the physiology of the Schaffer collateral synapse, the connection between CA3 and CA1 pyramidal cells, has been extensively studied. Within the same preparation, however, the mossy fiber synapse, the connection from dentate granule cells to CA3 pyramidal cells, has received much less experimental attention. This synapse differs in many striking ways from that of the CA1 region: as examples, it has a strikingly different synaptic bouton morphology, a high peptide content, and a form of synaptic plasticity that differs in many regards from the more extensively studied form found in area CA1. This synapse, therefore, offers an ideal opportunity to examine aspects of synaptic physiology different from those at CA1 synapses, and thus further our understanding of CNS synaptic transmission in general.

B. Opioidergic Action in the Nervous System

It has been well known for some time that morphine, a derivative of opium, is an effective analgesic compound that also has strong addictive potential that is seen as a side effect of pain management as well as after abuse by recreational users (Jaffe, 1990). As such, the discovery in 1973 of receptors that bound opiate agonists (Pert & Snyder, 1973; Simon, Hiller & Edelman, 1973; Terenius, 1973) was of great importance as it opened the door for extensive research into the mechanisms underlying these phenomena. The receptors were found by using the active and inactive forms of stereospecific opiate antagonists and examining their inhibition of opiate agonist binding in different combinations, an idea put forward by Goldstein some years earlier (see Goldstein, 1976 for review). It was not until two years later, however, that the first endogenous activators of these receptors were sequenced (Hughes, Smith, Kosterlitz, Fothergill, Morgan & Morris, 1975). These peptides, in synthesized form, dose dependently mimicked the effects of morphine in inhibiting the contraction of the smooth muscle of the gut myenteric plexus or mouse vas deferens and their effects were inhibited by the opioid receptor antagonist naloxone. Thus, met- and leu- enkephalin, the first of the opioid peptides, were discovered. Extensive subsequent research has revealed a number of different endogenous opioid peptides and receptors (Cox, 1982; Akil, Watson, Young, Lewis, Khachaturian & Walker, 1984; Hökfelt, 1991; Brownstein, 1993; Reisine & Bell, 1993).

The opioid receptors fall into three basic families: mu, delta and kappa receptors. Each one displays different affinities and selectivities for the opioid peptides in the brain, but they all fall into the seven transmembrane spanning, G protein coupled superfamily of receptors. The resultant cellular actions upon

stimulation of these receptors by their opioid agonists in the nervous system has been studied in great detail since the seventies not only in the classical gut myenteric plexus and mouse vas deferens preparations, but also the spinal cord, dorsal root ganglia and various cell lines (see North, 1986; McFadzean, 1988 for review). Activation of both the mu and delta subtypes increase potassium conductance (Williams, Egan & North, 1982; Werz & Macdonald, 1983). At the cell body, from where the recordings are made, such actions hyperpolarize the cell, which serves to reduce its firing. Much work has indicated that opiates depress neurotransmitter release (Jessell & Iversen, 1977; Starke, 1977; Mudge, Leeman & Fischbach, 1979; Konishi, Tsunoo & Otsuka, 1981) and while hyperpolarization of the cell body would effectively reduce that cell's release by reducing its firing, in many cases it would appear that the opioidergic action is not at the cell body. At the nerve terminal increased K^+ current could shorten the action potential and reduce release by indirectly reducing calcium entry (Cherubini & North, 1985). On the other hand, other evidence has shown that mu and delta receptor activation can also decrease a calcium conductance (Hescheler, Rosenthal, Trautwein & Schultz, 1987; Shimahara & Icard-Liepkalns, 1987; Schroeder, Fischbach, Zheng & McCleskey, 1991; Seward, Hammond & Henderson, 1991), which, if occurring at the terminal, would directly inhibit release. Although there has been a recent report that the activation of kappa receptors can also enhance potassium current (Grudt & Williams, 1993), extensive research has shown that the activation of kappa receptors reduces calcium current through voltage sensitive calcium channels (Werz & Macdonald, 1984; Macdonald & Werz, 1986; Gross & Macdonald, 1987). While this reduction of calcium current has no effect on resting membrane potential, it quite clearly could depress transmitter release. It has also been reported, however, that kappa receptor activation can inhibit release without affecting calcium influx into the

terminal (Kato, Chapman & Bickness, 1992), but this was done indirectly by imaging calcium fluxes with fura-2.

Opioid action in the CNS subserves a variety of different, critical physiological systems. Opioids and their receptors are found in abundance in the spinal cord, especially in the upper layers of the dorsal horn where much C-fiber innervation and hence noxious stimulus input occurs (Basbaum & Fields, 1984), and can act here to modulate pain sensations (Duggan & North, 1984; Dickenson, 1991). Sites in the brainstem as well, especially the periaqueductal gray matter and rostroventral medulla, are especially sensitive to opioids which engage descending pain control mechanisms (Basbaum & Fields, 1984; Fields, 1987). Opioid action at other brainstem sites leads to the behavioral withdrawal effects seen when the opioid (e.g. heroin) action is ceased either passively or by chasing with the antagonist naloxone (Bozarth & Wise, 1984). In the mesolimbic dopamine system, on the other hand, opioid action leads to feelings of euphoria, and are implicated in the brain's natural reward system (Wise, 1989; Koob, 1992). The cardiovascular system, as well, is greatly affected by opioids, which can have both pressor and depressor effects depending on a host of conditions including site of administration and specific receptor targeted (Akil *et al.*, 1984).

Despite all this information on the actions of opioid peptides, encompassing cellular actions at one end and behavioral effects at the other, some very fundamental questions remain elusive. Although the distributions of the peptides and their receptors are known in much detail, one confusing aspect is the mismatch between these two distributions. In many cases peptidergic receptors are detected at sites where there is no indication of the presence of fibers containing the corresponding peptide that could produce a local action at the receptor; the reverse mismatch is also common (see Herkenham, 1987 for review). That peptides can diffuse large distances and thus may not require a

precise match between receptor and peptide has been shown in experiments by Duggan and colleagues using antibody covered microprobes inserted into the spinal cord (Duggan & Hendry, 1986; Duggan, Hope, Jarrott, Schaible & Fleetwood-Walker, 1990; Duggan, Schaible, Hope & Lang, 1992). They found that peptides released by stimulation of primary afferents can diffuse throughout the spinal cord. These data have led, in part, to the idea that peptides may not act in the precise one to one mode typical of fast neurotransmitters, for example glutamate, but instead can diffuse some distance and act more as local hormones.

This highlights one critically fundamental question about peptides in general and opioid peptides in particular: what are their endogenous synaptic actions? While a wealth of information is known about the synaptic actions of various fast neurotransmitters, only two cases of endogenous synaptic actions of peptides have been demonstrated in vertebrates (Konishi *et al.*, 1981; Jan & Jan, 1982). Both of these were done in the peripheral nervous system (PNS) where the ease of access and simplicity of connections lent themselves to the analysis of these effects.

The work of L. Y. Jan and Y. N. Jan in the bullfrog sympathetic ganglion established the concept of how peptidergic transmission works. Here both B and C type cells receive cholinergic synaptic input from B and C type preganglionic fibers, respectively. The released acetylcholine leads to both a fast depolarization, mediated by nicotinic receptors, and a slow depolarization, mediated by muscarinic receptors. Stimulation of the preganglionic fibers, however, also leads to a late slow depolarization in both cell types with a time course of minutes that is not affected by cholinergic antagonists, and they found that this effect could be mimicked by exogenous leutenizing hormone releasing hormone (LHRH). Furthermore, by using a specific antagonist they were able to show that this depolarization was due to a LHRH-like peptide that is contained

in and released by the C fibers only, although, unlike the acetylcholine, high frequency stimulation is required for its release. That an effect of the endogenously released peptide can be seen in the B cells, even though they receive no direct synaptic input from the LHRH-like peptide containing fibers, established that as a neurotransmitter this peptide is able to diffuse and act at some distance from its site of release. The study of Konishi et al. (1981) on the guinea pig sympathetic ganglion was similar in nature, and demonstrated that an enkephalin-like peptide acted at presynaptic sites to reduce the release of acetylcholine.

These studies have led to a generalized concept of the action of peptides: they require high frequency stimulation for release, can act at large distances from their site of release and have effects that are much slower in time course than typical fast neurotransmitters. This concept, while generalized to the entire nervous system, is based largely on what has been seen in PNS preparations. Experiments described in chapter 3 elucidate mechanisms of peptide action in the CNS at the mossy fiber synapse.

C. Long-Term Potentiation

The persistent enhancement in synaptic responses seen after a brief train of high frequency stimulation (tetanus) to presynaptic fibers was originally described by Bliss and colleagues over two decades ago (Bliss & Gardner-Medwin, 1973; Bliss & Lømo, 1973) and is now known as long-term potentiation (LTP). Since then it has been studied extensively as it provides not only an extremely accessible model for activity dependent synaptic modifications, but also a possible cellular mechanism underlying certain forms of learning and memory (Teyler & DiScenna, 1986; Squire, 1992). By far the most extensive research has focused on LTP in area CA1 of the hippocampus, and as a result many of the properties of LTP at the Schaffer collateral-CA1 synapse (CA1 LTP) have been described and elucidated (Madison, Malenka & Nicoll, 1991; Bliss & Collingridge, 1993; Nicoll, Wyllie, Manabe & Perkel, 1993). These include associativity, cooperativity and, possibly, synapse specificity.

This CA1 form of LTP is associative in that synapses receiving a stimulus that on its own would not induce LTP, will nonetheless exhibit LTP if their input is temporally paired with a strong, independent tetanus to the same cell (Gustafsson & Wigström, 1986). Cooperativity exists because two weak independent tetani, either one of which would not induce LTP on its own, can induce LTP when given simultaneously (Lee, 1983). LTP was believed to be synapse specific because of past experiments that demonstrated that only those synapses active during tetanic stimulation exhibited LTP (Andersen, Sundberg, Sveen & Wigström, 1977). Recently, however, this notion has been challenged by Schuman and Madison (1994) who examined this question with finer resolution. By recording intracellularly from two CA1 pyramidal cells as close as 25μm apart, they were able to demonstrate that when an input to one of the cells was

paired with depolarization, the input to the second cell, although not paired, still exhibited LTP. This effect was not seen if the two cells were separated by more than 300μm.

Many studies, beginning with two in 1983 (Collingridge, Kehl & McLennan, 1983; Lynch, Larson, Kelso, Barrionuevo & Schottler, 1983), led to the implication of N-methyl-D-aspartate (NMDA) receptor activation as a necessary event and calcium as a necessary signalling molecule for CA1 LTP induction. Recent experiments employing fluorescence-activated calcium chelators have even specified the precise time window around stimulation during which the calcium is necessary (Malenka, Lancaster & Zucker, 1992). While these studies have contributed to the general belief that calcium is necessary for LTP, they are nonetheless susceptible to the argument that the calcium is simply necessary as a permissive factor for LTP. The implication that calcium is actually mediating events necessary for LTP is made more strongly by experiments using flash photolysis to raise calcium levels in CA1 pyramidal cells and demonstrate that this is sufficient to induce potentiation (Malenka, Kauer, Zucker & Nicoll, 1988), and others demonstrating that the amount of LTP obtained at a synapse onto a CA1 pyramidal cell correlates with the amount of calcium entry into that cell (Perkel, Petrozzino, Nicoll & Connor, 1993). The nature of the events mediated by calcium is still under intense experimental scrutiny, although to this point much attention has focused on the actions of a number of kinases that have been implicated, including Ca^{++}/calmodulin kinase II and protein kinase C (Malenka, Kauer, Perkel, Kelly, Nicoll & Waxham, 1989; Malinow, Schulman & Tsien, 1989), and tyrosine kinase (O'Dell, Kandel & Grant, 1991). Quite recently it has been suggested that the postsynaptic calcium influx results in the production of diffusible messengers, in particular nitric oxide (NO) or carbon monoxide (CO), that may act in retrograde fashion to mediate their effects in the presynaptic

terminal (Böhme, Bon, Stutzmann, Doble & Blanchard, 1991; O'Dell, Hawkins, Kandel & Arancio, 1991; Schuman & Madison, 1991; Haley, Wilcox & Chapman, 1992; Zhuo, Small, Kandel & Hawkins, 1993). While we have not looked at CO-synthase inhibitors, in our hands, NO-synthase inhibitors have not been effective at blocking LTP (unpublished observations), although there are reports that NO-dependent LTP is only present under certain conditions (Gribkoff & Lum-Ragan, 1992; Haley, Malen & Chapman, 1993; Williams, Li, Nayak, Errington, Murphy & Bliss, 1993) which raises the possibility that our experimental protocol is not one that gives NO-dependent LTP.

NMDA receptor activation is universally accepted as necessary for CA1 LTP. Therefore, it was a great advancement in the field when Ascher and colleagues elucidated the role of magnesium in the functioning of the NMDA receptor (Nowak, Bregestovski, Ascher, Herbet & Prochiantz, 1984). These results made it clear that the NMDA receptor provided the basis for the associativity, cooperativity and, to some degree, synapse specificity of CA1 LTP. When bound by glutamate, the NMDA receptor does not immediately pass ions because it is blocked by extracellular magnesium. This block, however, is voltage dependent so that if the postsynaptic membrane potential is depolarized sufficiently (to about -35 mV) when glutamate is present, the magnesium block is relieved and sodium and calcium can enter the cell. Thus, associativity arises when one synaptic input is sufficiently strong to depolarize the postsynaptic membrane at a second synapse so that even the small amount of glutamate provided there by a concurrent weak input is sufficient to open the NMDA channel and allow calcium to enter and trigger LTP. Similarly, cooperativity occurs when smaller depolarizations induced by each of two independent inputs sums to provide sufficient depolarization to relieve the magnesium block. The synapse specificity of CA1 LTP is provided by the NMDA receptor's dependence

on glutamate *and* depolarization: only at those synapses that were active will glutamate be present to allow the NMDA channel to open. The results of Schuman and Madison (1994) suggest that this specificity is lost somewhat, but they attribute this phenomenon to the actions of NO as a diffusible retrograde messenger, which would be a step in the cascade leading to CA1 LTP that is downstream from activation of the NMDA receptor. Thus, the initial inductive trigger would still be synapse specific even if CA1 LTP itself was less so.

The design of the NMDA receptor, therefore, makes it a coincidence detector of activity in both the pre and postsynaptic cell. As such, it is ideally suited to be a molecular mechanism underlying the theoretical modification rule proposed by Donald Hebb (1949) in a hypothesis made two decades before the discovery of its cellular manifestation (Bliss & Gardner-Medwin, 1973; Bliss & Lømo, 1973):

> *When an axon of cell A... repeatedly or persistently takes part in firing [a cell B], some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased.*

While actual firing of cell B may not be strictly required, the general idea is quite correct and has led to the term "Hebbian" to describe a synapse where such a modification rule holds. The Schaffer collateral-CA1 synapse outlined above is one such example. As far as is known, the CA3-CA3 recurrent collateral (part of the associational-commissural (assoc-com) fibers) synapse is identical to that of the Schaffer collaterals and, thus, also Hebbian (Zalutsky & Nicoll, 1990). The mossy fiber-CA3 synapse, the subject of most of the work described in chapters 3-5, however, is strikingly different.

The work of Harris and Cotman (1986) established that mossy fiber LTP is independent of the NMDA receptor. Indeed, autoradiographic studies by Monaghan and Cotman (1985) suggest that the NMDA receptor is not even present at this synapse. The hypotheses concerning a mechanism for mossy fiber LTP fall into essentially two categories. One group has proposed that the distinction between mossy fiber LTP and CA1 NMDA-dependent LTP is actually quite minimal and that the same mechanisms underlie the two types of potentiation. The major difference, they maintain, is that at the mossy fiber synapse the source of the calcium into the postsynaptic cell necessary to trigger LTP is through the voltage gated calcium channel instead of through the NMDA receptor as at synapses in area CA1 (Johnston, Williams, Jaffe & Gray, 1992). Thus, manipulations and drugs that prevent postsynaptic depolarization would interfere with mossy fiber LTP.

Much evidence, however, suggests that the differences between mossy fiber and CA1 LTP are more profound. First, two groups have found that mossy fiber LTP is associated with a change in paired-pulse facilitation (Staubli, Larson & Lynch, 1990; Zalutsky & Nicoll, 1990). Paired-pulse facilitation is the enhanced response to a second stimulus given shortly (e.g., 40 ms) after a first. It has been proposed that this facilitation is due to residual calcium in the presynaptic terminal(Katz & Miledi, 1968), and it has been shown to be modified by manipulations that alter transmitter release (for review, see Zucker, 1989). Therefore, this interaction between mossy fiber LTP and paired-pulse facilitation suggests a presynaptic modification for this form of LTP as distinguished from CA1 LTP (Manabe, Wyllie, Perkel & Nicoll, 1993). Reports using quantal analysis have been somewhat equivocal, although, in general, they corroborate this finding (Hirata, Sawada & Yamamoto, 1991; Yamamoto, Sawada & Kamiya, 1992; Xiang, Greenwood, Kairiss & Brown, 1994). Second, others have shown that

mossy fiber LTP is not associative (Kauer & Nicoll, 1988; Chattarji, Stanton & Sejnowski, 1989; Zalutsky & Nicoll, 1990). Zalutsky and Nicoll (1990) showed that doubling the stimulus strength of a tetanus that did not give LTP still fails to induce LTP, although doubling the number of stimuli in the tetanus does, demonstrating that mossy fiber LTP lacks cooperativity. Finally, Zalutsky and Nicoll (1990) took advantage of the two types of inputs onto a CA3 pyramidal cell to illustrate the differences between the two forms of LTP. At the assoc-com synapse, LTP was blocked by intracellular chelators of calcium and hyperpolarization of the postsynaptic membrane, consistent with the fact that it is formed with the same presynaptic fiber as at the Schaffer collateral-CA1 synapse. Similarly, extended dialysis of the intracellular milieu that accompanies whole cell recordings as well as extensive postsynaptic disruption caused by the fluoride ion used in the intracellular solution also prevented LTP at this synapse. In contrast, none of these manipulations or insults had any effect on mossy fiber LTP. Parts of these results have been duplicated by two other laboratories (Katsuki, Kaneko, Tajima & Satoh, 1991; Langdon, Johnson & Barrionuevo, 1993), and together they not only suggest a very different mechanism for mossy fiber LTP, but also suggest that it is occurring presynaptically. The chapters that follow address what this mechanism might be.

D. Cyclic-AMP and Synaptic Enhancement in the Nervous System

In the mid 1950s Sutherland and colleagues elucidated the mechanism of action of norepinephrine in producing glycogenolysis in liver cells that led to their proposal of the generalized scheme of signalling that they termed the second messenger system (Sutherland, 1972). The particular second messenger that they discovered at that time, later isolated by Lipkin and colleagues (Lipkin, Cook & Markham, 1959), was 3',5'-adenosine-monophosphate, or cyclic-AMP (cAMP). In the intervening years, second messenger systems in general and cAMP in particular have been implicated in myriad effects throughout the body. In particular, the nervous system makes widespread use of cAMP including some preparations where it has been implicated in enhancement of synaptic transmission.

The most extensively studied example of such an enhancement is sensitization of the gill withdrawal reflex in Aplysia. In this behavior, noxious stimulation of the tail results in a defensive withdrawal of the gill when the siphon is touched. Persistent noxious stimulation of the tail results in the release of serotonin from a modulatory neuron onto a siphon sensory neuron presynaptic terminal. This serotonin acts at a receptor to cause a facilitation of transmission between the siphon sensory neuron and the motor neuron responsible for the gill withdrawal by enhancing transmitter release (see Byrne, Zwartjes, Homayouni, Critz & Eskin, 1993; Hawkins, Kandel & Siegelbaum, 1993 for review). The action of serotonin is inhibited by blockers of cAMP dependent protein kinase (PKA), and mimicked by cAMP (Castellucci, Nairn, Greengard, Schwartz & Kandel, 1982; Siegelbaum, Camardo & Kandel, 1982). A K^+ channel sensitive to serotonin (S channel) has been demonstrated by Siegelbaum et al. (1982) that is also activated by intracellularly injected cAMP. Moreover, Shuster

et al. (Shuster, Camardo, Siegelbaum & Kandel, 1985), using excised patches, showed that the activity of these S channels could be reduced by exposing them to the catalytic subunit of PKA.

These data have led to the proposal that synaptically released serotonin, acting via PKA, reduces the S-type K^+ current thus prolonging the action potential and causing enhanced release (Hawkins *et al.*, 1993). This hypothesis, however, is not considered by all to be the complete story. There are data that raise objections to the simplicity of this hypothesis including some that suggest that other factors, such as protein kinase C, are also involved (Byrne *et al.*, 1993). Furthermore, recent experiments, in which voltage clamp of the sensory neuron terminal was achieved, suggest that an effect of serotonin on action potential duration can only minimally affect postsynaptic responses and that the main effect, although still via cAMP, must be on the release process itself (Klein, 1994). Thus, although the issue is not completely settled, there is clear evidence in this system that the cAMP cascade can cause presynaptic enhancement of transmission.

In this Aplysia tail shock preparation, the pattern of noxious stimulation can determine the duration of the synaptic enhancement. While any duration of enhancement appears dependent on cAMP, the longer forms may result from a distinct molecular change (Byrne *et al.*, 1993; Hawkins *et al.*, 1993). For example, classical conditioning can occur in Aplysia and indeed, in this case, the long-term enhancement is produced much more rapidly than with sensitization. The classical conditioning protocol involves stimulation that results in the temporal pairing of serotonin action at the siphon sensory neuron terminal (the unconditioned stimulus, US), with action potential induced calcium entry into the same terminal (the conditioned stimulus, CS). The former is induced by noxious stimulation of the tail, and the latter by touching the siphon. This

classical conditioning has several features in common with that seen in mammals including strict temporal correlation between the CS and US. It has been suggested that the effects of classical conditioning, including the temporal requirements of the CS and US, are mediated by an adenylyl cyclase sensitive to both calcium and G_s which detects the convergence of the stimuli (Abrams, Karl & Kandel, 1991). Thus the cyclase would serve not just as a necessary step to increased cAMP levels, but as a key point of regulation of the cAMP mediated synaptic enhancement.

The long duration of the enhancement produced by some patterns of stimulation requires protein synthesis (Montarolo, Goelet, Castellucci, Morgan, Kandel & Schacher, 1986; Schacher, Castellucci & Kandel, 1988; Castellucci, Blumenfeld, Goelet & Kandel, 1989) as well as activation of the nuclear cAMP response element (Dash, Hochner & Kandel, 1990). Furthermore, established enhancement is reversed by blockers of PKA (Castellucci *et al.*, 1982), implying that persistent PKA activity is responsible for the long-term enhancement. Thus, it has been proposed that the late phase of this enhancement results from gene expression changes to the PKA catalytic subunit that render it more susceptible to cAMP (Kandel & Schwartz, 1982). In area CA1 of the hippocampus it has also been demonstrated that blockers of PKA applied during the tetanic stimulation reduce a late form of enhancement seen a few hours after tetanus, as do blockers of protein synthesis (Frey, Krug, Reymann & Matthies, 1988; Frey, Huang & Kandel, 1993; Matthies & Reymann, 1993; Huang & Kandel, 1994). There is also one report that blockers of PKA affect traditional, early LTP (≤ 1hr) in area CA1 (Musgrave, Ballyk & Goh, 1993), although this is in contradiction with our results (see chapter 5, Fig. 30).

In the bullfrog sympathetic ganglia Kuba and Kumamoto (1986) have shown that adrenaline causes an enhancement of synaptic transmission. Since

adrenaline produces cAMP through activation of an adenylyl cyclase, they tested whether this was responsible for the enhancement. Indeed, the adrenaline-induced enhancement was mimicked by cAMP analogs, and cholera toxin, which irreversibly ADP ribosylates G_s thereby activating adenylyl cyclase. The enhancement also can be seen in the presence of the phosphodiesterase (PDE) inhibitors 3-isobutyl-1-methylxnthine (IBMX) and caffeine, presumably due to their blockade of endogenous cAMP breakdown. There is also a potentiation seen in this system after tetanic stimulation of the preganglionic fibers, raising the possibility that cAMP is the mediator of this use-dependent enhancement. Arguments against this possibility are that tetanus-induced potentiation is dependent on presynaptic calcium entry and is enhanced at lower temperatures (Koyano, Kuba & Minota, 1985), both of which are not the case for adrenaline-induced enhancement. Also, tetanus induced potentiation was not blocked by staurosporine (Minota, Kumamoto, Kitakoga & Kuba, 1991), an inhibitor of PKA. Minota et al. (1991) did not, however, show that staurosporine could block the adrenaline-induced enhancement, and thus they had no positive control to show that the staurosporine was effective. As for the dependence on calcium, the adenylyl cyclase that is sensitive to both calcium and G_s (Tang, Krupinski & Gilman, 1991) could explain the difference: a tetanus would use calcium to activate the cyclase and the cAMP cascade whereas adrenaline would use G_s. Thus a role for the cAMP cascade in bullfrog sympathetic ganglia LTP remains a possibility.

A cAMP cascade has also been implicated in long-term facilitation (LTF) at the crayfish neuromuscular junction (Dixon & Atwood, 1989). Both forskolin and IBMX cause an enhancement of synaptic transmission, as does cholera toxin, thus implicating adenylyl cyclase and increased cAMP in the synaptic enhancement. While it was not tested whether this cAMP induced enhancement is pre- or

postsynaptic, quantal analysis of tetanus induced LTF revealed that it occurs presynaptically (Baxter, Bittner & Brown, 1985; Wojtowicz, Parnas, Parnas & Atwood, 1988). That this would also be the case for the cAMP induced enhancement, indeed that the cAMP cascade is the mechanism responsible for the tetanus induced LTF was suggested in elegant experiments by Dixon and Atwood (1989) who showed that inhibitors of PKA and adenylyl cyclase specifically block LTF induced by tetanic stimulation of the excitor axon. The enhancing effects of forskolin alone at this synapse, however, are transient, suggesting that perhaps some signal in addition to cAMP is required for truly *long-term* facilitation. Intriguingly, the long lasting phase of LTF at the crayfish neuromuscular junction has been shown to develop even in the absence of calcium and sodium ions during the tetanus (Wojtowicz & Atwood, 1988; Wojtowicz *et al.*, 1988). This is a critical distinction between LTF at the crayfish neuromuscular junction and LTP in the hippocampus. As discussed in chapter 1, section C, LTP in area CA1 depends critically on postsynaptic calcium; at the mossy fiber synapse, although postsynaptic calcium is not necessary, removing extracellular calcium during a tetanus prevents LTP as shown by Ito and Sugiyama (1991) and ourselves in chapter 4.

Overall, there is strong precedent for cAMP mediated enhancement of neurotransmission at a variety of synapses. The many similarities between the mossy fiber system of the hippocampus and some invertebrate and peripheral preparations, including its peptide content as well as the presynaptic nature of its LTP, invite intriguing speculations concerning mossy fiber LTP and the cAMP cascade. Aside from the proposed involvement in the late stage of enhancement in area CA1 of the hippocampus mentioned above, however, there is to date no evidence for a cAMP mediated LTP within the mammalian CNS.

E. Anatomy of the CA3 Region of the Hippocampus

The axons of the dentate granule cells of the hippocampus are referred to as mossy fibers and were first described in the late 1800's by Golgi. They have been subsequently studied extensively by many others (see Amaral & Witter, 1989 for review) including much early work by Ramon y Cajal, who named them mossy fibers because of their similar appearance to the mossy fibers of the cerebellum. Their trajectory is predominantly in the transverse direction across the hippocampus in a band that courses through the entire CA3 region of the hippocampus proper extending to the border of area CA1 (see Blackstad, Brink, Hem & Jeune, 1970; Gaarskjaer, 1978; Gaarskjaer, 1978; Gaarskjaer, 1986 for review). At this stage, termed the end bulb, the fibers then turn and extend some distance in the longitudinal direction out of the plane of a transverse slice such as is used for *in vitro* electrophysiological recordings. The transverse band is known as *stratum (s.) lucidum* and primarily occupies a region on the apical side and immediately adjacent to the CA3 pyramidal cell body, although a minor band can extend a short distance from the dentate gyrus on the basilar side of the CA3 pyramidal cell layer in *s. oriens* (see Fig. 1 and 2).

Upon leaving the dentate granule cell, the mossy fiber branches extensively within the hilus before sending a primary axon into *s. lucidum.* Typically 5 to 12 primary collaterals can be seen and these can then branch further into secondary and tertiary collaterals all contained within the hilus although sometimes crossing its entire extent (Claiborne, Amaral & Cowan, 1986). Amaral and colleagues demonstrated that while the collaterals do extend into the granule cell layer, this is rare and they do not enter the molecular layer of the dentate gyrus under normal conditions. These collaterals have many small varicosities that make *en passant* asymmetric excitatory synapses onto dendritic

FIGURE 1. Drawing of the anatomy of the hippocampal transverse slice used for in vitro recordings.

In the dentate gyrus, perforant path (pp) fibers cross the hippocampal fissure (hf) and enter the hippocampus where they synapse on the dendrites of the granule cells in stratum (s.) moleculare (a proportion also contact the distal dendrites of both CA1 and CA3 cells). The granule cells occupy s. granulosum and send their axons (mossy fibers; mf) through the hilus to area CA3. The basilar dendrites of the CA3 pyramidal cells occupy s. oriens, while the cell bodies are in s. pyramidale. The apical dendrites extend first into a proximal region termed s. lucidum which is the termination zone for the mossy fibers, and then into s. radiatum and s. moleculare in that order. The axons of the CA3 pyramidal cells either leave the hippocampus via the fimbria, contact the CA1 pyramidal cells as the Schaffer collaterals (sch), or send recurrent collaterals (the associational-commissural fibers; a/c) back onto CA3 cells synapsing in s. oriens and s. radiatum. The order within the CA1 region is essentially the same except that there is no region equivalent to s. lucidum and the apical dendrites extend immediately into *s. radiatum*. The axons leaving the pyramidal cells (ax) travel to the subiculum in the white matter layer termed the alveus. Adapted from Madison (1983).

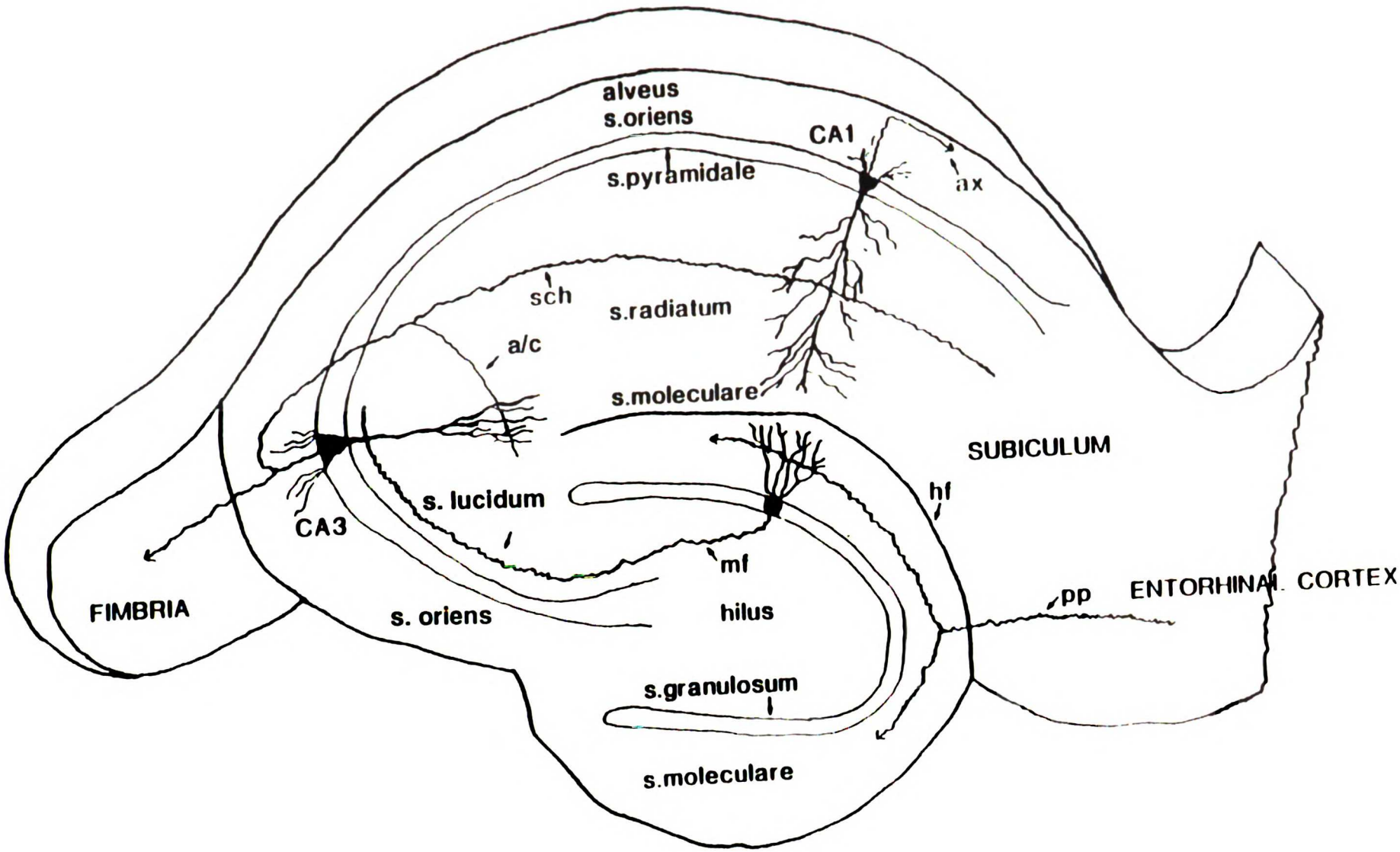
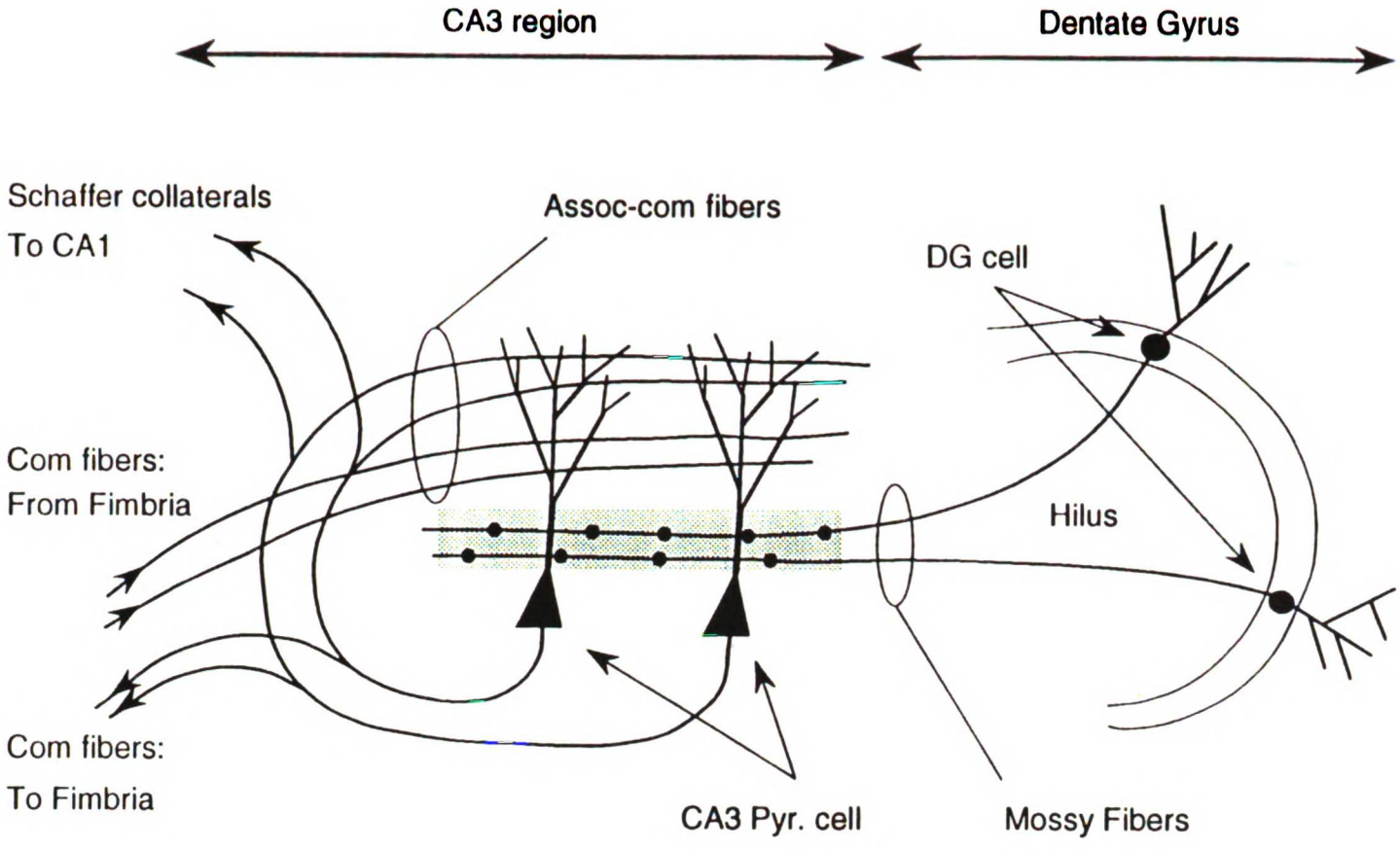

alveus
s.oriens
CA1
s.pyramidale
ax
sch
s.radiatum
a/c
s.moleculare
SUBICULUM
s. lucidum
hf
CA3
mf
pp
ENTORHINAL CORTEX
FIMBRIA
s. oriens
hilus
s.granulosum
s.moleculare

FIGURE 2. Schematic of the mossy fiber and associational-commissural connections of the hippocampus.

The axons of the dentate granule (DG) cells are called mossy fibers, and they synapse onto the proximal apical dendrite region of CA3 pyramidal (Pyr.) cells (indicated by shading). They are the only synapses made onto this portion of the dendrites. The associational-commissural (Assoc-com) fibers originate from CA3 pyramidal cells ipsilaterally or contralaterally (Com fibers). These fibers form synapses either in s. oriens (see Fig. 1) onto the basal dendrites of CA3 pyramidal cells or in s. radiatum (see Fig. 1) onto apical dendrites that are distal to s. lucidum. The axons of CA3 pyramidal cells also send a collateral to synapse onto cells in area CA1 (Schaffer collaterals).



= *S. Lucidum*: region of uniquely mossy fiber inputs to CA3 pyr. cells

shafts of local interneurons, possibly including basket cells. Less common and usually at the end of the collaterals are larger swellings, similar to what is seen in the CA3 region only smaller, that seem to contact the thorny excrescences of mossy cells of the hilus.

The parent mossy fibers, as they pass through *s. lucidum*, give rise to synaptic expansions that form large terminal boutons of about 2-10μm diameter and engulf the thorny excrescences of the proximal apical dendrite of the CA3 pyramidal cells to form calycal-like synapses (Hamlyn, 1961). An axon of any one dentate granule cell has been estimated to have about 14 such synaptic expansions along its transverse trajectory in *s. lucidum* and most likely somewhat more along its longitudinal extent beyond the end bulb (Claiborne *et al.*, 1986). These authors point out that this would suggest a very low interconnectivity between dentate granule cells and CA3 pyramidal cells compared, for example, to CA3-CA3 interconnectivity. Furthermore, the convergence is rather high since there are 0.8-1 x 10^6 granule cells and only about 160,000 CA3 pyramidal cells in the rat hippocampus.

The mossy fibers are unmyelinated axons and this, along with a small diameter, results in extremely slow conduction velocities. The parent mossy fiber is about 1μm in diameter and the hilar collaterals are closer to 0.2μm (Claiborne *et al.*, 1986). The drastic change in diameter from a 1μm axon to the huge terminal bouton has been proposed to lead to terminal invasion delays and even complete failures that may be the source of observed asynchrony in the mossy fiber input to CA3 pyramidal cells (Langdon, Johnson & Barrionuevo, 1993). Aside from being unmyelinated and having huge terminal boutons, the mossy fibers are distinguished in the hippocampus because of their high concentration of zinc, which leads to their being dramatically labeled with the Timm stain, and of enkephalin, dynorphin and their precursors (Gall, Brecha,

Karten & Chang, 1981; McGinty, Henriksen, Goldstein, Terenius & Bloom, 1983; McLean, Rothman, Jacobson, Rice & Herkenham, 1987). The transmitter mediating fast excitatory transmission at the mossy fiber-CA3 synapse, however, appears to be glutamate based on a number of pieces of evidence: its presence and release upon stimulation, the presence of high affinity uptake sites, (Crawford & Connor, 1973; Storm-Mathisen, Leknes, Bore, Vaaland, Edminson, Haug & Ottersen, 1983), the action of exogenous glutamate on CA3 cells (see chapters 3 and 5, Figs. 9 and 27, and Sawada & Yamamoto, 1984), and, most importantly, the potent block of synaptic transmission by the glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX; see Fig. 3). Under normal conditions in the *in vitro* slice preparation, the NMDA receptor antagonist 2-amino-pentovaleric acid (APV) has no effect on synaptic transmission (Harris & Cotman, 1986). It has also been shown, using immunocytochemical techniques, that there is, at best, a low level of NMDA receptor binding in *s. lucidum* (Monaghan & Cotman, 1985). While these data are far from conclusive, they have nonetheless led to the general assumption that fast excitatory transmission at the mossy fiber synapse is mediated entirely by glutamate receptors of the non-NMDA type. Although there has been no conclusive evidence to the contrary, one study has suggested that an NMDA component to mossy fiber synaptic responses can be detected electrophysiologically (Isaacson & Nicoll, 1990).

The recipient CA3 pyramidal cells are the source of the Schaffer collateral projection to the pyramidal cells of area CA1 of the hippocampus as well as the assoc-com recurrent projection back onto the pyramidal cells of area CA3. Both of these projections contain myelinated fibers (Ishizuka, Weber & Amaral, 1990), and therefore, even though their diameters are roughly equivalent to those of mossy fibers (Ishizuka *et al.*, 1990), the conduction velocity of Schaffer and assoc-

com projections far exceeds that of the mossy fibers. The recurrent collaterals synapse on the basal dendrites of the CA3 cells in *s. oriens* and on the apical dendrites distal to *s. lucidum* in the region termed *s. radiatum* . They do not appear to synapse on the most distal dendrites of the CA3 cells in *s. lacunosum-moleculare,* which instead receives input from the perforant path. As far as is known, and consistent with the fact that the two projections arise from the same cell, the assoc-com synapses are identical to those of the Schaffer collaterals in CA1. The presence of these recurrent collaterals in area CA3, however, raise problems when doing electrophysiological recordings in this area. If synaptic activity onto a CA3 pyramidal cell results in that cell firing an action potential, that action potential will result in synaptic excitation of other CA3 pyramidal cells increasing the likelihood of firing an action potentials in those cells, which would lead to this cycle repeating itself. Thus the possibility exists for epileptiform activity if this cycle of firing is not kept in check. Under normal conditions inhibitory synaptic action prevents such activity (Miles & Wong, 1987), but it is therefore extremely difficult to use inhibitory antagonists when studying this system. Not as dramatic but equally troubling is the possibility of recording disynaptic inputs in CA3 pyramidal cells when stimulating the dentate granule cells of *s. granulosum* (see Figs. 1 and 2), a problem compounded by inhibitory antagonists (Miles & Wong, 1987). While in the absence of such antagonists disynaptic inputs are unlikely (Langdon *et al.*, 1993), the anatomy of this region, as described above, makes the identification of monosynaptic inputs very difficult. The asynchrony of mossy fiber inputs and the drastic difference in conduction velocities between mossy fibers and assoc-com fibers preclude the use of such standard procedures for determining monosynapticity as frequency following and latency measurements (see Sah & Nicoll, 1991). For this reason a large part of the work described herein was done using extracellular field

recordings. A negative waveform recorded extracellularly indicates current flow into cells in the region of the electrode, a current sink. Since the mossy fiber synapses are the only excitatory input to CA3 pyramidal cells in the region of *s. lucidum* (see Fig. 2), a negative waveform detected by a recording electrode placed in this region indicates that these synapses are active. When stimulating dentate granule cells, if disynaptic inputs to CA3 pyramidal cells do occur, then they will synapse in *s. radiatum* or *s. oriens* (see Fig. 2), and the resultant current flow at the site of a recording electrode placed in *s. lucidum* will be outward, a current source. In chapter 4, when determining at what site calcium is necessary for mossy fiber LTP, this problem with single cell recording is avoided altogether by blocking any synaptic activity postsynaptically.

CHAPTER TWO

METHODS

Standard procedures for preparing and maintaining guinea pig hippocampal slices were used and all animals were handled in accordance with UCSF animal care guidelines. Three to four week old guinea pigs were anaesthetized with halothane and decapitated. The cranium was opened and the brain removed and placed immediately in ice cold artificial cerebrospinal fluid (ACSF) bubbled with 95% O_2/ 5% CO_2. The ACSF contained (in mM): 119 NaCl, 2.5 KCL, 1.3 $MgCl_2$, 2.5 $CaCl_2$, 26 $NaHCO_3$, 1 NaH_2PO_4, and 10 Glucose, and was equilibrated with 95% O_2/ 5% CO_2. The two hippocampi were individually dissected out from the underlying thalamus and overlying cortex of each hemisphere. The hippocampi were placed on an agar block which was then glued (Krazy glue) onto a plexiglass cutting platform. The platform was placed in a well containing ice cold bubbled ACSF on the base of a vibratome (Ted Pella Co.). Transverse hippocampal slices 400-500 μm thick were cut and placed in a holding chamber for at least 1 h to equilibrate. Immediately prior to recording, a slice would be transferred to a standard superfusing chamber with a flow rate of 1.5-2.5ml/ min and held immobilized between two nylon nets (see Nicoll & Alger, 1981). Experiments were done at room temperature or 30-32 °C where specifically mentioned. Field recordings were made with electrodes filled with 1-3M NaCl. Intracellular electrodes were filled with 2M potassium methyl sulphate and had resistances of 70-90 MΩ. The internal solution for whole cell experiments consisted of (in mM): 140 CsGluconate, 10 CsHEPES, 0.2 EGTA, 2 MgATP, 0.3 Na_3GTP, 8 NaCl. This low level of internal chloride was used in order that the chloride reversal potential be approximately -70mV. Evoked excitatory postsynaptic currents (EPSCs) were then recorded from the CA3 cells while holding at the chloride reversal potential in order to reduce contamination by inhibitory postsynaptic currents (IPSCs). This allowed us to avoid using picrotoxin to block γ-amino-n-butyric acid (GABA) currents, which would have

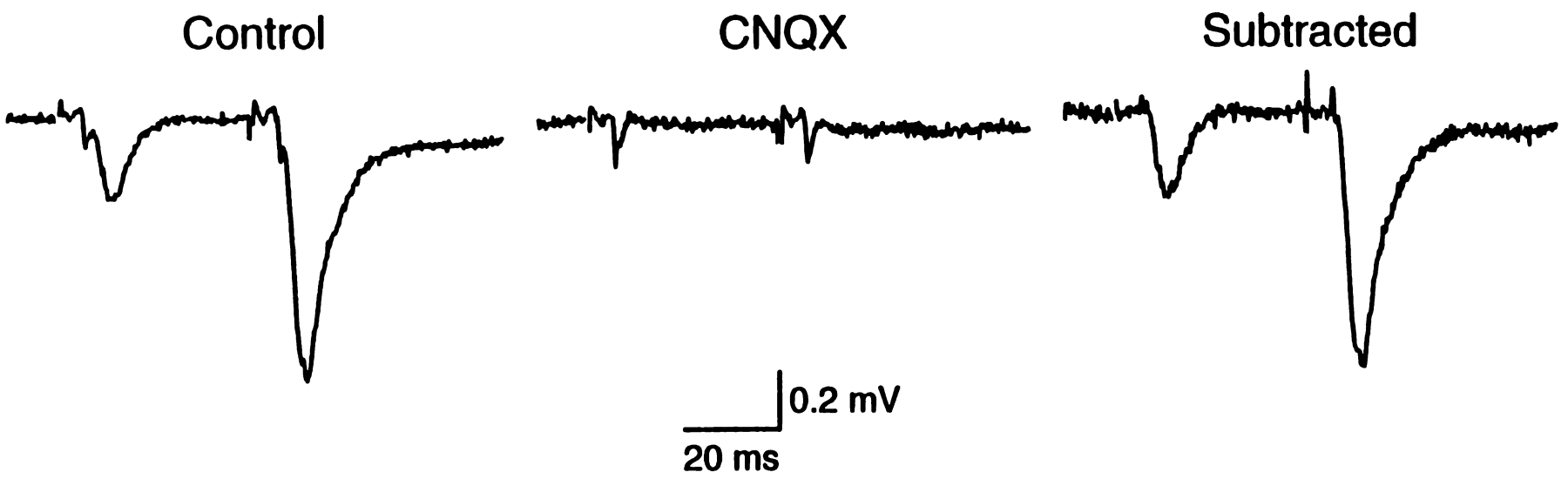
increased the possibility of recording disynaptic inputs (Miles & Wong, 1987). The whole cell electrodes typically had resistances of 4-7 MΩ. The data were recorded with either an Axoclamp 2A or an Axopatch-1D (Axon Instruments). Responses were filtered at 1-5 kHz, digitized at 3-5 kHz on a TL-1 interface (Axon) and collected on a 286 IBM compatible computer. A modified version of pCLAMP that allowed for on-line monitoring of response sizes was used for analysis.

In a transverse section of the hippocampus, the mossy fibers, more so than the Schaffer collateral projection, tend to leave the plane of the slice. For this reason one cannot always record a synaptic response when stimulating in the granule cell layer of the dentate gyrus and recording in CA3. Furthermore, with intracellular and whole cell experiments there is the possibility of contamination of a synaptic response from axons other than those of the mossy fibers, which can contribute to the recorded synaptic response. In order to maximize the presence of a mossy fiber input, therefore, at the outset of all experiments the stimulating electrode (bipolar stainless steel) was placed in *stratum (s.) lucidum* and a field electrode was used to record at different places in the granule cell layer of the dentate gyrus in order to find the best antidromic field potential. The stimulating electrode was then placed at this site for the experiment, although some minor adjustments during the intracellular or whole cell recording were occasionally still necessary to further improve the mossy fiber excitatory postsynaptic potential (EPSP) or EPSC. Furthermore, as mentioned above, GABA inhibitors were never used in order to minimize disynaptic inputs (Miles & Wong, 1987), and contributions of IPSCs were minimized during whole-cell experiments by holding the membrane potential at the Cl^- reversal potential, which was typically -60 to -75 mV.

For field recordings, the restricted anatomy of the mossy fiber input serves to define clearly mossy fiber inputs because a current sink occurs in the region of synaptic input and a source in regions distant from the input region. Therefore, when recording in *s. lucidum*, a monosynaptic mossy fiber input appears as a negative waveform that reverses to a positivity as the recording electrode is moved from *s. lucidum* to *s. radiatum*. Care was taken to minimize the occurrence of positivities following the monosynaptic mossy fiber negativity, as these may reflect disynaptic activation of remote excitatory synapses.

Extracellular recordings sometimes revealed a fiber volley response that was highly variable from slice to slice and often overlapped with the synaptic response. Therefore, at the end of all field experiments 20μM 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) or occasionally 6,7-Dinitroquinoxaline-2,3-dione (DNQX) was added to the bath to assess the fiber volley component of the response (Fig. 3). This manipulation also assured that in every experiment the recorded response was due to synaptic transmission. The non-synaptic, fiber volley component of the recorded response that remained in the presence of CNQX was then subtracted from all traces in the analysis. Unless otherwise mentioned, all figures of extracellular traces shown in this thesis have had the fiber volley component subtracted out. This component could often be a large fraction of the e.p.s.p. in response to the first of paired stimuli, especially after manipulation-induced depression. Therefore, in chapters 3 and 4 (except when indicated, see Fig. 14), where the experiments often involved manipulation-induced depression, we analyzed the amplitude of the second response because the larger synaptic component allowed for more accurate and less variable measurements. Elsewhere the response amplitude to the first pulse was analyzed.

FIGURE 3. Subtraction of fiber volley component from recorded response. In the first panel (Control) is shown a mossy fiber response typical of those used throughout these studies recorded with an extracellular field pipette. The response shows marked facilitation to pulses delivered 40 ms apart as is typical of mossy fibers. The middle panel (CNQX) shows the remaining response after the addition of 20 μM CNQX to the bath. This is then subtracted from the control response to give the subtracted, purely synaptic response shown in the third panel (Subtracted).


Control
CNQX
Subtracted
0.2 mV
20 ms

When an associational-commissural (assoc-com) input was used, a second stimulating electrode was placed in *s. radiatum* to the CA1 side of the recording electrode and far away from *s. lucidum* to avoid activating mossy fibers, and the initial slope was analyzed. For recordings in CA1, 100μM picrotoxin was added to the ACSF to block GABAergic transmission and the CA3 region was removed by knife cut to avoid rebound bursting activity.

Baseline transmission was monitored at 0.025-0.1 Hz, and when paired pulses were used, they were given 40 ms apart. A standard long-term potentiation (LTP) inducing tetanus consisted of 4 trains of 100Hz stimulation for 1 second, separated by 20 seconds and given at baseline stimulus strength, although where mentioned this pattern was altered. All LTP-inducing tetani to the mossy fibers were given in the presence of 25 μM D-APV to block N-methyl-D-aspartate (NMDA) dependent, non-mossy fiber LTP. All values are expressed as means ± s.e.m.

Most drugs used were made up as stock solutions and added to the ACSF at the source of the perfusion system. These drugs were the following: dynorphin A (1-17) (Bachem); 8-(4-Chlorophenylthio)-adenosine-3',5'-cyclic monophosphorothioate, Sp isomer (Sp-8-CPT-cAMPS), 8-(4-Chlorophenylthio)-adenosine-3',5'-cyclic monophosphorothioate, Rp isomer (Rp-8-CPT-cAMPS) (BioLog); KT5720 (BioMol); {N-[2-((3-(4-Bromophenyl)-2-propenyl)amino)ethyl]-5-isoquinoline, HCl} (H-89), forskolin, 1,9-dideoxyforskolin (Calbiochem); D(-)-2-amino-5-phosphonovaleric acid (D-APV) (Cambridge Research Biochemicals); naloxone HCl (Endo Labs); Pituitary Adenylyl Cyclase Activating Peptide (PACAP-27) (Peptide Inc.); ω-Agatoxin-IVA (Peptides International); nor-Binaltorphimine 2HCl, 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), 6,7-Dinitroquinoxaline-2,3-dione (DNQX), Sp-adenosine 3',5'-cyclic monophosphothioate triethylamine salt (Sp-cAMPS), Rp-adenosine 3',5'-cyclic

monophosphothioate triethylamine salt (Rp-cAMPS), N^6-[2-(3,5-Dimethoxyphenyl)-2-(2-methylphenyl)-ethyl] adenosine (DPMA) and S-(3-Dimethylaminopropyl)isothiourea dihydrochloride (Dimaprit) (Research Biochemicals Inc.); [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO), [D-$Pen^{2,5}$]-enkephalin (DPDPE), ethylene glycol-bis(β-amino-ethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 5-Hydroxytryptamine hydrochloride (5-HT), isoproterenol, kynurenic acid, nifedipine, U69,593 and ω-Conotoxin-GVIA (Sigma); (1S,3R)-1-Aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD), (RS)-α–Methyl-4-carboxyphenylglycine ((RS)-MCPG) (Tocris Neuramin). KT5720, DPMA, 1,9-dideoxyforskolin and forskolin were made up in DMSO (0.1% final concentration). 5-HT was made up in H_2O with 1% ascorbic acid. MCPG was either made up as a 100mM stock in 110mM NaOH or dissolved directly into the ACSF by sonication.

On occasion, glutamate (Sigma) was delivered using an iontophoretic injection unit (World Precision Instruments, Inc.). The iontophoretic pipette was filled with glutamate made up at 200mM in distilled water and adjusted to pH 8. A picospritzer (General Valve Corp.) was used to deliver nor-Binaltorphimine (nor-BNI) and dynorphin by puff application. In these cases the puffer pipettes were filled with either 1μM nor-BNI or 5μM dynorphin A (1-17) diluted from stock solutions into ACSF.

CHAPTER THREE

Actions of Dynorphin at the Mossy Fiber-CA3 Synapse

INTRODUCTION

The discovery that a variety of small peptides are specifically present within neurons that also contain classical neurotransmitters has led to the concept of coexistence of transmitters (Hökfelt, 1991). The functional implications of this concept have been most thoroughly developed in invertebrates (Kupferman, 1991) and in the vertebrate peripheral nervous system (PNS) (Jan & Jan, 1982; Jan & Jan, 1983; Lundberg & Hokfelt, 1983). Here it has been shown that the action of peptides differs from that of classical transmitters in that high frequency stimulation is required for peptide release, their action is long lasting and they act at considerable distances from their site of release. The widespread occurrence of the colocalization of peptides with classical neurotransmitters within neurons of the central nervous system (CNS) has led to the assumption that the model developed in the PNS also applies to the CNS. The functional correlates of coexistence in the CNS, however, have been difficult to establish unambiguously as, indeed, has been any role of peptides as synaptic messengers in the CNS. We have used the well characterized mossy fiber pathway in the hippocampus to test for any physiological role of peptides.

As pointed out by Paton (1958), the classical criteria necessary to satisfy in order to prove that a given substance acts endogenously as a neurotransmitter are: i, that the substance is present in the system in question, ii, that the substance can be released in a specific manner, iii, that exogenous application of the substance mimics the endogenous action, iv, that there is a manner of removing the substance, and v, that specific antagonists can block the endogenous effect. It has been well established that the amino acid glutamate acts as a fast neurotransmitter at the mossy fiber-CA3 synapse. The mossy fibers have been shown to contain and release glutamate (Crawford & Connor, 1973;

Storm-Mathisen *et al.*, 1983); application of glutamate or related agonists produces inward current (see Figs. 9 and 27 and Sawada & Yamamoto, 1984); and the non-N-methyl-D-aspartate (NMDA) receptor antagonist CNQX blocks the synaptic response (Fig. 3). The mossy fibers also contain opioid peptides, in particular dynorphin (Gall *et al.*, 1981; McGinty *et al.*, 1983; McLean *et al.*, 1987). The evidence that the opioid peptide dynorphin acts in this system as a neurotransmitter, however, is incomplete. It has been shown that dynorphin can be released upon stimulation of the mossy fibers (Terrian, Johnston, Claiborne, Ansah-Yiadom, Strittmatter & Rea, 1988; Wagner, Evans & Chavkin, 1991) and there are peptidases that effectively remove the peptide, but no endogenous action has been demonstrated. We find that activation of mossy fibers, in addition to generating the well studied glutamatergic fast excitatory postsynaptic potential (EPSP), also generates a long lasting inhibition of neighboring mossy fiber synapses by a presynaptic action of synaptically released dynorphin. This released dynorphin can also act back on the synapses from which it was released and in so doing modify the threshold of long-term potentiation (LTP) at those synapses. This is evidenced when brief tetani, which are below threshold for mossy fiber LTP, generate LTP in the presence of opiate receptor antagonists. Furthermore, the presynaptic inhibition by dynorphin is more pronounced on synapses expressing LTP. These results establish dynorphin as a synaptic transmitter at glutamatergic mossy fiber synapses, albeit with a distinctly different mode of action than the fast neurotransmitter glutamate. They also demonstrate an important interaction between this peptidergic system and the mechanisms governing the generation and expression of LTP. Some of these results have previously appeared in press (Weisskopf, Zalutsky & Nicoll, 1993).

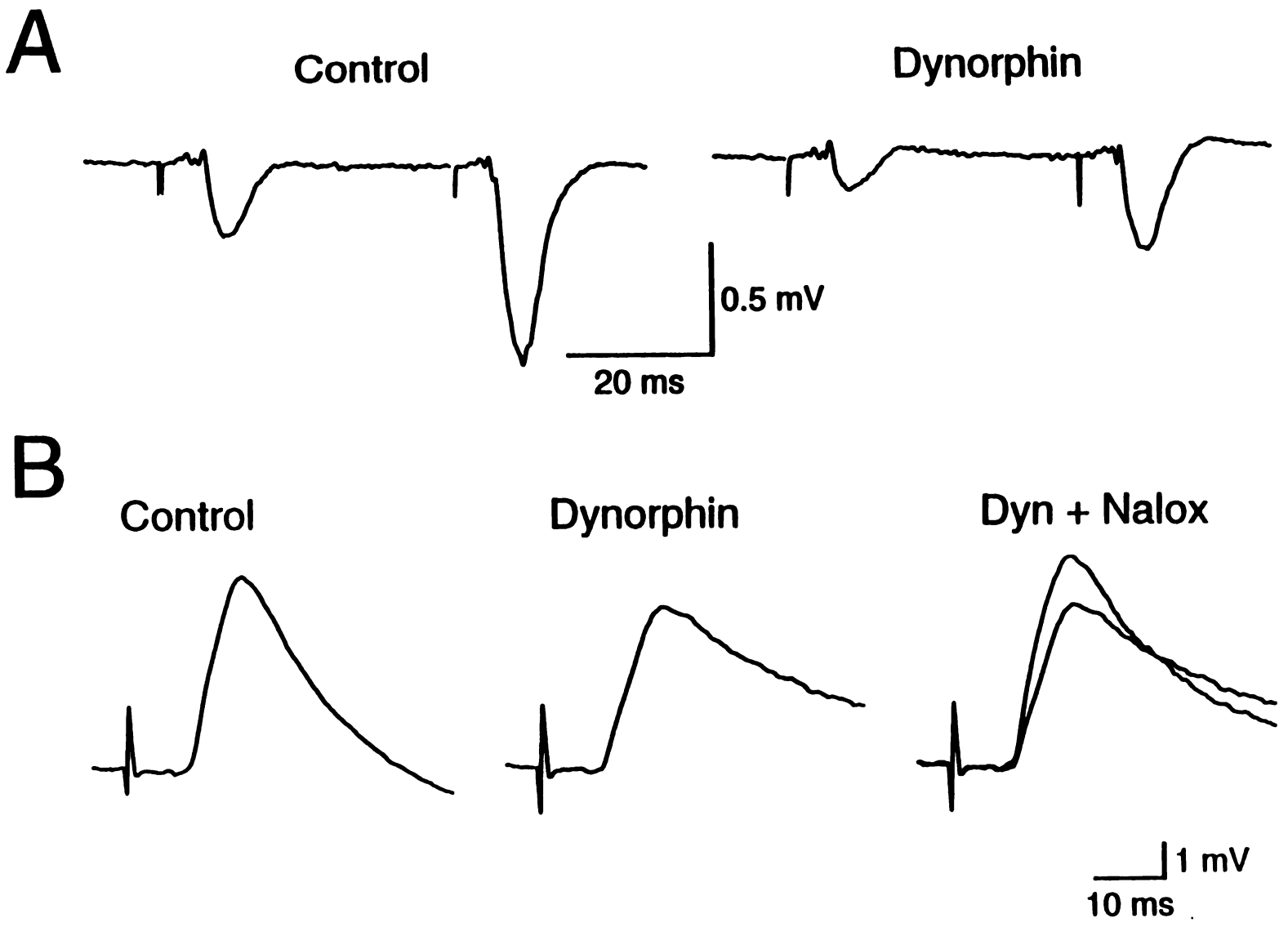
RESULTS

Dynorphin inhibits mossy fiber synaptic transmission

Our initial finding using extracellular field potential recordings, as illustrated in figure 4, was that bath application of 100-500nM of the opioid peptide dynorphin A (1-17), henceforth simply dynorphin, potently inhibited mossy fiber synaptic transmission. In contrast to past studies which reported either a variable (Iwama, Ishihara, Satoh & Takagi, 1986) or no effect (Caudle & Chavkin, 1990) of dynorphin at mossy fiber synapses, our results were extremely reproducible and consistent. The inhibitory effect was seen in 49 out of 51 slices tested and averaged 40 ± 2% (n = 51) (see Figs. 5B, 6A, 9B and 13). With intracellular recordings as well, dynorphin could be seen to reduce the rate of rise (25 ± 8%, n=6) and peak of the EPSP (Fig. 4B). In addition, inhibitory poststnaptic potentials (IPSPs) evoked by mossy fiber stimulation, as seen following the EPSP in figure 4B, were also reduced by dynorphin. Some studies have reported non-opioid receptor mediated actions of some kappa receptor agonists (Alzheimer & Ten Bruggencate, 1990), however all of the present effects of dynorphin were reversed by the opioid receptor antagonist naloxone indicating that the effects were, indeed, mediated by activation of opioid receptors (Fig. 4B). Simple washout of dynorphin, without the use of antagonists, was more difficult and could take up to 1 hour. Dynorphin did not significantly change the membrane potential (+0.25 ± 1.1 mV, n=6) nor the input resistance (+1.5 ± 7.4%, n=6) of CA3 pyramidal cells.

The anatomy of the CA3 region of the hippocampus allows for the simultaneous testing of drug effects on two different synapses, those of the mossy fibers and those of the associational-commissural (assoc-com) fibers,

FIGURE 4. Inhibitory action of dynorphin on mossy fiber synaptic responses. **A.** Mossy fiber responses recorded using extracellular field recordings are depressed by dynorphin (500 nM). **B.** Intracellular recordings of MF responses were also depressed by dynorphin (500 nM) and this was reversed by the opioid receptor antagonist naloxone (10 μM) (Nalox), applied for 7 minutes. The post-dynorphin EPSP (Dynorphin) is also shown superimposed on the post-naloxone EPSP for reference (Dyn + Nalox). Each record is the average of 20 traces.


A
Control
Dynorphin
0.5 mV
20 ms
B
Control
Dynorphin
Dyn + Nalox
1 mV
10 ms

within the same preparation that share the same postsynaptic cell and differ only in the presynaptic element. Thus, it is of interest that the action of dynorphin was found to be entirely specific for responses to stimulation of the mossy fibers (Fig. 5). This figure also demonstrates that the effects of dynorphin were slow, both in onset and washout. Full recovery from the effects of dynorphin could take up to 1 hour.

Dynorphin activates presynaptic Kappa 1 opioid receptors

Experiments were then carried out to characterize the receptor subtype responsible for the dynorphin inhibition of mossy fiber responses. Based on the action of selective agonists and antagonists, opioid receptors have been divided into three types—mu, kappa and delta (Goldstein, 1987). Consistent with the idea that dynorphin is a selective kappa agonist (Chavkin, James & Goldstein, 1982), dynorphin, at concentrations which depressed mossy fibers, had no disinhibitory action in the CA1 region (n = 3). Such a disinhibition would result in an increased ability of a given synaptic input to produce an action potential in the postsynaptic cell. This is manifested by an increase in the population spike recorded extracellularly in the pyramidal cell layer which results from the synchronous action potential discharge of many cells in response to synaptic stimulation. Such disinhibition is a well known result of mu opioid (Nicoll, Siggins, Ling, Bloom & Guillemin, 1977; Zieglgansberger, French, Siggins & Bloom, 1979; Neumaier, Mailheau & Chavkin, 1988) and delta opioid (Lupica & Dunwiddie, 1991) receptor activation in the CA1 region. Furthermore, we found that the selective kappa antagonist nor-Binaltorphimine (nor-BNI) (Takemori, Ho, Naeseth & Portoghese, 1988) (100 nM) completely blocked the inhibitory effect of dynorphin (Fig. 6A). At these same concentrations, nor-BNI had no

FIGURE 5. Specificity of action of dynorphin for mossy fibers.

A. Sample records of simultaneously recorded mossy fiber (MF) and associational-commissural (assoc-com) responses from before (Control) and after (Dynorphin) the application of 250 nM dynorphin. Each record is the average of 10 traces. **B.** Comparison of dynorphin's effects on MF (closed circles) and assoc-com (open circles) field potentials recorded from the same slice (n = 5). The MF inputs were strongly inhibited by a 5 min application of dynorphin (100-250 nM; solid bar), while the initial slope of the assoc-com inputs was unaffected.

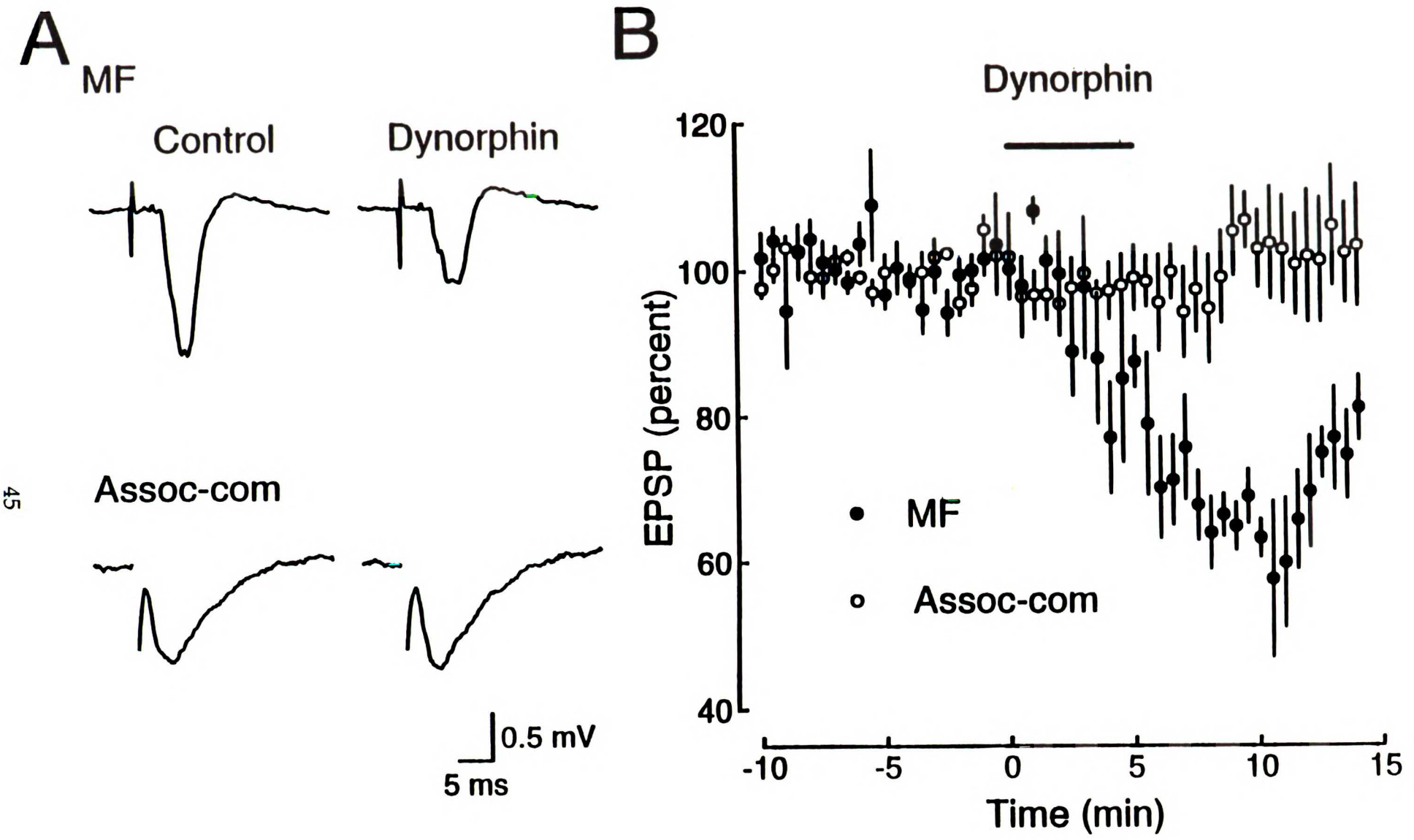
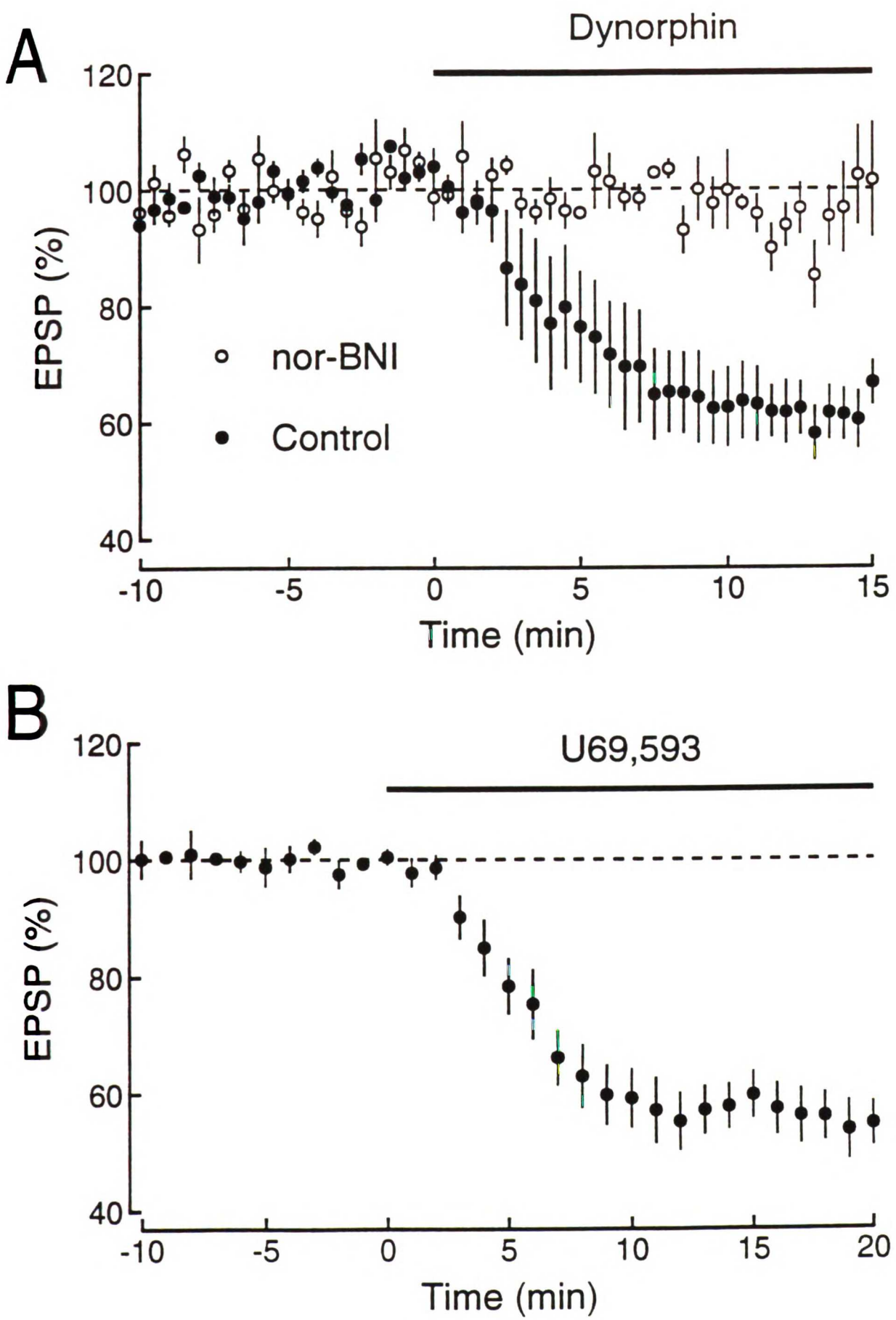

A
MF
Control
Dynorphin
Assoc-com
0.5 mV
5 ms
B
Dynorphin
120
100
80
60
40
EPSP (percent)
MF
Assoc-com
-10
-5
0
5
10
15
Time (min)

FIGURE 6. Kappa 1 receptor-mediated depression of mossy fiber synaptic transmission.

A. Comparison of the effect of 500 nM dynorphin (solid bar) on mossy fiber synaptic responses in control conditions (filled circles, n = 4) and in the presence of the kappa 1 antagonist nor-Binaltorphimine (Nor-BNI, 100 nM; open circles, n = 4). Nor-BNI completely blocks the effects of dynorphin. **B.** The kappa 1 selective agonist, U69,593 (300 nM; solid bar) also depresses mossy fiber synaptic transmission (n = 5).


A
Dynorphin
120
100
80
60
40
EPSP (%)
nor-BNI
Control
-10
-5
0
5
10
15
Time (min)
B
U69,593
120
100
80
60
40
EPSP (%)
-10
-5
0
5
10
15
20
Time (min)

effect on the mu and delta receptor-mediated disinhibitory action in the CA1 region caused by the selective agonists DAMGO (Gillan & Kosterlitz, 1982) (100 nM) (n = 3) and DPDPE (Mosberg, Hurst, Hruby, Gee, Yamamura, Galligan & Burks, 1983) (1 μM) (n = 3), respectively. This concentration of nor-BNI was also without effect on membrane potential or input resistance of CA3 pyramidal cells (n = 4). Taken together, these results strongly suggest that the inhibition of mossy fiber responses is mediated by kappa receptors. The action of nor-BNI, however, further suggests that the effects of dynorphin are specifically mediated by the kappa 1 receptor since nor-BNI has been shown to exhibit specificity for this subtype of kappa receptor (Zukin, Eghbali, Olive, Unterwald & Tempel, 1988). We have confirmed this by demonstrating that U69,593 (300 nM) (Fig. 6B), a kappa agonist that has been used to define kappa 1 sites (Zukin *et al.*, 1988; Nock, Giordano, Cicero & O'Connor, 1990), depressed mossy fiber responses, an effect which was blocked by nor-BNI. This depression (43 ± 4%) was equivalent to that seen with dynorphin, and subsequent application of dynorphin produced no further depression (Fig. 7). These findings with the kappa 1 selective ligands U69,593 and nor-BNI are somewhat surprising in that while kappa binding sites are abundant in *stratum (s.) lucidum* of the guinea pig (McLean *et al.*, 1987), kappa 1 binding sites have not been detected (Wagner *et al.*, 1991). Our physiological results, however, indicate that kappa 1 receptors must be present in the mossy fiber pathway of the guinea pig and, when activated, depress synaptic transmission.

When mossy fiber responses were depressed in the presence of 500nM dynorphin, brief puff application of 1 μM nor-BNI at the site of the mossy fiber-CA3 synapse in *s. lucidum*, but not at the site of stimulation in the granule cell layer of the dentate gyrus, resulted in a reversal of the depressant effect of dynorphin (Fig. 8). This was done in three experiments and indicated that

FIGURE 7. Dynorphin does not depress mossy fiber transmission in the presence of U69,593.

A. Application of 300 nM U69,593 causes a typical depression of mossy fiber synaptic transmission. Subsequent application of 500 nM dynorphin has no further effect. **B.** Sample records of control mossy fiber responses from the same experiment before addition of U69,593 to the bath (Control), in U69,593 only (U69,593), and after the subsequent addition of dynorphin as well (Dynorphin). All records are averages of 10 responses.

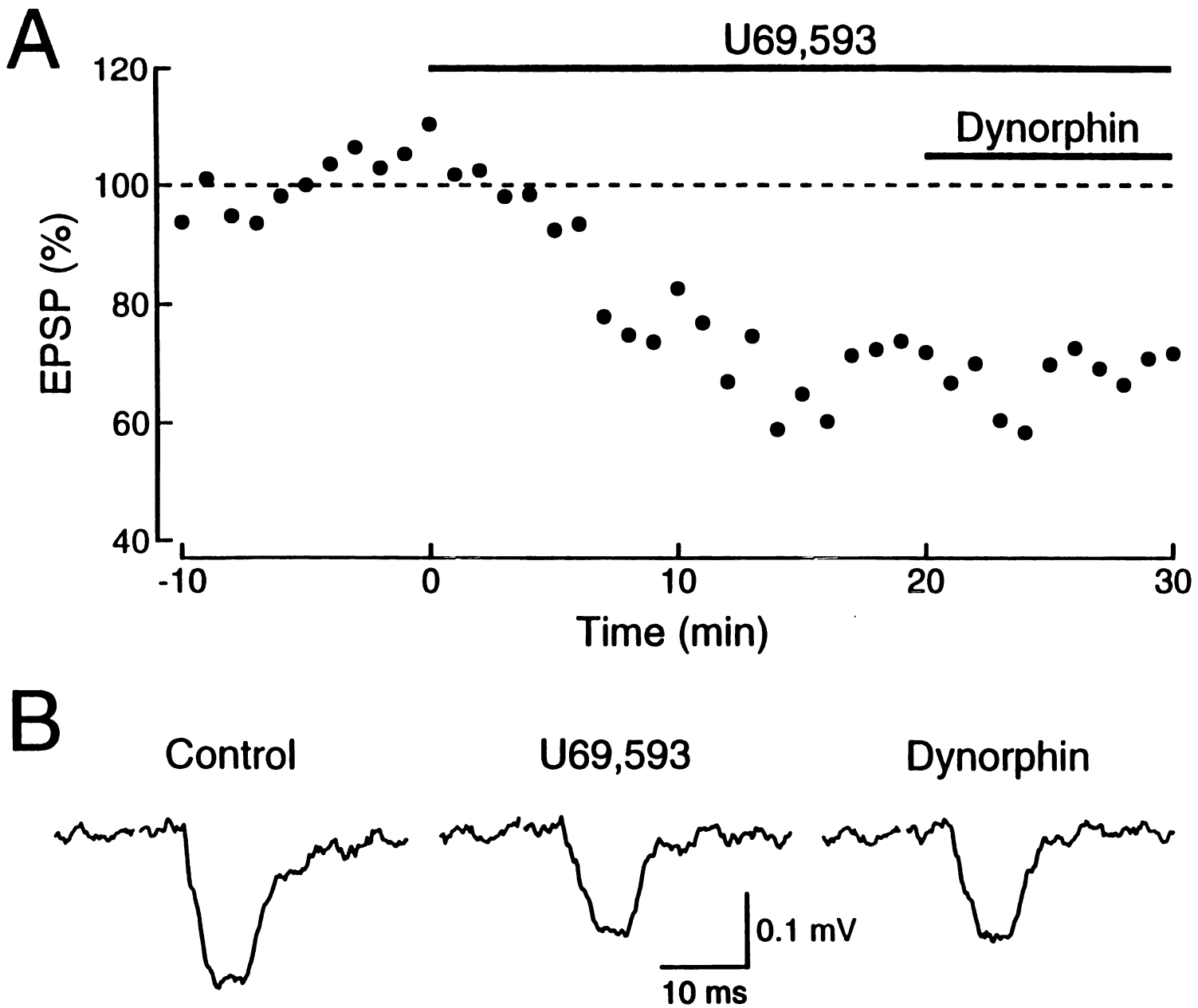
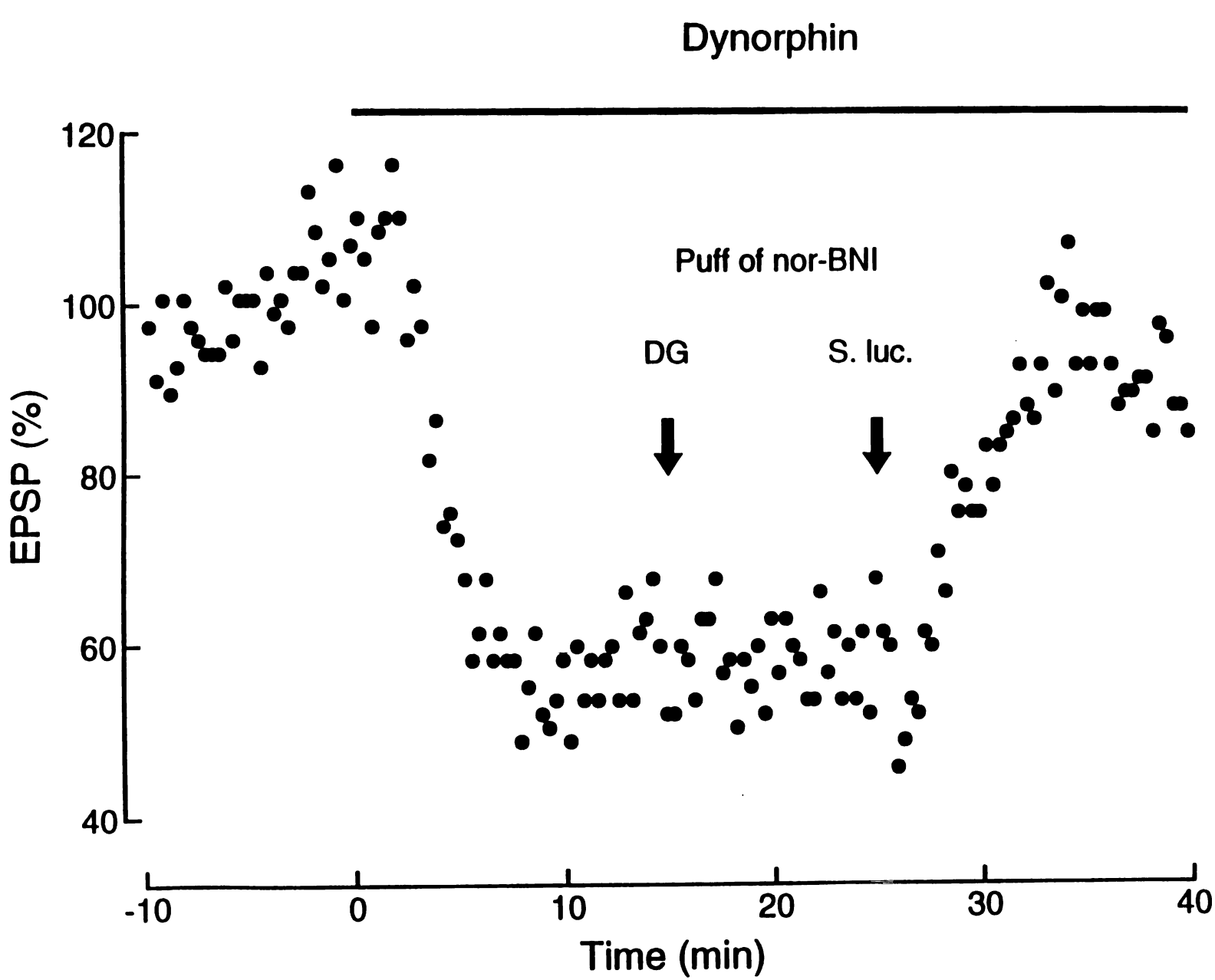

A
U69,593
Dynorphin
120
100
80
60
40
EPSP (%)
-10
0
10
20
30
Time (min)
B
Control
U69,593
Dynorphin
0.1 mV
10 ms

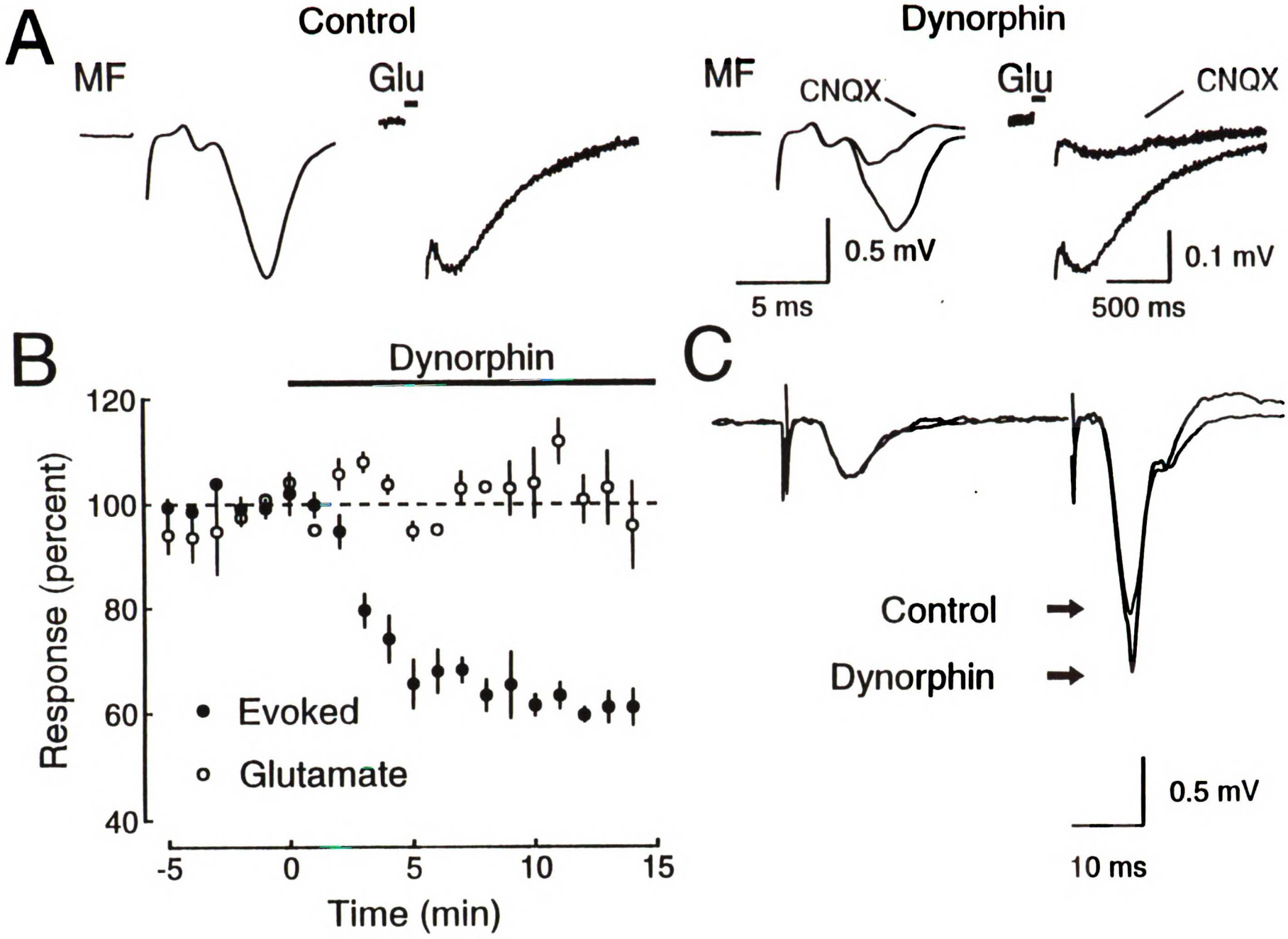
FIGURE 8. Dynorphin acts at the mossy fiber-CA3 pyramidal cell synapse. Five puff applications of Nor-BNI (1 μM, 500 ms, 2 bar) separated by 1 s each given to the slice surface at the stimulating electrode in the dentate gyrus (DG) had no effect on dynorphin-induced depression. The identical application given at the recording electrode in *s. lucidum* 10 min later (S. luc.) rapidly reversed the depression.


Dynorphin
Puff of nor-BNI
DG
S. luc.
EPSP (%)
120
100
80
60
40
-10
0
10
20
30
40
Time (min)

dynorphin was exerting its inhibition at the synapse between the mossy fiber and CA3 pyramidal cell. This depression, however, could result from an action at one of two sites: dynorphin could depress synaptic transmission by inhibiting the postsynaptic action of glutamate or by inhibiting the release of glutamate from the presynaptic terminal. To distinguish between these possibilities we compared the effects of dynorphin on responses to glutamate applied in *s. lucidum* from one barrel of a double barreled pipette and to mossy fiber synaptic responses evoked by granule cell stimulation, both recorded from the adjacent barrel. While dynorphin had its typical depressant action on the mossy fiber synaptic response it had no effect on the response to iontophoretically applied glutamate (Fig. 9A). Both the synaptic and glutamate responses, however, were susceptible to CNQX applied to the bath (Fig. 9A). This differential action of dynorphin is summarized for 4 slices in figure 9B. The lack of effect of dynorphin on the responses to exogenously applied glutamate is consistent with a presynaptic action.

Further evidence for a presynaptic action of dynorphin comes from the analysis of paired-pulse facilitation. Paired pulse facilitation is a phenomenon seen at many chemical synapses, including mossy fiber synapses (Figs. 3 and 4A), where the response to the second of two stimuli (pulses) delivered with a short interval (40 ms in Figs. 3 and 4A) is enhanced with respect to the first. This facilitation reflects a presynaptic enhancement in transmitter release (Zucker, 1989), and manipulations that decrease transmitter release increase paired-pulse facilitation (Katz & Miledi, 1968; Mallart & Martin, 1968; Creager, Dunwiddie & Lynch, 1980). We therefore examined the effects of dynorphin on paired-pulse facilitation. In these experiments, the stimulus strength was increased after the effects of dynorphin had stabilized so that the amplitude of the response to the first pulse in the presence of dynorphin matched the amplitude of the control

FIGURE 9. Dynorphin decreases transmitter release from mossy fiber synapses. **A.** Sample records of responses to mossy fiber stimulation (MF) or iontophoretically applied glutamate (Glu; solid bar) evoked alternately in the same slice before (Control) and 10 minutes after (Dynorphin) the addition of dynorphin (500 nM) to the Ringer. A double barreled micropipette was used, one for recording and the other for iontophoretic application of glutamate, to ensure that glutamate was applied to the recording site. The recording barrel contained 3 M NaCl and the other barrel was filled with glutamate (200 mM in distilled water, pH 8) for iontophoresis (400 nA, 100 msec, no retaining current). MF and glutamate (Glu) sample records are averages of 15 and 5 responses, respectively. To illustrate the efficacy of antagonists on both types of responses, the unsubtracted records are shown. Recordings obtained after addition of 20 μM CNQX to the Ringer are superimposed on the post-dynorphin traces (CNQX). **B.** Comparison of responses in the same slice to mossy fiber stimulation (evoked; closed circles) and iontophoretically applied glutamate (100-800 msec, 200-800 nA, no retaining current) (open circles) in *s. lucidum.* Dynorphin (500 nM; solid bar) depressed the evoked responses, but had no effect on the iontophoretic responses (n = 4). **C.** Increase in paired pulse facilitation after dynorphin. Sample records are averages of 5 traces before (control) or 10 minutes after the addition of dynorphin (500 nM) to the Ringer.


A
Control
MF
Glu
Dynorphin
MF
CNQX
Glu
CNQX
0.5 mV
5 ms
0.1 mV
500 ms
B
Dynorphin
120
100
80
60
40
Response (percent)
Evoked
Glutamate
-5
0
5
10
15
Time (min)
C
Control
Dynorphin
0.5 mV
10 ms

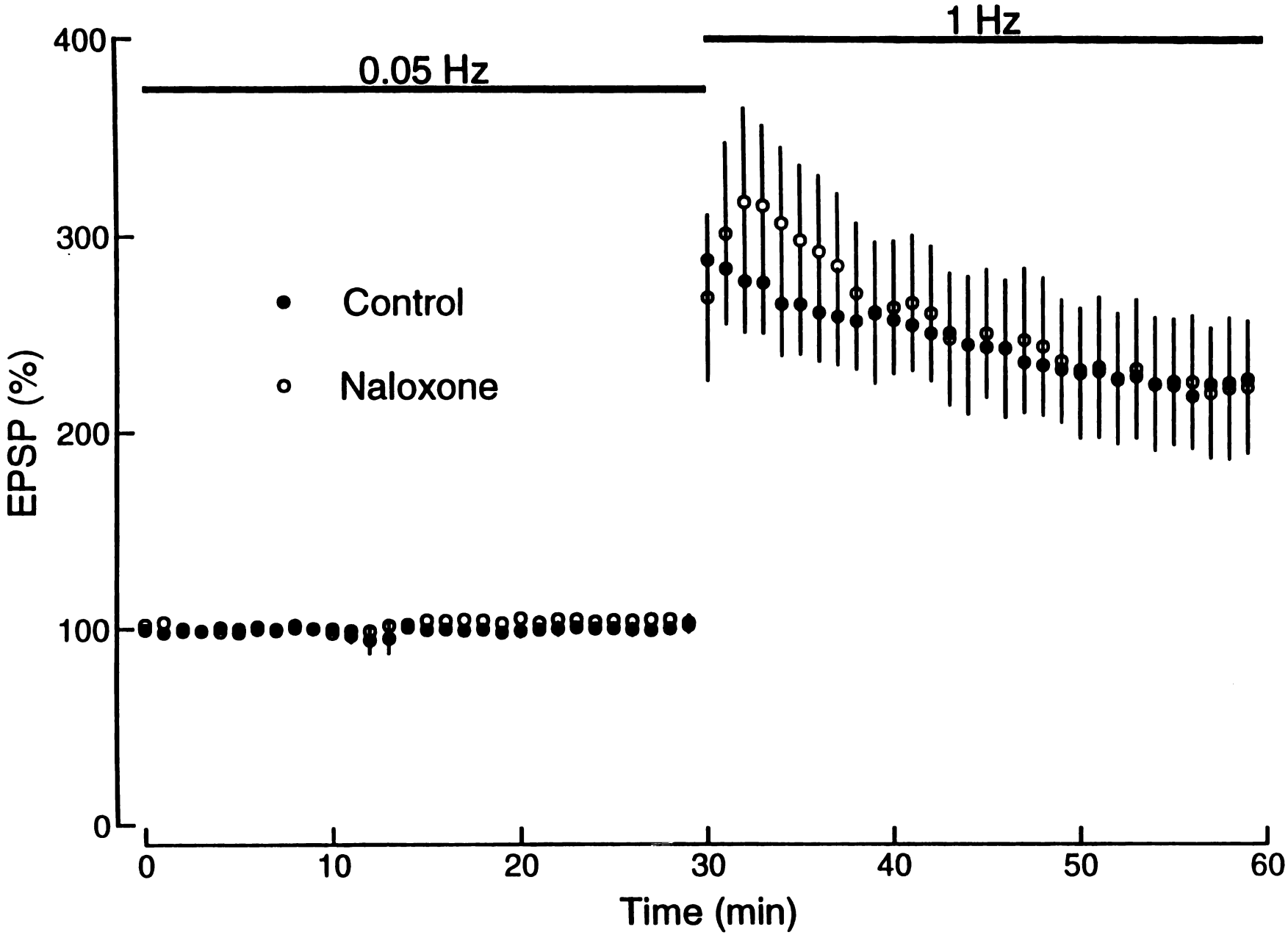
response to the first pulse. This is done in order to avoid possible non-linear effects that might result from the altered response size. An example is illustrated in figure 9C, which shows that during the action of dynorphin the facilitation evoked by paired pulses is enhanced. The results from 10 slices showed that the inhibition is associated with a 17.7 ± 7.5% increase in facilitation ($p<0.05$, two tailed paired sample Student t test). This finding suggests that kappa receptors are present on mossy fiber synaptic terminals and that their activation reduces glutamate release. This could also explain the selective inhibitory action of dynorphin on IPSPs evoked by mossy fiber stimulation (Fig. 4B). If kappa receptors are also on the mossy fiber terminals of collaterals that synapse onto inhibitory interneurons, then the drive of the interneurons by mossy fibers, but not by other inputs, would also be expected to be selectively depressed by dynorphin. Our findings are in accord with numerous other studies indicating a presynaptic location of kappa receptors (McFadzean, Lacey, Hill & Henderson, 1987; Gannon & Terrian, 1991; Gauchy, Desban, Krebs, Glowinski & Kemel, 1991; Kato *et al.*, 1992; Pinnock, 1992; Wagner, Caudle & Chavkin, 1992).

Dynorphin mediates heterosynaptic depression

Given the presence of dynorphin in the mossy fibers (Gall *et al.*, 1981; McGinty *et al.*, 1983; McLean *et al.*, 1987) and the action of this peptide applied exogenously, we reasoned that it might be possible to elucidate an endogenous synaptic action of opioid peptides at this CNS synapse. At baseline stimulation frequency of 0.05 Hz, application of naloxone had no effect on EPSPs in 5 of 7 slices. Furthermore, the increase seen in mossy fiber responses upon increasing the stimulation frequency from 0.05 to 1 Hz in control conditions was not different from the increase seen in the presence of naloxone (Fig. 10). These

FIGURE 10. Effect of naloxone on stimulation at low frequencies.

Mossy fiber synaptic transmission shows marked facilitation when the stimulation frequency is increased from 0.05 Hz (lower bar) to 1 Hz (upper bar). The presence of naloxone (10 μM) in the bath (open circles, n = 7) has no significant effect on this facilitation seen in control conditions (filled circles, n = 7), which suggests that dynorphin is not released in significant quantities with this protocol.

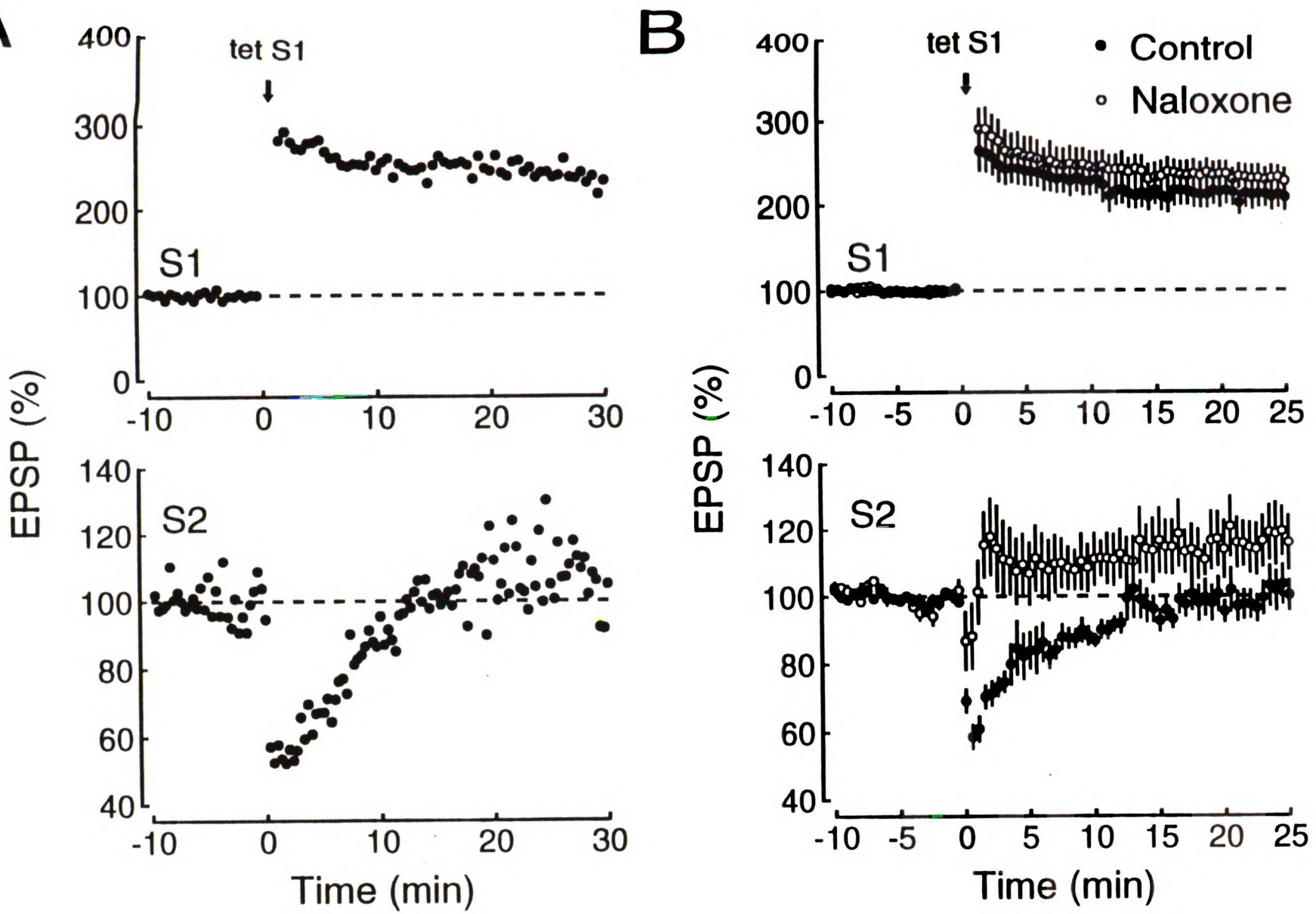

1 Hz
0.05 Hz
400
300
200
100
0
Control
Naloxone
EPSP (%)
0
10
20
30
40
50
60
Time (min)

results suggest that significant amounts of peptide are not released from these synapses at frequencies of up to 1 Hz. It had been noted before, however, that when one of two independent mossy fiber pathways is tetanized at higher frequencies, a transient depression lasting 10-20 minutes is seen in the untetanized pathway (Bradler & Barrionuevo, 1990; Zalutsky & Nicoll, 1992). We repeated this finding and an example of this heterosynaptic depression is shown in figure 11A. One possible mechanism for this depression is that dynorphin is released from the tetanized synapses and acts to depress nearby synapses. If this were the case, then such a tetanus given in the presence of the opioid receptor antagonist naloxone should not produce an associated heterosynaptic depression. Therefore, two groups of slices, one with and one without naloxone were compared. As can be seen in figure 11B, naloxone blocked the heterosynaptic depression and actually unmasked a small facilitation which probably reflects a small subset of fibers in the control pathway that were also activated by the tetanus to the other pathway. Our finding that mossy fiber LTP is not blocked by naloxone contradicts some previous reports. One group performed experiments using *in vivo* recording techniques (Derrick, Weinberger & Martinez, 1991; Derrick, Rodriguez, Lieberman & Martinez, 1992) which introduces many differences that might be responsible for the discrepancy. The other report used *in vitro* techniques (Martin, 1983) as we did, and we do not have an explanation for the observed differences. It is important to note, however, that although our result concerning the effect of naloxone on mossy fiber LTP is negative, we do have the concurrent positive control: while naloxone did not block LTP, it did effectively block the heterosynaptic depression (Fig. 11B).

The depression by exogenous dynorphin seen previously was the result of long duration bath application and, hence, the time course of its action did not

FIGURE 11. Heterosynaptic depression of mossy fiber responses is blocked by the opiate receptor antagonist naloxone.

A. A standard tetanus was given to one pathway (S1) at time 0. The responses to this input exhibit LTP. Monitored concurrently were the responses to a second input that did not receive any stimulation during the tetanus to the other pathway (S2). These responses exhibit a transient depression. **B.** Comparison of the effects of naloxone (10 μM) on both the tetanized (S1) and untetanized (S2) inputs in a group of control slices (filled circles; n = 15) and a group of slices that had naloxone (10 μM) added to the bath (open circles; n = 8). LTP is seen in both cases, but the transient depression is blocked by naloxone. We do not know what causes the very brief post-stimulus inhibition that persists in the presence of naloxone (S2: open circles), but it is clearly not opioid mediated. The typical decaying component to mossy fiber LTP (Zalutsky & Nicoll, 1990, 1992) is not apparent in these figures because the second of paired pulses is plotted. In all experiments, two pathways that did not show any cross-pathway facilitation were chosen to minimize the number of fibers common to both inputs.


A
B
tet S1
tet S1
Control
Naloxone
S1
S2
S1
S2
EPSP (%)
EPSP (%)
Time (min)
Time (min)
400
300
200
100
0
140
120
100
80
60
40
-10
-5
0
5
10
15
20
25
30

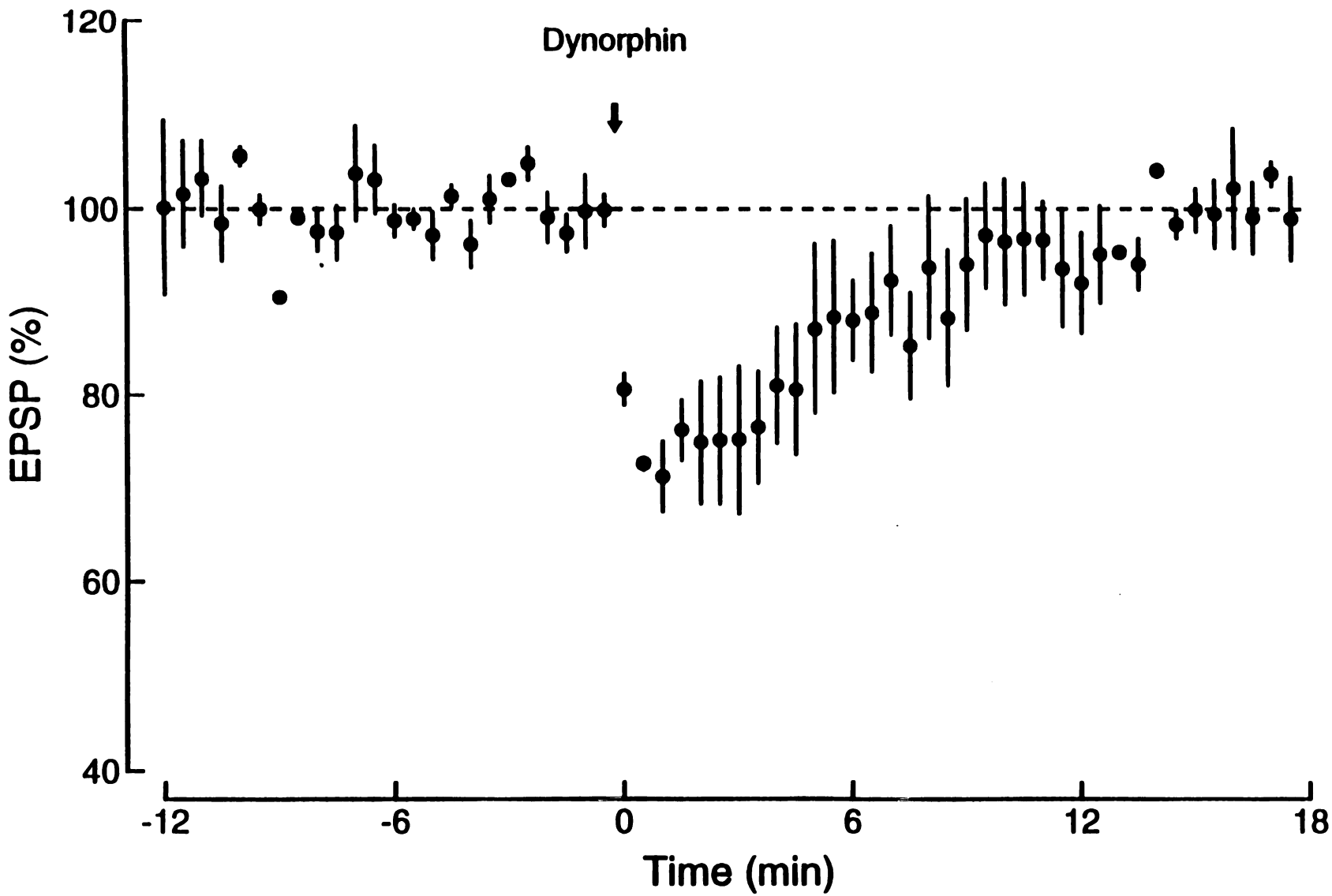
resemble that of the heterosynaptic depression seen after mossy fiber tetanus. Shown in figure 12, however, is the result of a brief puff application of dynorphin in *s. lucidum*. The time course of this depression is quite similar to that of the heterosynaptic depression, which is the expected result if dynorphin mediates the heterosynaptic depression. The long duration of both the heterosynaptic depression and that following puff application of dynorphin is likely due to the slow clearance of peptide because application of antagonists rapidly reverses the action of dynorphin (see Fig. 8). This suggests that at least some degree of dynorphin's action is due to multiple binding episodes by a single molecule after it has been released.

Dynorphin inhibits the induction and expression of mossy fiber LTP

It is clear that the mossy fiber LTP induced by the prolonged tetanus used to examine heterosynaptic depression (in the presence of the NMDA receptor antagonist D-APV) was not blocked by naloxone, contrary to some previous reports (Martin, 1983; Derrick *et al.*, 1991; Derrick *et al.*, 1992). In fact, the LTP may actually have been enhanced to a small extent (Fig. 11B), although not as much during the first 10-20 minutes as might be expected if dynorphin were also causing a homosynaptic depression. It is not clear exactly why this happens, although if the mechanism underlying the early intense potentiation were not as sensitive (or sensitive at all) to dynorphin this would mask the effects of dynorphin. The strong modulation of mossy fiber responses by endogenously released dynorphin, however, still raised the possibility that, under the right conditions, synaptically released dynorphin could modulate mossy fiber LTP. Therefore, we tried to avoid the early intense potentiation after a tetanus in order to have a more sensitive assay to test the possibility that synaptically released

FIGURE 12. Puff application of dynorphin.

Five puff applications of 5 μM dynorphin (200 ms, 2 bar) given to the slice surface at the recording electrode in *s. lucidum* produces a transient depression with a time course that closely resembles that seen during heterosynaptic depression (n = 3).



dynorphin may act homosynaptically and inhibit the induction of mossy fiber LTP. To do this we selected a tetanus protocol (5-10 pulses at 100 Hz repeated at 20 sec intervals 4 times) that in most cases failed to generate LTP. In the same preparation nor-BNI was then added to the bath, which had no effect on baseline transmission (+2.6 ± 2.7%, $p>0.05$), and the same stimulus was repeated. In the presence of nor-BNI, LTP could be evoked by the same tetanus that initially failed to evoke LTP. An example is shown in figure 13A and a summary graph of 11 such experiments is shown in figure 13B. In a separate set of control slices a second tetanus was given in the absence of nor-BNI and no LTP was seen (Fig. 13C). This ensures that the LTP observed in figure 13A and B resulted from the action of nor-BNI. In 10 of the experiments illustrated in figure 13B, a second mossy fiber pathway was monitored and the heterosynaptic inhibition observed in this pathway was inhibited by nor-BNI. The results presented above indicate that synaptically released dynorphin can prevent the induction of mossy fiber LTP.

Previous studies have proposed that the expression of mossy fiber LTP, which is independent of NMDA receptors, may be mediated by an increase in glutamate release because paired-pulse facilitation is decreased after LTP induction (Staubli *et al.*, 1990; Zalutsky & Nicoll, 1990). Since dynorphin also acts presynaptically, we wondered whether synapses that had undergone LTP might have an altered sensitivity to the action of dynorphin. In this experiment two independent pathways were examined. After LTP had been evoked in one pathway the stimulus strength was decreased so that the response was similar in size to the control pathway. Under these conditions the tetanized pathways were on average potentiated to 294 ± 39% (measured at 10 to 15 min) of control. Dynorphin was then applied and the inhibition compared in the two pathways. As shown in figure 14, which summarizes the results from 8 experiments,

dynorphin produces a larger inhibition of synapses expressing LTP (18.3 ± 4.9%, $p<0.05$, two tailed paired sample Student t test). This effect, as well as the demonstrated ability of dynorphin to act back on the synapses from which it was released (Fig. 13), would suggest that following a tetanus in the presence of naloxone there should be a transient enhanced potentiation compared to control conditions that mirrors heterosynaptic depression. However, this was not seen (Fig. 11B), suggesting that some aspect of the early phase of mossy fiber LTP prevents the action of dynorphin. More directly, the results of figure 14 also indicate that the action of dynorphin depends upon the prior activity of the synapse.

FIGURE 13. Synaptically released dynorphin blocks the induction of mossy fiber LTP.

A. Example experiment in which after baseline responses were stable for 10 minutes a series of brief tetani (10 impulses, 100 Hz, repeated 4 times at 20s intervals) was presented at the test stimulation strength (1st Tet). This failed to induce LTP and subsequently nor-BNI (100 nM; solid bar) was applied to the bath. In the presence of nor-BNI the identical stimulation (2nd Tet) then succeeded in evoking LTP. **B.** Compilation of 11 such experiments with the tetani ranging from 5-10 impulses. **C.** In a separate group of 5 slices recorded in a manner identical to those in **B**, no nor-BNI was applied and the identical stimulation was given again after a 10 min stable baseline. For all these experiments, if test responses, averaged between 5-15 min after the first tetanic stimulation, deviated <10% from pre-stimulation levels, then the stimulation was accepted as sub-threshold for generation of LTP. If larger than 10% the experiment was terminated. The interval between the tetanus and the first subsequent test stimulus varied up to 30 seconds, thus the data points immediately following a tetanus are not an accurate representation of post-tetanic potentiation. Furthermore, points that exceeded the scale of the figure are not shown.

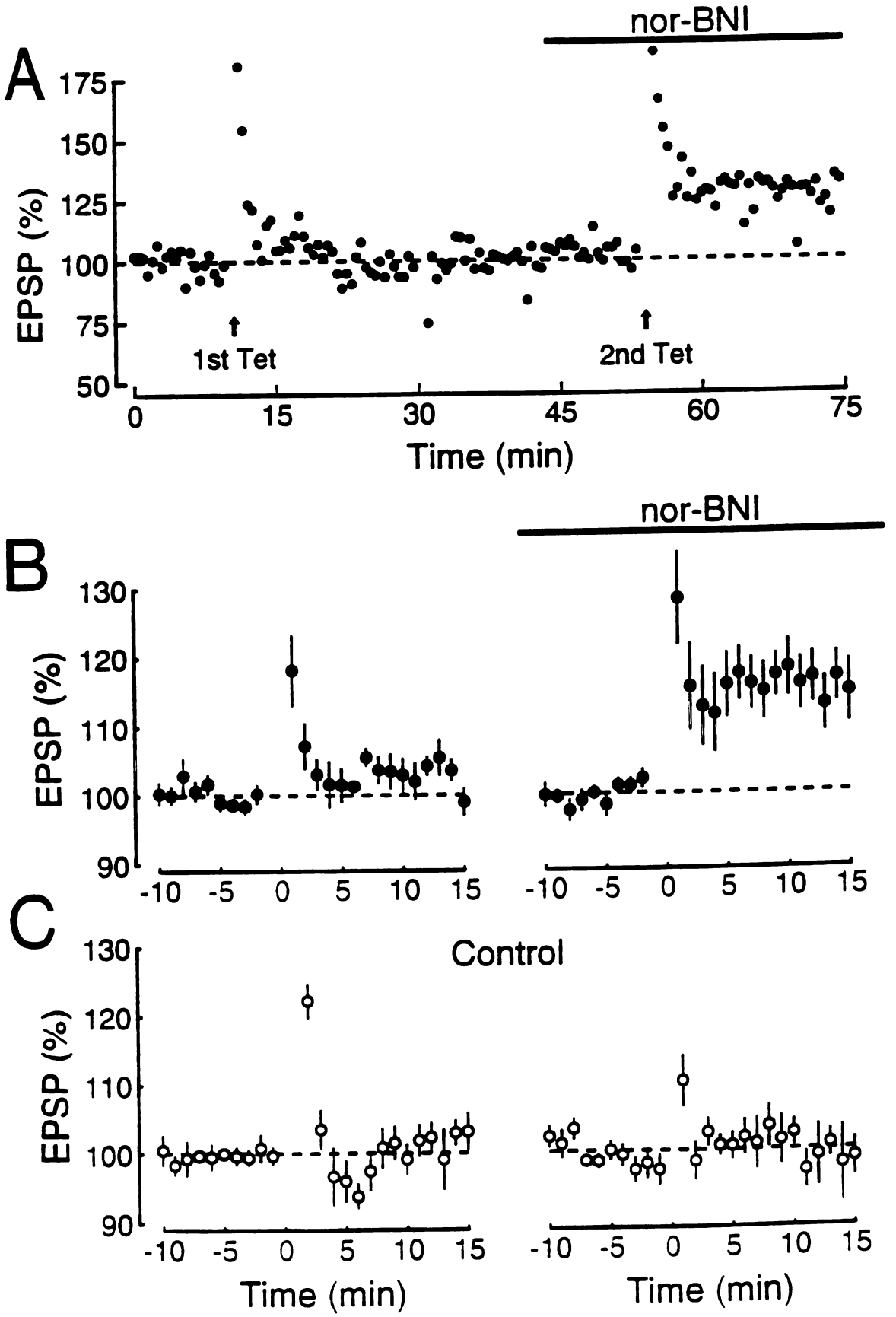
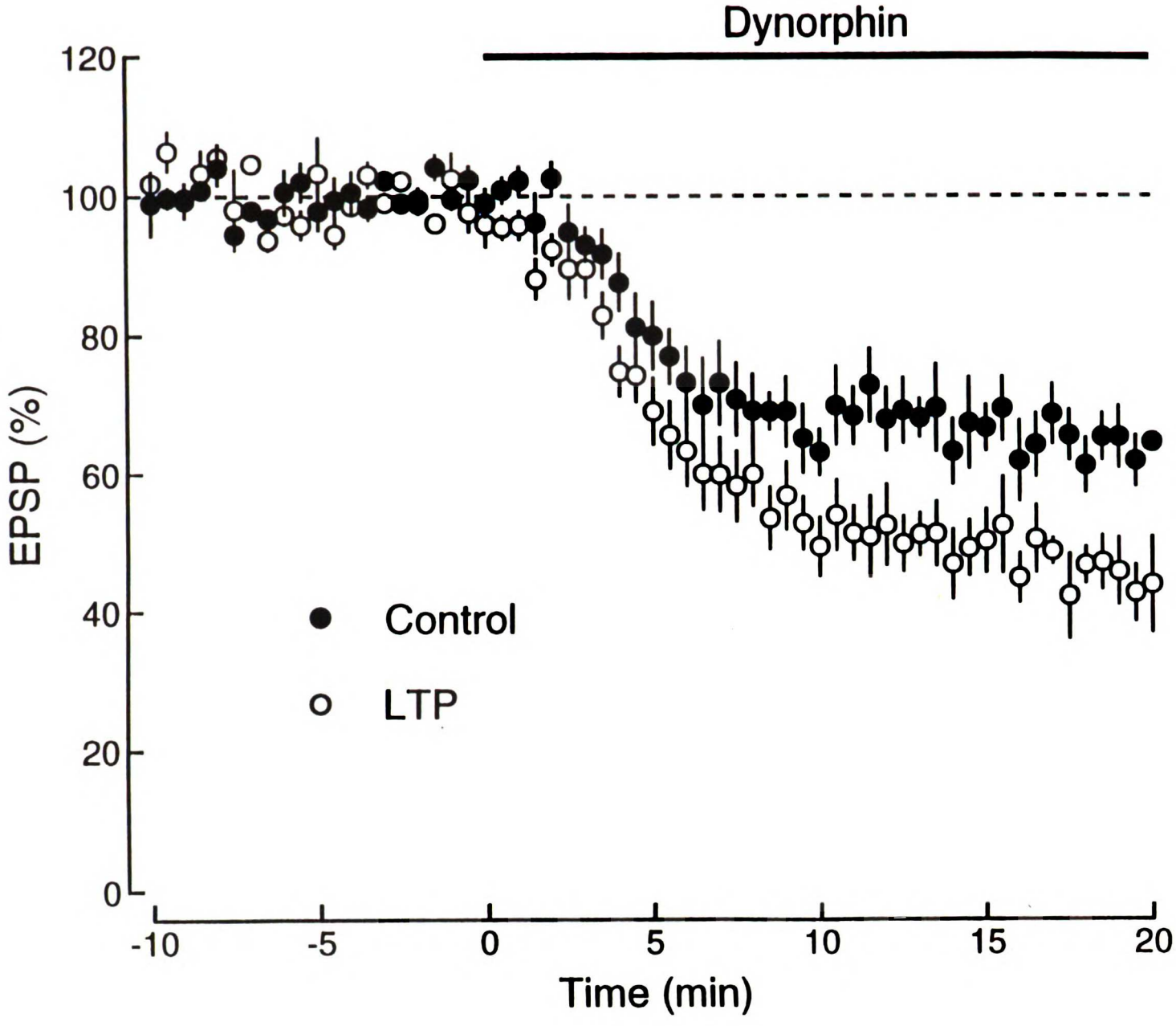


FIGURE 14. Dynorphin is more effective on mossy fiber synapses expressing LTP.

Two mossy fiber pathways in one slice were concurrently monitored. One of these was given a standard LTP inducing tetanus and the other was not. Once responses stabilized, the stimulus strengths were adjusted so that each pathway gave approximately identical responses. After a stable 10 min baseline period, dynorphin (500 nM; solid bar) was applied and the responses of both the previously tetanized (open circles) and untetanized (filled circles) pathways were monitored (n = 8). In this series of experiments analysis of the second EPSP of a pair, although suggestive, did not show a convincing difference in sensitivity to dynorphin of tetanized and untetanized pathways. Thus, it was necessary to analyze the first EPSP of a pair in order to demonstrate clearly an increased sensitivity to the effects of dynorphin. This more pronounced differential effect of dynorphin on the response to the first EPSP supports the notion that dynorphin is counteracting the effects of LTP (i.e. the enhancement seen with LTP is greater on the first pulse and the depression seen with dynorphin is greater on the first pulse).


Dynorphin
120
100
80
60
40
20
0
EPSP (%)
Control
LTP
-10
-5
0
5
10
15
20
Time (min)

DISCUSSION

This study has taken advantage of the anatomical simplicity of the hippocampal mossy fiber system to elucidate the role of the opioid peptide dynorphin at this synapse. We have demonstrated that dynorphin causes a pronounced and reliable depression of mossy fiber synaptic responses while having no effect on assoc-com responses. Through the use of the specific kappa1 agonist, U69,593, and antagonist, nor-binaltorphimine, we have attributed this depression to an action of dynorphin at kappa1 receptors. This is somewhat surprising given past anatomical evidence that suggested that kappa1 binding was low in *s. lucidum* of the guinea pig (Wagner *et al.*, 1991), however the present physiological data clearly demonstrate a kappa1 receptor presence. Consistent with much previous work on dynorphin (McFadzean *et al.*, 1987; Gannon & Terrian, 1991; Gauchy *et al.*, 1991; Kato *et al.*, 1992; Pinnock, 1992; Wagner *et al.*, 1992) we have found the action of dynorphin to be presynaptic, acting at the mossy fiber terminal to reduce transmitter release. This is not only evidenced by the experiments with iontophoretic glutamate application and paired-pulse facilitation, but is also suggested by the demonstrated enhancement of the depressant effect of dynorphin on mossy fiber synapses expressing LTP (Fig. 14). The wealth of data indicating a presynaptic locus for mossy fiber LTP (Staubli *et al.*, 1990; Zalutsky & Nicoll, 1990; Xiang *et al.*, 1994 and see chapter 4), would imply that a manipulation that interacts with that of LTP must also be presynaptic. Indeed, much of the previous work on the action of dynorphin has implicated the N-type calcium channel (Gross & Macdonald, 1987; Adamson, Xiang, Mantzourides, Brammer & Campbell, 1989; Bean, 1989; Xiang, Adamson, Brammer & Campbell, 1990) as the ultimate target affected by dynorphin. Therefore, given the above mentioned interaction between mossy fiber LTP and

the actions of dynorphin, a clear hypothesis is that the expression of LTP at the mossy fiber synapse is mediated by alterations of the N-type calcium channel. This hypothesis is addressed in the following chapter.

Further experimentation in this study has provided compelling evidence that the opioid peptide dynorphin is a neurotransmitter in this system. First we have shown that the transient depression of mossy fiber synaptic responses seen for 10-20 minutes after the tetanic stimulation of a second independent set of mossy fibers is blocked by the opioid antagonist naloxone. Second, delivery of dynorphin by puff application in *s. lucidum* in order to mimic a point release of the peptide such as would occur endogenously after tetanization at the synapse resulted in a transient depression of responses that closely resembled the endogenous effect. These data, together with the previously well established presence and releasability of dynorphin at these synapses (Terrian *et al.*, 1988; Wagner *et al.*, 1991), satisfy Paton's criteria for a neurotransmitter and demonstrate for the first time an endogenous synaptic action of a neuropeptide within the CNS. The findings that (i) its release requires high frequency stimulation, (ii) its duration of action is prolonged, perhaps due to slow clearance of the peptide, and (iii) it can act at a distance are similar to the peptidergic transmission characterized in the PNS and discussed in chapter 1, section B (Konishi *et al.*, 1981; Jan & Jan, 1982; Jan & Jan, 1983; Lundberg & Hokfelt, 1983). The heterosynaptic depression mediated by dynorphin that we have demonstrated also bears resemblance to the invertebrate, Aplysia, in which it has been shown that, aside from sensitization, noxious stimulation of the tail can also result in transient depression of the gill motor neuron response. It has been suggested that the tail stimulation releases FMRFamide from interneurons that then acts at the sensory neuron terminal to depress transmitter release, by both

increasing K^+ current and decreasing Ca^{2+} current (Mackey, Glanzman, Small, Dyke, Kandel & Hawkins, 1987).

What might be the behavioral role of the peptidergic transmission we have described at the mossy fiber synapse? Many studies indicate that opiate receptor activation impairs cognitive function and, conversely, that naloxone enhances performance in a variety of learning and memory tasks (Gallagher, 1988; Decker & McGaugh, 1991). Of particular interest, are studies that focus on the CA3 region of the hippocampus in particular and demonstrate effects specifically on spatial memory, for which the hippocampus has been implicated (Nadel, 1991). Electrical stimulation of the dentate gyrus impairs the ability of rats to learn the position of a food reward in a radial maze retrieval task (Collier & Routtenberg, 1984). This stimulation-induced impairment was prevented by systemic administration of naloxone prior to the task. Furthermore, another study demonstrated that dynorphin, applied directly into the hippocampus, also results in impairments of spatial working memory in a radial maze task that are dose dependent (McDaniel, Mundy & Tilson, 1990). In light of these behavioral results and the connection often proposed between LTP and learning and memory, the relationship we found between this mossy fiber peptidergic system and LTP in these same synapses is quite intriguing. The relative requirements for peptide release and LTP are such that synaptically released dynorphin during a tetanus can inhibit the induction of LTP, thereby regulating the threshold for LTP induction in this system. Thus, dynorphin may be an endogenous negative regulator of learning capacity.

At the mossy fiber-CA3 synapse the action of dynorphin at neighboring synapses produces a frequency dependent lateral inhibition. Such a function is widely used to accomplish important tasks almost everywhere nervous systems exist, from the eye of the crab *Limulus* to the human sensory nervous system,

where it increases our ability to distinguish two distinct points of pressure applied to the skin: sensory acuity. In the mossy fiber system of the hippocampus this function, which would serve to focus potentiation into the CA3 region, may be of equal importance. Some theories of learning and memory hypothesize that the CA3 region of the hippocampus is a site of associative memory formation based on the high degree of interconnection between CA3 pyramidal cells together with the restricted input from dentate granule cells (Marr, 1971; McNaughton & Nadel, 1990; Rolls, 1990). In computer simulations of such associative learning matrices, a critical element for achieving high memory capacity and a low rate of error upon retrieval, is that the inputs to the system be as unique as possible (Kohonen, Lehtiö, Rovamo, Hyvärinen, Bry & Vainio, 1977; Kohonen, Oja & Lehtiö, 1981). The frequency dependent lateral inhibition mediated by dynorphin that we have demonstrated here, would enhance the uniqueness of dentate granule cell input to CA3 pyramidal cells by depressing those fibers surrounding the true input that is being activated at higher frequencies. Our results demonstrating the greater sensitivity of synapses expressing LTP to the inhibitory action of dynorphin adds a conditional property to the action of dynorphin. This would aid in performing the lateral inhibition in that any surrounding fibers that may have undergone higher frequency stimulation in the recent past, and therefore be more likely to contaminate a unique input because their transmission is enhanced, would be correspondingly more effectively depressed. In addition, at a cellular mechanistic level, this suggests that dynorphin and mossy fiber LTP may share a common step, and this issue is addressed in the following chapters.

CHAPTER FOUR

The Role of Calcium and Calcium Channels in Mossy Fiber Synaptic Transmission and LTP

INTRODUCTION

As discussed in chapter 1, section C, it has been known since the experiments of Harris and Cotman (1986) that mossy fiber long-term potentiation (LTP) differs from the well studied potentiation of the CA1 region of the hippocampus, most notably in its independence of N-methyl-D-aspartate (NMDA) receptors. Since then, this lab (Zalutsky & Nicoll, 1990) and others (Katsuki *et al.*, 1991; Langdon *et al.*, 1993) have found that mossy fiber LTP, in addition to its NMDA receptor independence, differs in other striking ways from NMDA receptor-dependent LTP. Thus, it is independent of postsynaptic calcium and does not require depolarization of the postsynaptic cell (but see Williams & Johnston, 1989; Jaffe & Johnston, 1990); it does not exhibit the property of associativity or cooperativity (Kauer & Nicoll, 1988; Chattarji *et al.*, 1989; Zalutsky & Nicoll, 1992), in contrast to NMDA receptor-dependent LTP; and mossy fiber LTP, unlike NMDA receptor-dependent LTP, is associated with a change in paired pulse facilitation (Staubli *et al.*, 1990; Zalutsky & Nicoll, 1990), a process shown to be presynaptic. These findings are all consistent with a model in which both the induction and the expression of mossy fiber LTP is entirely presynaptic.

The likely role of presynaptic mechanisms in mossy fiber LTP focuses attention on the role of presynaptic calcium channels in synaptic transmission as well as LTP at this synapse. As discussed in chapter 1, section B, studies on the mechanism of action of dynorphin in a variety of systems have found it to have a highly specific presynaptic inhibitory action (McFadzean *et al.*, 1987; Gannon & Terrian, 1991; Gauchy *et al.*, 1991; Kato *et al.*, 1992; Pinnock, 1992; Wagner *et al.*, 1992), apparently acting primarily on the N-type calcium channel (Gross & Macdonald, 1987; Adamson *et al.*, 1989; Bean, 1989; Xiang *et al.*, 1990). Thus, the finding discussed in chapter 3 that dynorphin interacts with LTP is consistent

with a model in which the expression of mossy fiber LTP may occur at the level of a presynaptic N-type calcium channel. Both physiological and pharmacological studies on calcium channels have described at least four distinct subtypes of channels, L, N, P, and T (Bean, 1989; Hess, 1990; Tsien, Ellinor & Horne, 1991; Llinás, Sugimori, Hillman & Cherksey, 1992; Miller, 1993). These different channels are distinguished by a number of factors, most notably their activation and inactivation ranges, single channel conductances, and sensitivities to different antagonists. Of these, T is the only one considered a low voltage threshold channel. It is activated with depolarizations to between -60 and -70mV, but largely inactivates at voltages positive to -50mV, and has a single channel conductance of ~8pS. Of the high voltage threshold calcium channels, the L-type require the most depolarization for activation (to -10mV or beyond), then the N-type (positive to -20mV) and finally the more recently discovered P-type channels require depolarization to about -40 mV. While the conductance of L-type channels is rather large at ~25pS, N and P-types fall in the range of ~10-20pS. However, it has proved difficult if not impossible to separate clearly these channel types by their voltage sensitivity, kinetics and single channel conductances. The greatest distinctions come from their differential sensitivities to antagonists: L-type channels are blocked by dihydropyridines, N-type by the peptide toxin ω-Conotoxin GVIA (ω-CgTx) isolated from the mollusc *Conus geographus*, and P-type by the recently developed peptide toxin ω-Agatoxin IVA (ω-Aga-IVA) isolated from the funnel web spider *Agelenopsis aperta*. Thus these antagonists allow for a detailed dissection of the roles of the various calcium channel types in any given system. While it is quite well accepted that synaptic release from nerve terminals depends on presynaptic calcium influx (Zucker & Landò, 1986; Augustine, Charlton & Smith, 1987; Zucker, Delaney, Mulkey & Tank, 1991), the particular calcium channel type underlying that influx is less

established. A number of studies in various systems have implicated either L or N-type or both (Smith & Augustine, 1988), and with the recent isolation of ω-Aga-IVA, P-type channels have also been implicated in some systems (Luebke, Dunlap & Turner, 1993; Takahashi & Momiyama, 1993). Little is known, however, about which types of calcium channel control transmitter release at mossy fiber synapses, although it has been reported that the selective N-type calcium channel blocker ω-CgTx reduces synaptic transmission (Kamiya, Sawada & Yamamoto, 1988). In the present study we show that mossy fiber LTP is not dependent on postsynaptic calcium entry, but rather depends critically on presynaptic calcium entry. We also address the role of calcium channel subtypes at mossy fiber synapses in the release of glutamate, as well as in the induction and expression of LTP. These results have previously appeared in press (Castillo, Weisskopf & Nicoll, 1994a; Manzoni, Weisskopf & Nicoll, 1994).

RESULTS

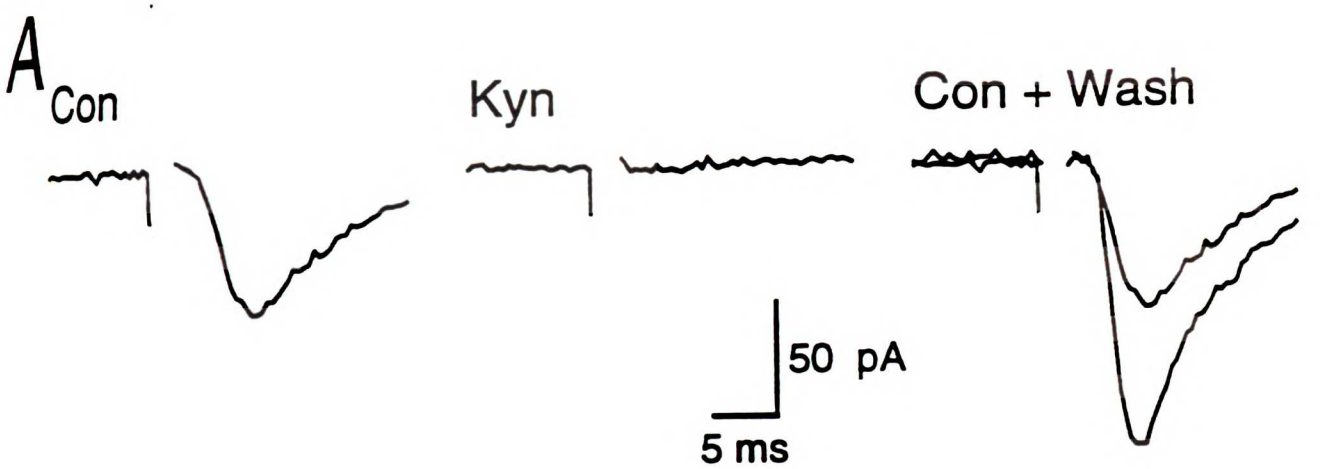
Evidence that mossy fiber LTP is dependent on presynaptic calcium

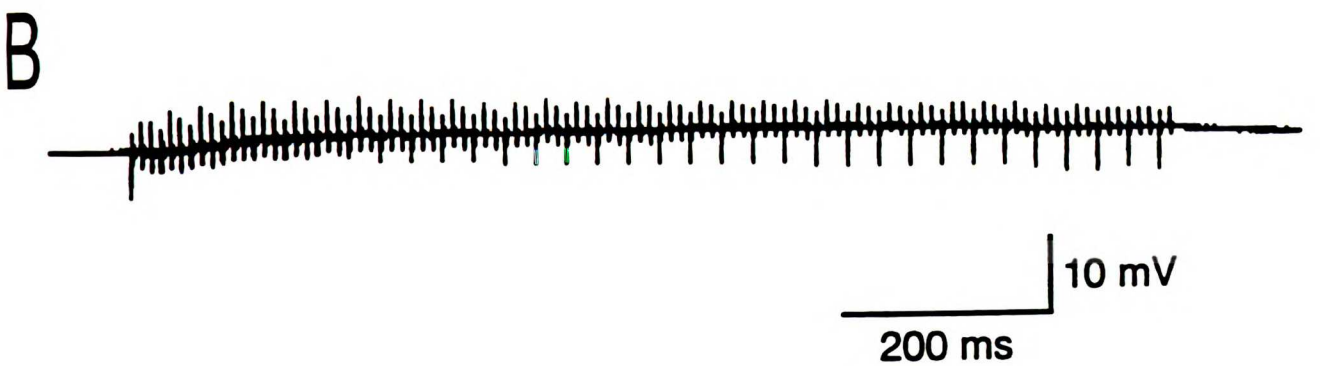
Clearly the previous data indicating that mossy fiber LTP is presynaptic (Staubli *et al.*, 1990; Zalutsky & Nicoll, 1990) would suggest that postsynaptic calcium is not necessary for the induction of mossy fiber LTP. We first addressed this question directly by tetanizing the mossy fibers during the blockade of both NMDA and non-NMDA glutamate receptors with the glutamate receptor antagonist kynurenate. Initial experiments were done using CNQX, however this antagonist proved too difficult to wash from the slice preparation presumably because of its high affinity for glutamate receptors. In contrast, kynurenate has a lower affinity than CNQX (Watkins, Krogsgaard-Larsen & Honoré, 1990) and can be quickly washed from the slice. We used concentrations of 10-20 mM and while this level of kynurenate should eliminate NMDA currents by blocking the glycine site (Kemp, Foster, Leeson, Priestley, Tridgett, Iversen & Woodruff, 1988), 25 μM D-APV was always present during LTP-inducing tetani to insure blockade. In experiments using both whole-cell patch clamp (Fig. 15) and field potential recordings (Fig. 16) we demonstrate that tetani given in the presence of kynurenate still generate mossy fiber LTP.

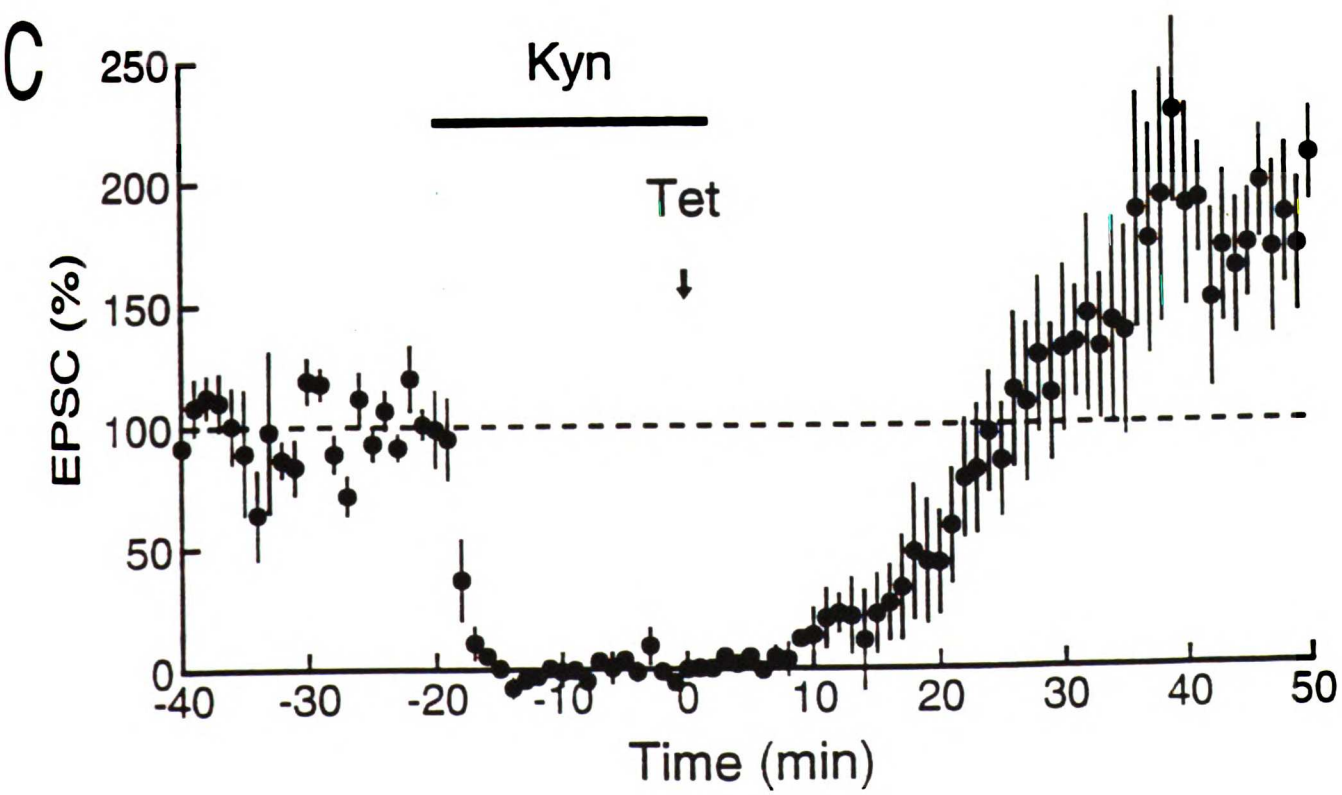
Typical voltage clamp traces from a whole-cell experiment are shown in figure 15A. After obtaining a stable baseline kynurenate was applied and the responses were quickly and completely blocked. The recording was then switched to current clamp recording mode so that the membrane potential could be monitored during the tetanus. As is evident (Fig. 15B), tetanization of the mossy fibers in this CA3 pyramidal cell caused only a 3 mV envelope of depolarization which was not only well below threshold for Na^+-dependent

FIGURE 15. Kynurenate-induced blockade of mossy fiber synaptic transmission, recorded with whole-cell pipettes, fails to block mossy fiber LTP.

A. Typical records of mossy fiber responses from whole-cell voltage clamp recordings in a CA3 pyramidal cell before addition of 20 mM kynurenate (Con), during kynurenate application (Kyn), and after a standard tetanus in kynurenate and washout of the drug (Wash). Each trace is the average of 10 responses. The membrane potential was held at -75 mV. **B.** Example of the response of the same pyramidal cell as in A to 100 Hz stimulation for 1 sec in kynurenate as recorded in current-clamp at -75 mV. **C.** Time course of whole cell experiments in which a tetanus was delivered in the presence of 20 mM kynurenate (solid bar; n = 4).


A
Con
Kyn
Con + Wash
50 pA
5 ms


B
10 mV
200 ms

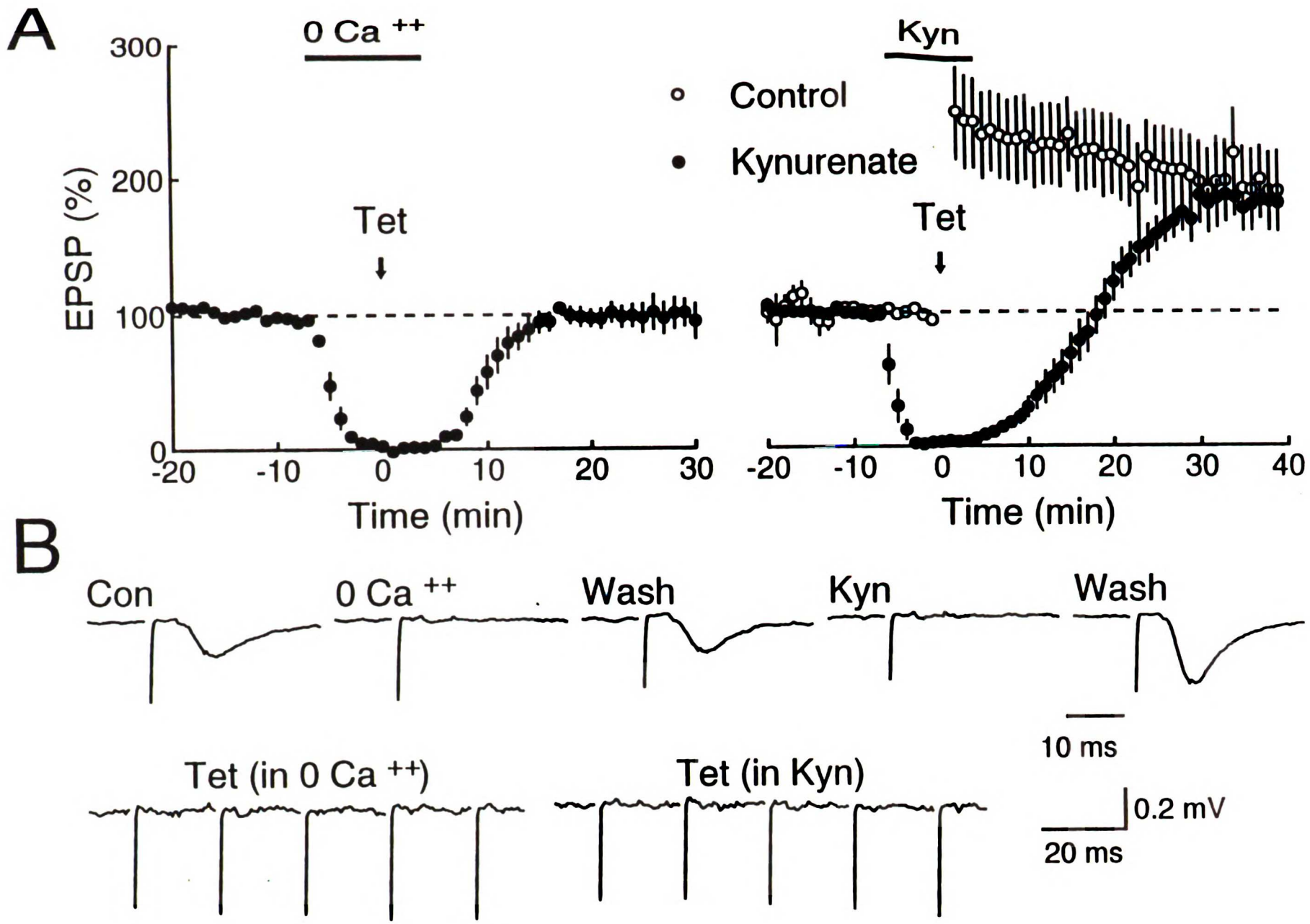

C
Kyn
Tet
EPSC (%)
250
200
150
100
50
0
-40
-30
-20
-10
0
10
20
30
40
50
Time (min)

action potential generation but is even further removed from activation of high threshold calcium channels. Following washout of the antagonist the responses clearly exhibited LTP, ruling out a role for postsynaptic high threshold voltage sensitive calcium channels. These results and those obtained in 3 other cells are plotted in figure 15C. In these experiments we also monitored responses to associational-commissural (assoc-com) stimulation and found that in the presence of kynurenate tetanization of these fibers also caused no change in the membrane potential, indicating that the abolition of synaptic transmission was widespread. These findings strongly support previous results (Zalutsky & Nicoll, 1990) indicating that mossy fiber LTP is entirely independent of calcium entry into the postsynaptic cell either through calcium channels or through non-NMDA glutamate channels that are calcium permeable, and clearly mechanistically quite distinct from the Hebbian, NMDA-dependent LTP of the CA1 region of the hippocampus.

The experiments discussed above, while ruling out postsynaptic calcium entry in mossy fiber LTP induction, do not rule out a role for calcium altogether. To address the role that calcium might play in mossy fiber LTP we attempted to repeat the observations of Ito and Sugiyama (1991) who, using extracellular field potential recording, reported that mossy fiber LTP was blocked by removal of extracellular calcium but was unaffected by blockade of non-NMDA receptors. After recording stable baseline synaptic responses, we removed calcium from the extracellular bathing medium by switching to a solution containing 0 Ca^{++}, 6 mM Mg^{++}, and 100 μM EGTA ("0 Ca^{++}"); when the field potential synaptic responses had disappeared, LTP-inducing tetani were applied. In each of 5 experiments following return to the control solution, responses returned to baseline values (Fig. 16A) indicating that in the absence of extracellular calcium the induction of mossy fiber LTP is blocked. In the same experiments we

FIG **URE 16.** Blockade of mossy fiber synaptic transmission by removal of $[Ca^{++}]_e$, but not by kynurenate, blocks mossy fiber LTP.

A. After extracellular field potentials were stable for at least 10 min, the Ringer was switched to one containing 0 mM Ca^{++}, 6 mM Mg^{++} and 100 μM EGTA (0 Ca^{++}, solid bar), henceforth referred to as '0 Ca^{++}'. Once responses were completely blocked, 4 tetani at 50 or 100 Hz (for 1 s, separatd by 20 s) were given and the Ringer returned to normal (n = 5). After responses had restabilized for > 20 min, 10-20 mM kynurenate was added to the bath (Kyn, solid bar). Once responses were completely blocked, the identical tetani were repeated and the kynurenate washed out. In two additional cases, the tetani were given in kynurenate without a prior 0 Ca^{++} period, and these are included in the analysis. Superimposed on the averaged responses subjected to tetanus in kynurenate (filled circles) are a set of experiments obtained during this study where a standard tetanus (100 Hz) was given in control conditions (open circles; n = 6). **B.** Typical records of extracellular mossy fiber field responses before switching to 0 Ca^{++} (Con), in the 0 Ca^{++} Ringer (0 Ca^{++}), after tetanus in 0 Ca^{++} and return to normal Ringer (Wash), during kynurenate application (Kyn), and after tetanus in kynurenate and subsequent washout of the antagonist (Wash). Each trace is the average of 10 responses. Also shown are the first five stimuli of a tetanus given in 0 Ca^{++} ['Tet (in 0 Ca^{++})'] and one given in kynurenate ['Tet (in Kyn)']. No restoration of synaptic responses was observed at any time during the 4, 1 second tetani.



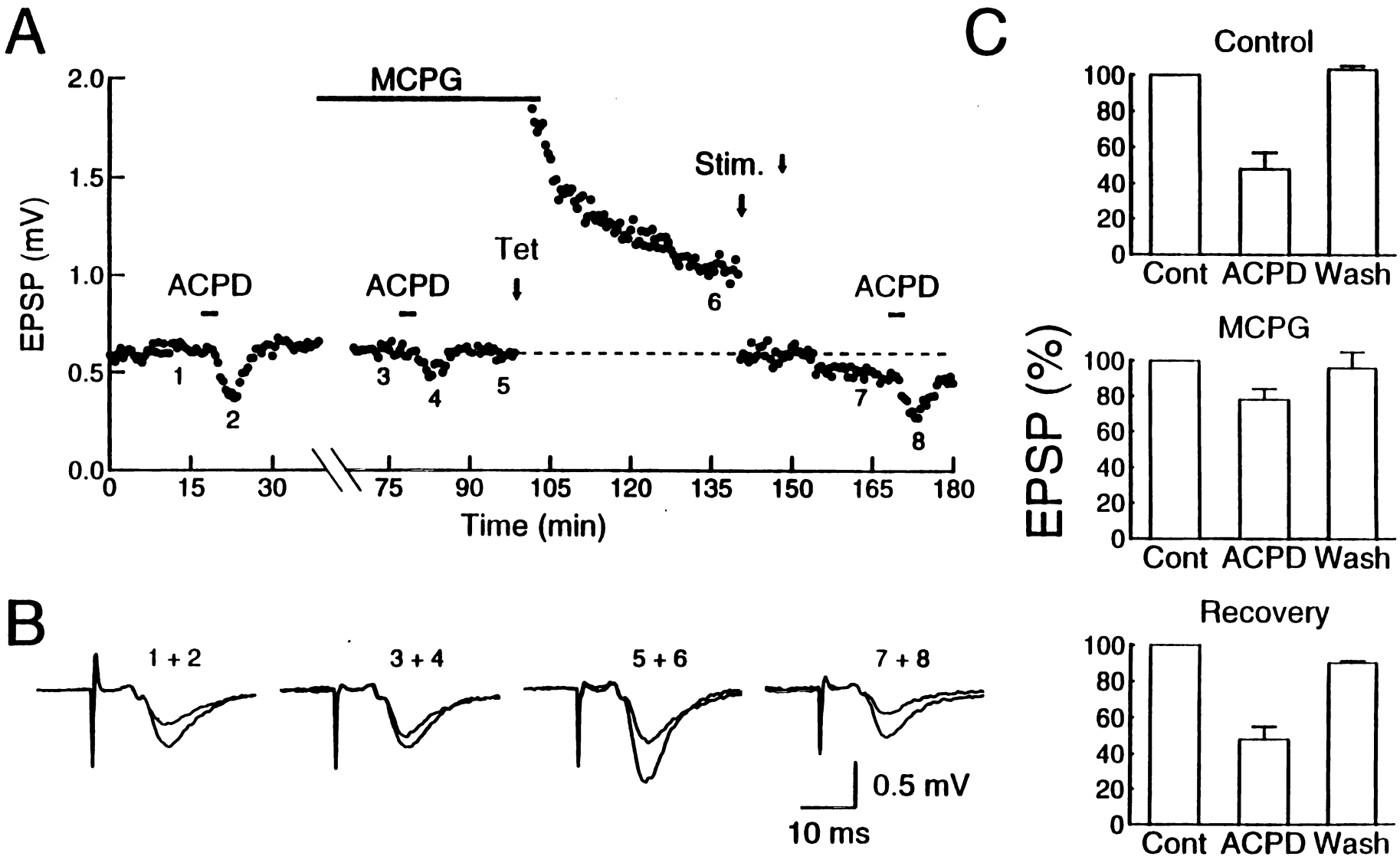
compared this presynaptic blockade of synaptic transmission to the postsynaptic blockade of non-NMDA glutamate receptors with kynurenate for their effects on LTP. If subsequent tetanus in kynurenate induced LTP, as one would expect given the whole cell experiments, this would not only serve as a control that these slices could exhibit LTP, but it would also highlight the difference between the effects of presynaptic and postsynaptic calcium entry. Although application of 10-20 mM kynurenate quickly blocked synaptic transmission, if the mossy fibers were tetanized robust LTP was, indeed, observed upon washout of kynurenate (n = 7) (Fig. 16A). In separate experiments, no tetanus was given during the blockade and the responses returned to baseline values (n = 5, data not shown), indicating that kynurenate application alone did not have any potentiating effects on the responses. Superimposed on the LTP induced in the presence of kynurenate is LTP evoked in a series of control experiments obtained during this study (n = 6). Despite the blockade of non-NMDA receptors the magnitude of LTP was indistinguishable from control (control = 194 ± 30%, Kyn = 185 ± 12%, P>0.05, Student's t-test). The field potentials from a typical experiment are shown in figure 16B. The "0 Ca^{++}" and kynurenate solutions completely blocked the synaptic response, and, importantly, during the tetanus in either solution there was no recovery of synaptic transmission in the mossy fiber pathway (Fig. 16B). The complete failure to induce any restoration of evoked synaptic responses during the tetanus in kynurenate, both in the field as well as the whole-cell experiments, eliminates any problems of interpretations associated with potential disynaptic inputs and strongly suggests that postsynaptic activation of ionotropic glutamate receptors is not necessary for the induction of mossy fiber LTP.

It remains, however, a possibility that glutamate released during the tetanus could act at presynaptic or postsynaptic metabotropic glutamate

receptors to induce LTP. Traditionally this has been a difficult issue to address because of the lack of a good metabotropic glutamate receptor antagonist. The recent discovery of a new such antagonist, (RS)-a-methyl-4-carboxyphenylglycine (MCPG) (Eaton, Jane, Jones, Porter, Pook, Sunter, Vdvarhelyi, Roberts, Salt & Watkins, 1993), has raised the possibility of addressing this question. Indeed, it has recently been proposed by one group that metabotropic glutamate receptor activation is a necessary step in LTP induction at mossy fiber synapses and that MCPG can block this LTP (Bashir, Bortolotto, Davies, Berretta, Irving, Seal, Henley, Jane, Watkins & Collingridge, 1993). Therefore, in separate experiments, we tested the action of MCPG on mossy fiber LTP. To ensure that the MCPG was effective in our system, we also examined its effects on the presynaptic depressant action of the metabotropic glutamate receptor agonist aminocyclopentane dicarboxylate (ACPD) (Baskys & Malenka, 1991). As can be seen from the example in figure 17A and B, MCPG (1mM) was effective at antagonizing the depression caused by ACPD (5μM), yet LTP could still be induced even though the MCPG had been present for over 30 minutes. Subsequently the MCPG could be washed out and the ACPD-induced depression returned. A summary of the effects of MCPG on the ACPD-induced depression from four such experiments is shown in figure 17C. In these same slices, however, LTP evoked by one tetanus at 100 Hz for 1 second could still be induced and the potentiation averaged 207 ± 27% (measured at 35 min), which was not different from the LTP evoked in a separate set of control slices (232 ± 32%, n=4). We then also tested the effects of MCPG on LTP alone in experiments using a 'blind' protocol. Control responses were obtained before a coded vial was given to the experimenter that contained either control artificial cerebrospinal fluid (ACSF) or ACSF with MCPG (1mM). After washing this in for at least 20 minutes a tetanus of 100Hz for 250 milliseconds was given and at

FIGURE 17. MCPG inhibits ACPD induced depression, but not LTP, at mossy fiber synapses.

A. Single experiment illustrating the effect of 5 μM ACPD (short bars labeled ACPD) on mossy fiber synaptic responses in control conditions, in MCPG (1 mM; long bar labeled MCPG) 40 min after it was added, and more than 60 min after washout. The ACPD effect was reversibly reduced in the presence of MCPG. A tetanus (1x 100 Hz for 1 s) was given more than 60 min after addition of MCPG (arrow, Tet), yet LTP was not blocked. After LTP had stabilized the stimulus was reduced (Stim. ↓) such that the responses were of similar magnitude to those during the control period. **B.** Sample records from the experiment illustrated in **A.** The numbers above the records correspond to the time at which they were taken as indicated in **A.** Each record is the average of 5 responses. **C.** Averaged data from four experiments like that in **A** indicating response levels in ACPD (5-10 μM for 4 min; ACPD), and after washout of ACPD (Wash) as compared to 10 min stable pre-ACPD baseline levels (Cont) for the following three cases: Top row — before addition of MCPG (Control), middle row — at least 20 min after MCPG was added to the Ringer's (0.5-1 mM; MCPG), and bottom row — at least 40 min after washout of MCPG (Recovery).


A
MCPG
Stim.
Tet
ACPD
ACPD
ACPD
EPSP (mV)
Time (min)
B
1 + 2
3 + 4
5 + 6
7 + 8
0.5 mV
10 ms
C
Control
MCPG
Recovery
EPSP (%)
Cont ACPD Wash

least 15 minutes later the solution was returned to the control. The reduced tetanus protocol was used to increase the likelihood of MCPG antagonizing LTP. After the series was completed the code was broken and the results analyzed. It was immediately clear, however, that MCPG had not blocked LTP. Measured at 35 minutes after the tetanus, control LTP was 153 ± 7% (n = 6) of baseline, and in MCPG it was 144 ± 7% (n = 6). Thus we concluded that, in our system, the metabotropic antagonist MCPG does not block mossy fiber LTP.

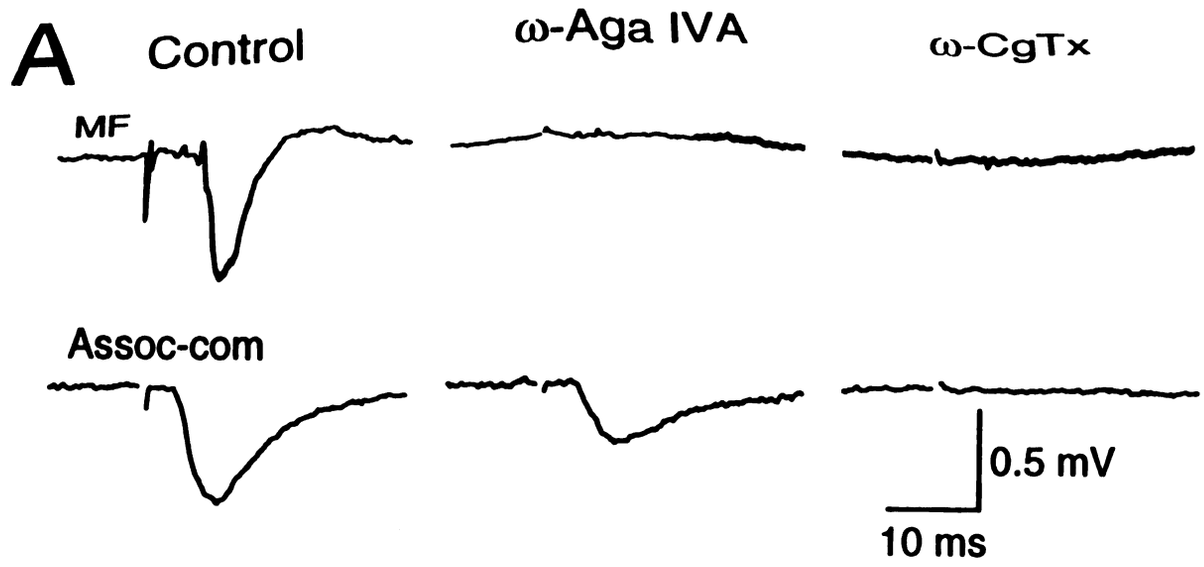
Overall, these results emphasize the importance of presynaptic calcium in both transmitter release and mossy fiber LTP. We have, therefore, undertaken a pharmacological analysis of the types of calcium channels involved in mossy fiber synaptic transmission and the effect of selective calcium channel blockers on mossy fiber LTP.

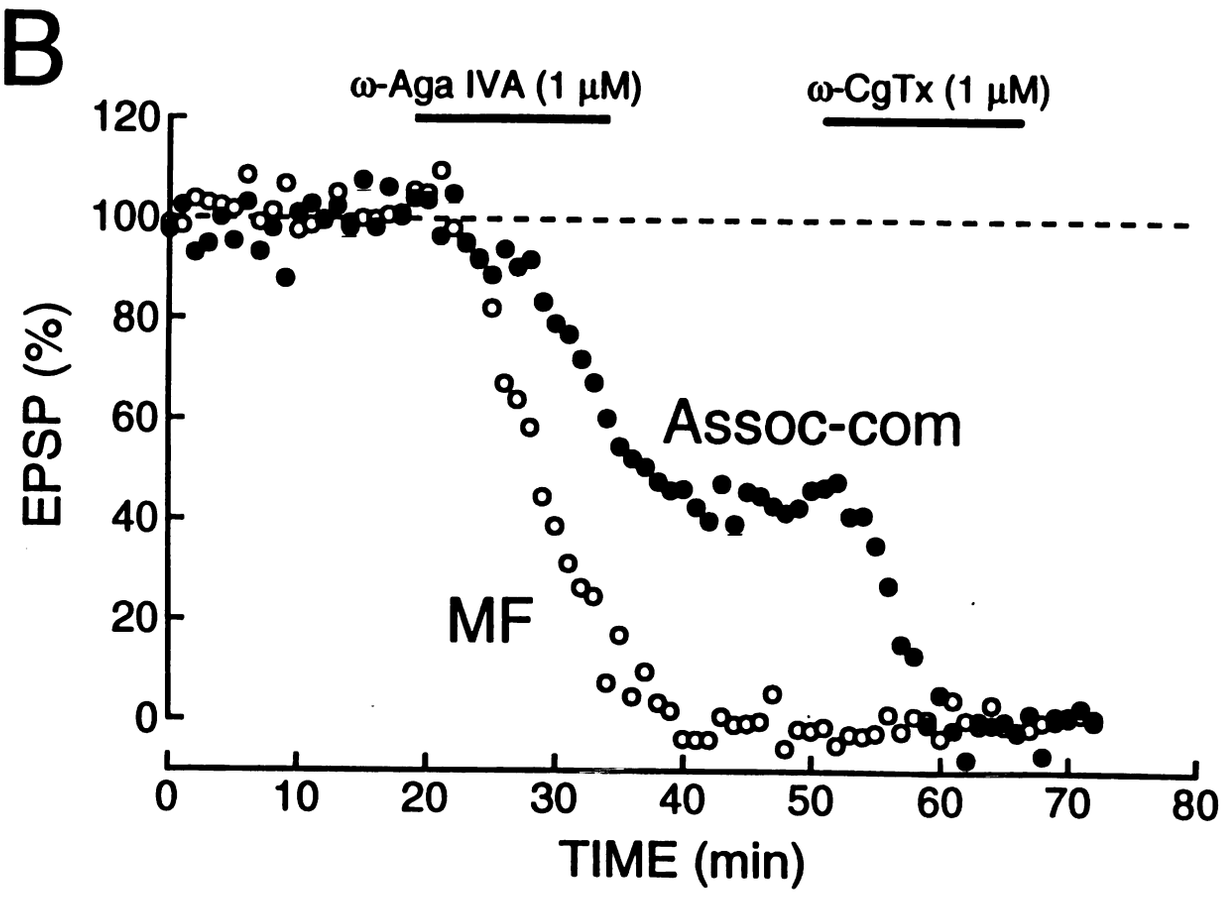
Effect of calcium channel blockers on mossy fiber and associational-commissural transmission

Figures 18-21 are taken from Castillo et al. (1994a) and demonstrate the dependence of release at assoc-com and mossy fiber synapses on different subtypes of calcium channels. Three calcium channel blockers that are reported to be relatively selective for L, N, and P were used. These included nifedipine to block L-type channels (Fox, Nowycky & Tsien, 1987; Aosaki & Kasai, 1989), ω-CgTx to block N-type channels (Fox *et al.*, 1987; Aosaki & Kasai, 1989; Plummer, Logothetis & Hess, 1989; Cox & Dunlap, 1992), and ω-Aga-IVA to block P-type channels (Mintz, Adams & Bean, 1992; Mintz, Venema, Swiderek, Lee, Bean & Adams, 1992). Nifedipine, even at concentrations as high as 30 μM had only minor effects on synaptic transmission at either type of synapse. On average it reduced mossy fiber responses by 13 ± 3% (n = 5). The case shown in figure 18

FIGURE 18. Effects of ω-Aga-IVA and ω-CgTx on mossy fiber and associational-commissural field potentials.

A. Sample records of the field potentials which are plotted in **B**. Each trace represents the average of 4 responses during control, after application of ω-Aga-IVA (1 μM) and after subsequent application of ω-CgTx (1 μM). **B.** Time course of the changes in mossy fiber (MF; open circles) and associational-commissural (Assoc-com; filled circles) field potentials to which ω-Aga-IVA (1 μM) and ω-CgTx (1 μM) were applied successively for 15 minute periods.





demonstrates that ω-Aga-IVA potently inhibits both mossy fiber and assoc-com synaptic transmission, although more so the former. In this example ω-Aga-IVA (1 μM) reduced the assoc-com responses by approximately 50%, but at the same time completely blocked the mossy fiber response. This differential sensitivity was seen in all preparations. Overall, ω-Aga-IVA caused a 60 ± 5% (n = 5) reduction in the assoc-com responses (Fig. 19A1) and a 96 ± 1% (n = 15) (Fig. 19B1) reduction in the mossy fiber responses. The effects of ω-CgTx were slightly less as it was found to block 53 ± 3% (n = 6) of the assoc-com response (Fig. 19A2) and 75 ±2% (n = 15) of the mossy fiber response (Fig. 19B2). In the presence of both ω-CgTx and ω-Aga-IVA transmission was entirely blocked in the assoc-com pathway (n = 5) (Figs. 18 and 19A3) as well as in the mossy fiber pathway (n = 4) (Figs. 21B, 22B3). The above results indicate that, while the assoc-com and mossy fiber synapses are both sensitive to the two blockers, the assoc-com synapses are considerably less sensitive to ω-Aga-IVA.

Dose-response curves were generated to ensure that residual responses observed after stabilization in the presence of an antagonist were not due to incomplete block by the antagonist. As can be seen in figure 20A the lowest effective concentration of ω-Aga-IVA on the mossy fiber responses was 10 nM and the antagonism saturated at 250 nM. On the other hand, no detectable effect was seen on the assoc-com pathway at a concentration of 100 nM. The IC 50 for the mossy fiber responses sensitive to ω-Aga-IVA was 50 nM, while it was 190 nM for the assoc-com responses. With ω-CgTx (Fig. 20B) slight antagonism of the mossy fiber responses was observed at 10 nM, which saturated at 300 nM. The blocking action of these two toxins on the mossy fiber responses was reversible during prolonged washing (up to 3 hrs).

The combined antagonism of mossy fiber transmission by ω-CgTx and ω-Aga-IVA was considerably over 100% raising some question as to the specificity

FIGURE 19. Inhibitory effects of ω-Aga-IVA and ω-CgTx on associational-commissural and mossy fiber responses.

Normalized field potentials are plotted against time. Toxins were applied for 15 minutes. **A.** Partial inhibition by ω-Aga-IVA (n = 5) (A_1) or ω-CgTx (n = 6) (A_2) of associational-commissural (Assoc-com) field potentials. Combined application of both toxins completely blocked Assoc-com responses (n = 4) (A_3). **B.** While ω-Aga-IVA (n = 15) almost completely blocked mossy fiber (MF) responses (B_1), ω-CgTx (n = 15) induced a partial inhibition (B_2).

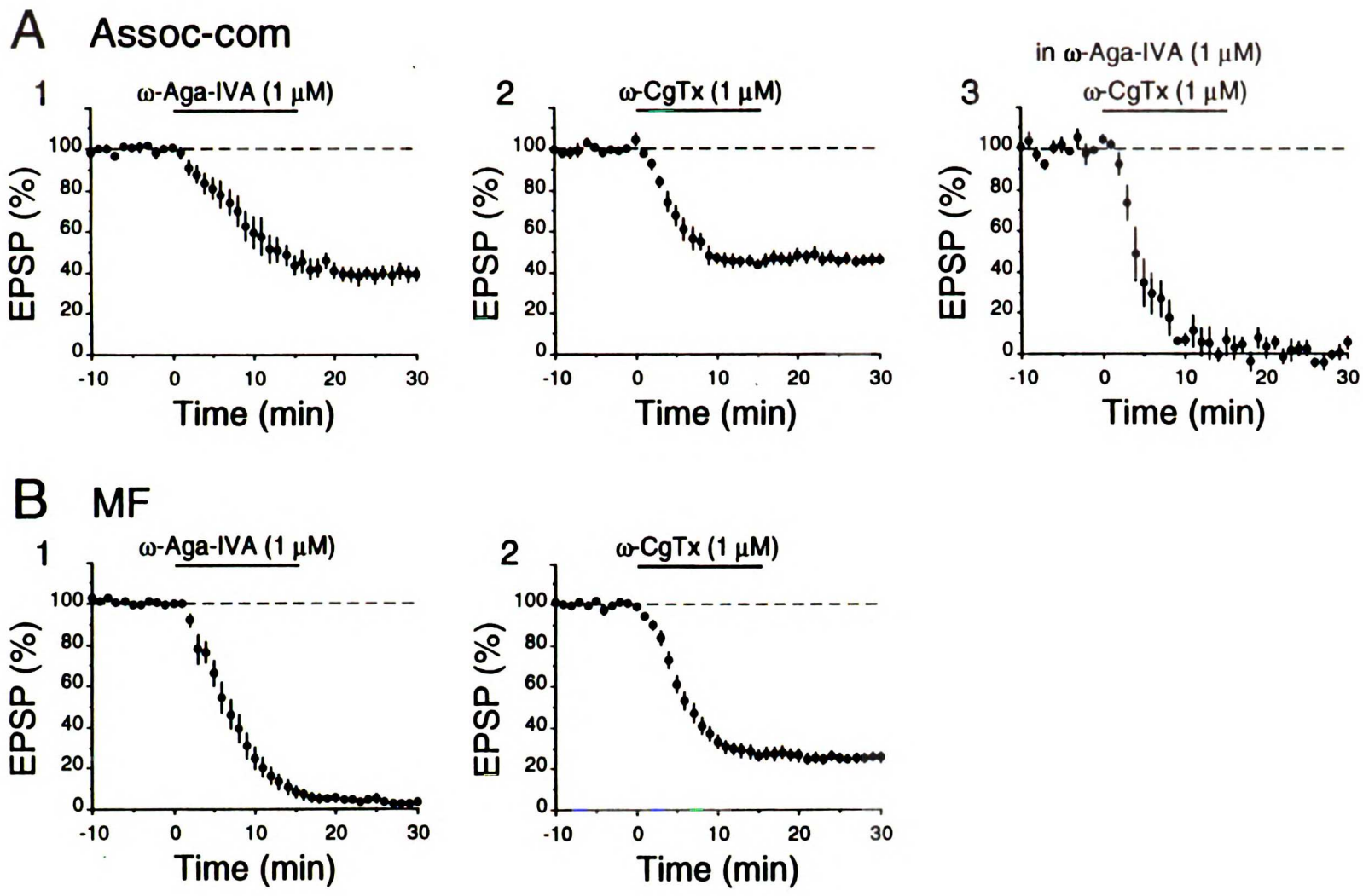
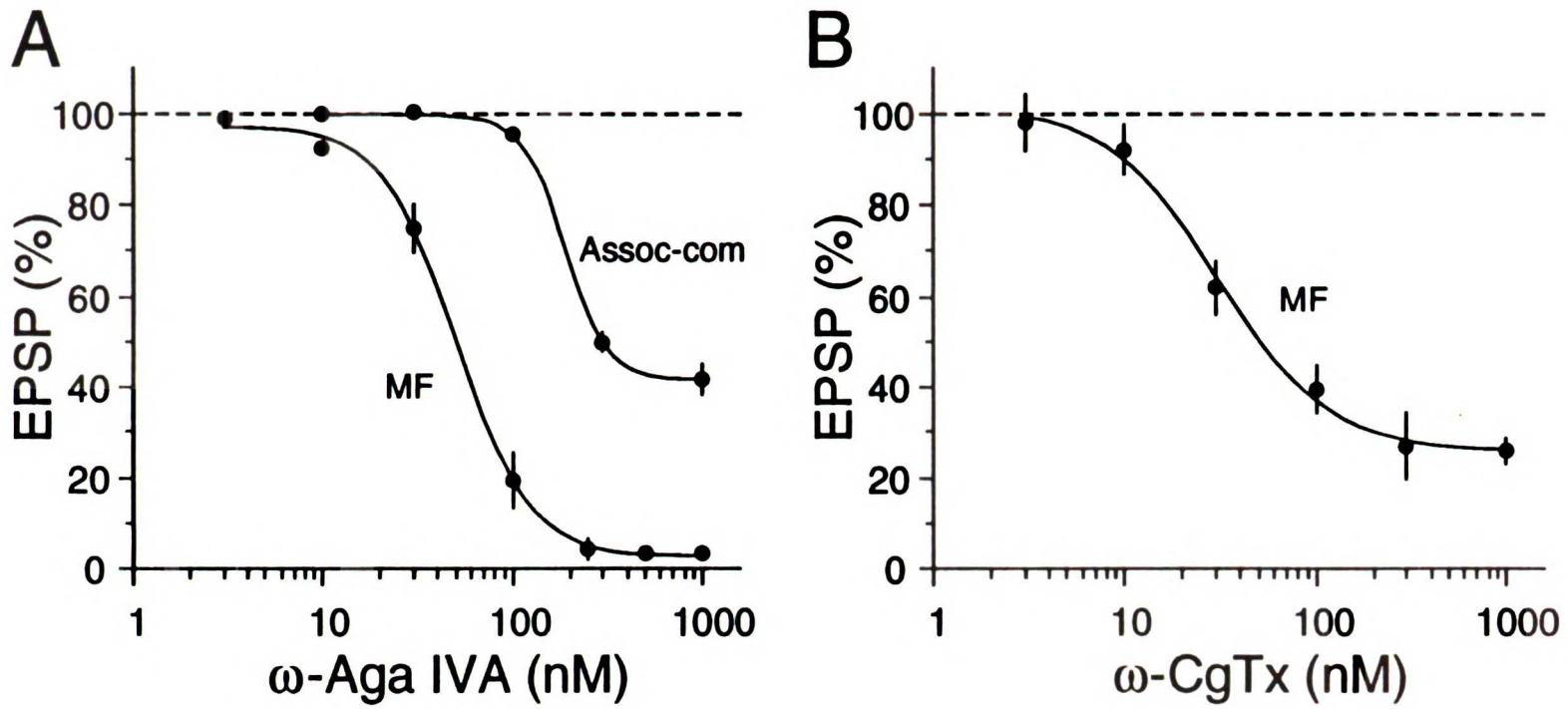

A
Assoc-com
1
ω-Aga-IVA (1 μM)
2
ω-CgTx (1 μM)
3
in ω-Aga-IVA (1 μM)
ω-CgTx (1 μM)
EPSP (%)
Time (min)
B
MF
1
ω-Aga-IVA (1 μM)
2
ω-CgTx (1 μM)

FIGURE 20. Dose-response relationship of ω–Aga-IVA and ω-CgTx on synaptic transmission.

A. Comparison of associational-commissural (Assoc-com) and mossy fiber (MF) responses to increasing concentrations of ω-Aga-IVA. **B.** Effect of increasing concentrations of ω-CgTx on MF responses. Each point represents mean ± s.e.m. of 4 to 15 measurements, expressed as a percentage of control responses. For each concentration the toxin was applied until the response had completely stabilized. The fitting curves were drawn according to $y= \{y_{max} - y_{min}/1+ (x/IC_{50})^n\} + y_{min}$, where y_{max} = response in absence of antagonist, y_{min} = response remaining in maximal antagonist concentration, x = concentration, IC_{50} = concentration of antagonist producing 50% inhibition of the response and n = slope.


A
100
80
60
40
20
0
EPSP (%)
Assoc-com
MF
1
10
100
1000
ω-Aga IVA (nM)
B
100
80
60
40
20
0
EPSP (%)
MF
1
10
100
1000
ω-CgTx (nM)

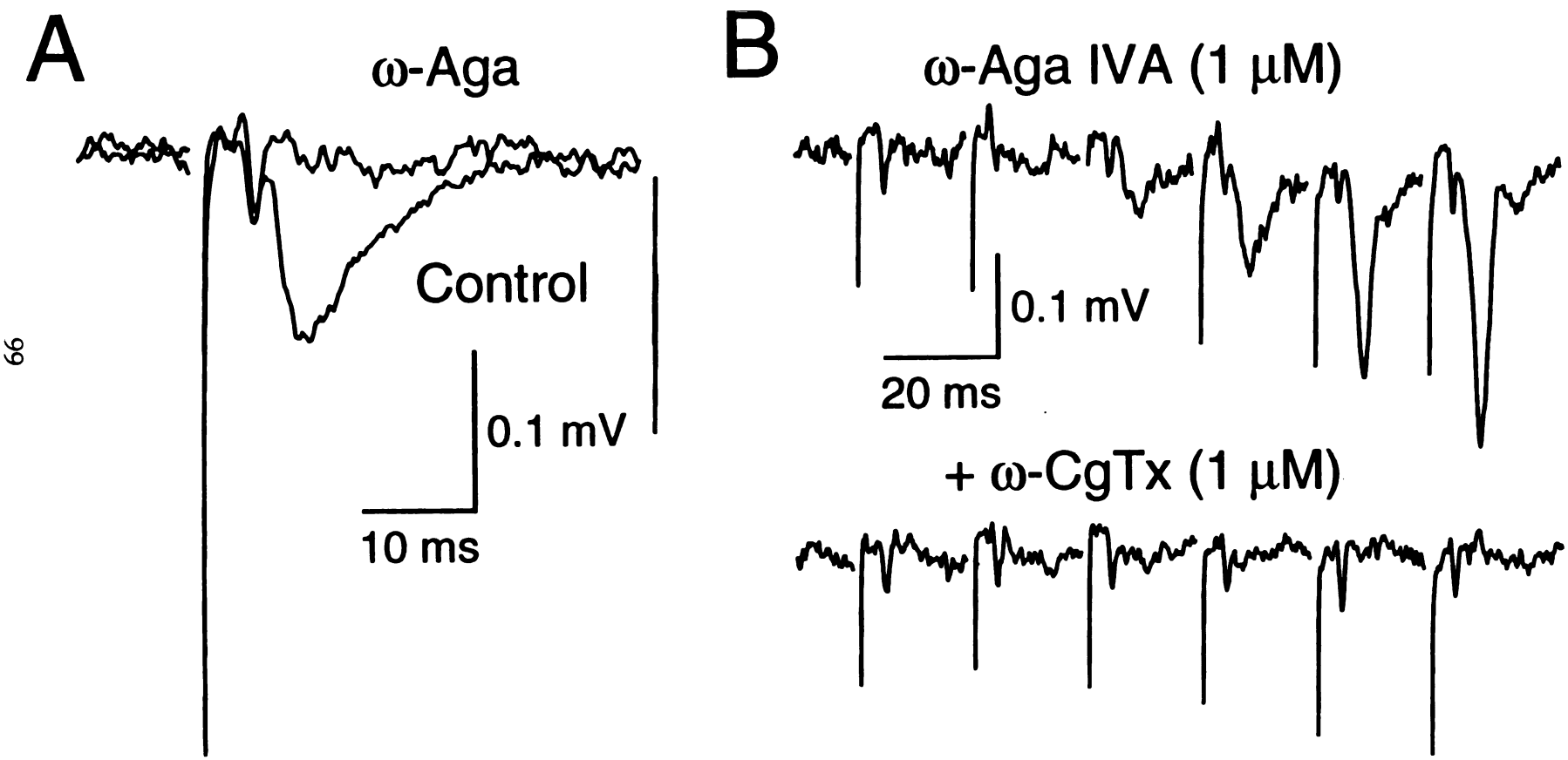
of the antagonists. This is a difficult question to resolve convincingly in a slice preparation, nonetheless, in order to begin to address this issue in our system, we examined a case in which ω-Aga-IVA completely blocked the mossy fiber response. In such a case, some response can be restored by the facilitation accompanying high frequency stimulation (Fig. 21). This restored transmission, which was typically even larger than the response at low frequency stimulation, is completely blocked by ω-CgTx (n = 5), implying that ω-CgTx sensitive channels were still present after the initial block by ω-Aga-IVA. In the presence of both toxins no restoration of transmission, either during prolonged tetani (1 sec) or afterward, occurred. A more direct analysis of this question was done in recent experiments by Wu and Saggau (1994) using the hippocampal slice preparation combined with calcium imaging, and the results indicated specificity of the two antagonists. They found, using the same concentrations of antagonists we used, that at CA3 to CA1 synapses ω-Aga-IVA blocked the same percentage of measured calcium transient whether or not ω-CgTx had previously been applied. A more likely explanation for the greater than 100% combined individual antagonist actions of ω-CgTx and ω-Aga-IVA, therefore, is a non-linear dependence of release on calcium. This issue is addressed in the following discussion section.

Effect of calcium channel blockers on mossy fiber LTP

Since the earlier results of this chapter highlight the dependence on calcium of mossy fiber LTP and reinforce other studies that indicate that the induction and expression of mossy fiber LTP is entirely presynaptic, the effects of calcium channel blockers on mossy fiber LTP were examined. Figures 22 and 23

FIGURE 21. High frequency stimulation in the presence of ω-Aga-IVA restores synaptic transmission.

Representative experiment in which ω-Aga-IVA (1 μM) completely blocked mossy fiber field potentials (**A**). However, transmission was restored during frequency facilitation (**B**, upper trace) by a 50 Hz train stimulation. This restored synaptic transmission was abolished by ω-CgTx (1 μM) (**B**, lower trace). Stimulus artifacts were blanked out for clarity.


A
ω-Aga
Control
0.1 mV
10 ms
B
ω-Aga IVA (1 μM)
0.1 mV
20 ms
+ ω-CgTx (1 μM)

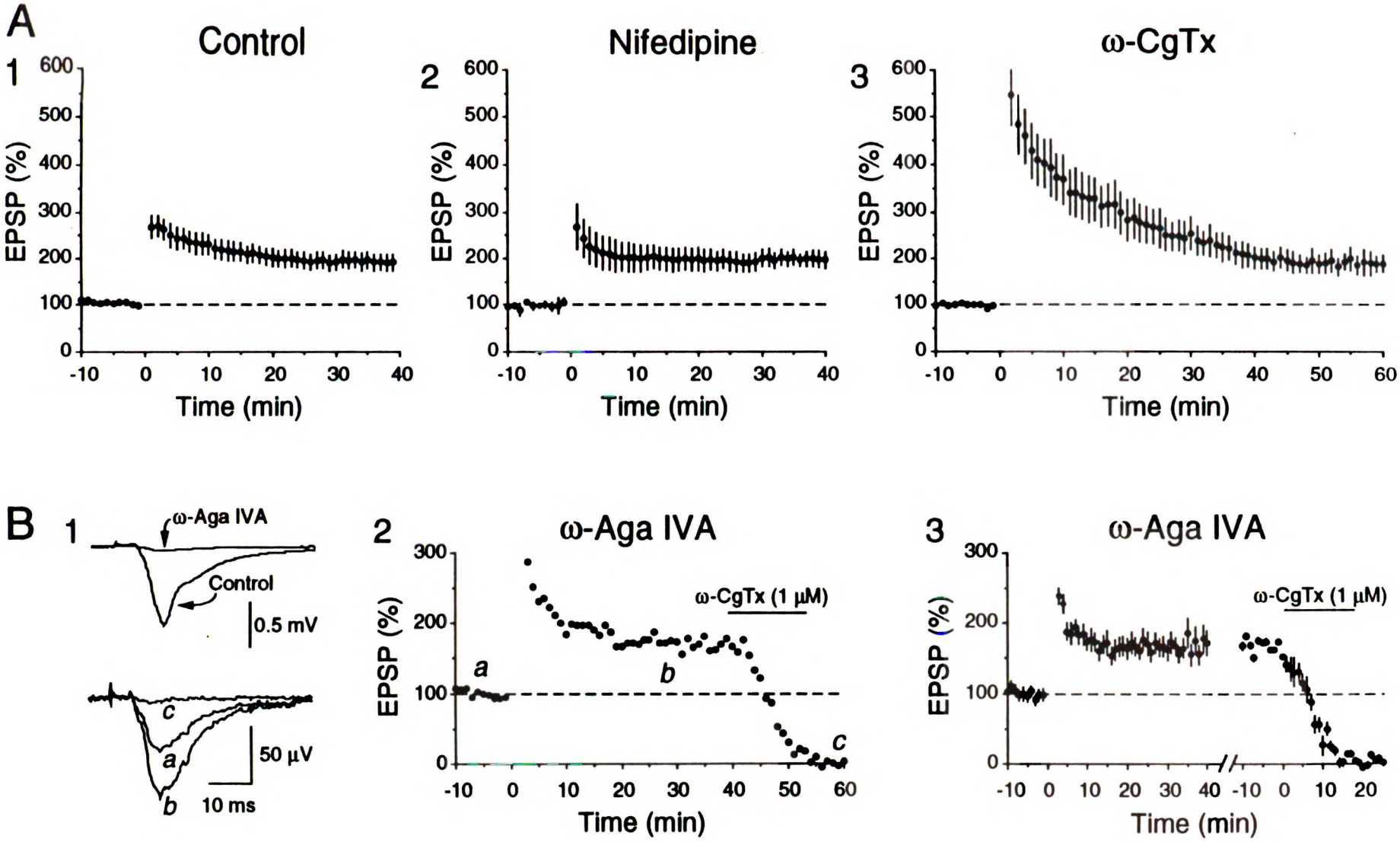
are taken from Castillo et al. (1994a) and demonstrate the results obtained with the same calcium channel blockers used above.

LTP induced in the presence of nifedipine was not significantly different from control, as can be seen by comparing the graphs in figures 22A1 and 22A2, indicating that L-type channels play no role in mossy fiber LTP. Although ω-CgTx reduced baseline transmission by 70%, LTP could still be elicited, indicating that N-type channels are also not required for LTP (Fig. 22A3). Interestingly, although without any obvious explanation, while the sustained LTP was no different from controls, the magnitude of the potentiation was considerably enhanced during the first 30 min. Finally, LTP could be obtained even in the presence of ω-Aga-IVA. Figures 22B1 and 2 show an experiment in which ω-Aga-IVA blocked 96% of the mossy fiber response. Nonetheless, tetanic stimulation elicited an LTP of normal magnitude. Subsequent application of ω-CgTx completely blocked the mossy fiber synaptic responses. Figure 22B3 summarizes all the experiments in which some detectable transmission remained in the presence of ω-Aga-IVA and shows that the magnitude of LTP was similar to that in control. In three other cases, in which ω-Aga-IVA completely blocked transmission, tetanization caused a long lasting restoration of synaptic responses in one slice and failed to restore transmission in the other two.

In most experiments the LTP inducing tetani were given after returning to the control solution, since the action of the toxins was shown to be irreversible over the time scale of these experiments. If the blocking action of the toxins were voltage dependent, it is conceivable that the enhanced response seen after the tetanus might be due to a tetanus-induced relief from some of the pre-existing block rather than the induction of LTP. If the tetanus was given in the continued presence of the toxins (n = 2 for each toxin), however, LTP still was observed.

FIGURE 22. Effects of calcium channel blockers on mossy fiber LTP.

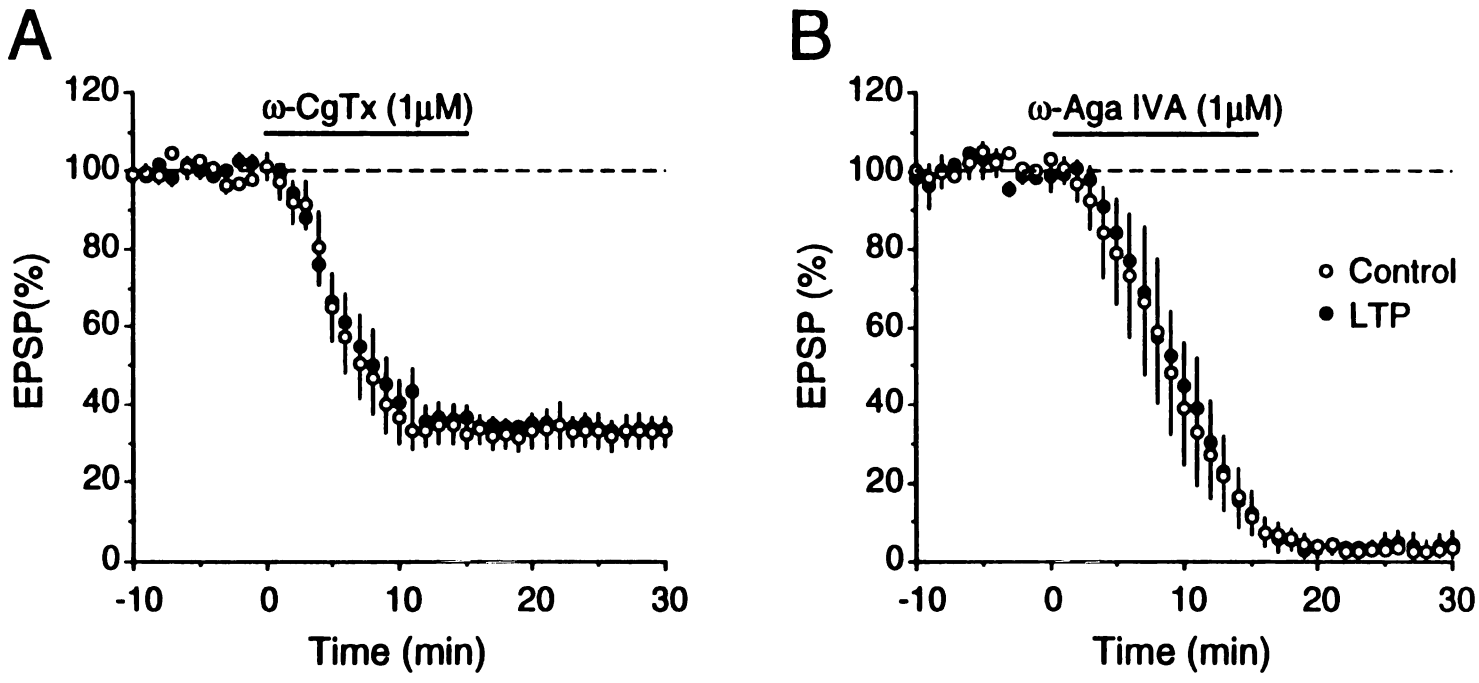
A. Mossy fiber field potentials are plotted against time. After baseline responses were stable for at least 10 min a standard tetanus was given in normal Ringer (Control, n = 9) (A_1), 30μM Nifedipine (n = 5) (A_2), or 1 μM ω-CgTx (n = 6) (A_3). **B.** Single experiment (B_1) showing that, while ω-Aga-IVA (1 μM) greatly reduced mossy fiber field potential responses (96%), LTP still was elicited (compare trace a to trace b in lower panel). These results are plotted in B_2. After LTP had stabilized ω-CgTx was applied and completely blocked synaptic transmission. The letters refer to the time at which the records illustrated in B_1 were obtained. Note that the trace labeled as a in the lower panel is the same as that labeled as ω-Aga-IVA in the upper panel but at a higher gain. Each trace is an average of 15 individual responses. B_3, summarizes 6 experiments using the same protocol as B_1.


A
Control
Nifedipine
ω-CgTx
1
2
3
EPSP (%)
Time (min)
B
ω-Aga IVA
Control
0.5 mV
50 µV
10 ms
a
b
c
ω-CgTx (1 µM)

These experiments clearly demonstrate that mossy fiber LTP is not dependent on one type of calcium channel. On the other hand, it could be that the expression of mossy fiber LTP is due to an up-regulation of both N- and P-type calcium channels in such a way that the relative contribution of N- and P-type calcium channels to synaptic transmission were changed. This possibility was tested by simultaneously comparing in the same slice the effect of either ω-CgTx or ω-Aga-IVA on a control pathway and one expressing LTP. The results are shown in figure 23 and indicate that the two toxins exert identical blocking effects on control and LTP expressing pathways. These results suggest that LTP is not associated with a change in the relative contribution of either N-or P-type calcium channels to synaptic transmission, although an identical up-regulation of both is still a possibility.

FIGURE 23. Mossy fiber LTP expression in ω-Aga-IVA and ω-CgTx.

Two separate mossy fiber pathways in one slice were concurrently monitored. Field potentials are plotted against time. One of these pathways was given a standard LTP-inducing tetanus (filled circles) and the other was not (open circles). Forty min after the tetanus and after decreasing the stimulus strength to the tetanized pathway, such that the amplitude of the responses in both pathways was of similar magnitude, either 1μM ω-CgTx (n = 4) or 1 μM ω-Aga-IVA (n = 3) was applied. These experiments did not show any difference in sensitivity to either toxin between tetanized and untetanized pathways.


A
ω-CgTx (1μM)
EPSP(%)
120
100
80
60
40
20
0
-10
0
10
20
30
Time (min)
B
ω-Aga IVA (1μM)
EPSP (%)
Control
LTP
Time (min)

DISCUSSION

In the present experiments two general issues were addressed. The first issue concerned the role of calcium in the induction of mossy fiber LTP and the second involved the role of calcium channel subtypes in mossy fiber synaptic transmission.

A role for presynaptic calcium entry in mossy fiber LTP

Using extracellular field potential recordings we have demonstrated that, although removal of extracellular calcium entirely blocked LTP induction, even during massive postsynaptic blockade of synaptic transmission with the glutamate receptor antagonist kynurenate tetanic stimulation of the mossy fibers results in LTP that is indistinguishable from that evoked under control conditions. While this strongly suggests that postsynaptic activity is unnecessary for mossy fiber LTP, we extended these results by using whole-cell patch clamp recordings in order to monitor postsynaptic activity directly. Using these techniques, we were able to demonstrate that, indeed, mossy fiber LTP is inducible even in the absence of postsynaptic activity. These results confirm and extend the results of Ito and Sugiyama (1991) and put a number of constraints on the mechanisms involved in the induction of mossy fiber LTP.

The results appear to exclude the necessity of entry of calcium into the postsynaptic cell, via either the non-NMDA receptor channel or via voltage sensitive calcium channels as proposed by Johnston and colleagues (Johnston *et al.*, 1992). This is because both of these pathways for calcium entry would require significantly more postsynaptic depolarization than was seen in the presence of kynurenate. This lack of depolarization after stimulation of either the

mossy fibers or the assoc-com fibers indicates a widespread suppression of activity within the slice which is critical in another sense. An objection that has often been raised in regards to intracellular or whole cell studies of the mossy fiber synapse is that there is always the possibility of having a disynaptic, and thus assoc-com rather than mossy fiber, input (see chapter 1, section A). When recording extracellular field potentials from *stratum lucidum*, the presence of a current sink serves to identify a monosynaptic mossy fiber input, but when recording from a single CA3 pyramidal cell, it is hard to categorically rule out a disynaptic input. The lack of activity in the presence of kynurenate, even during a tetanus, as demonstrated in this study indicates that the only fibers activated are the mossy fibers that are directly stimulated. They do not evoke postsynaptic depolarization, and thus cannot utilize this for generating LTP nor activating intervening neurons. Therefore, when the kynurenate is washed out, even if the recorded response is a disynaptic assoc-com input, the increased response size seen would have to be the result of an increased number of fibers recruited by a *potentiated* mossy fiber response at an earlier synapse rather than direct potentiation of the assoc-com synapse. Thus, this is strong evidence for the presynaptic nature of mossy fiber LTP.

There is still, however, the possibility that a postsynaptic action of glutamate at a metabotropic receptor could be playing a role. A number of different metabotropic glutamate receptors have been cloned, and shown to have a variety of effects in different areas of the nervous system. As a group they form a unique yet heterogeneous class of seven transmembrane region, G-protein coupled receptors that have been shown to lead to changes in inositol 1,4,5-triphosphate and/or cyclic-AMP (cAMP) levels (Nakanishi, 1992; Schoepp & Conn, 1993). In area CA1 of the hippocampus, activation of the metabotropic glutamate receptor with ACPD has been demonstrated to generate LTP under

certain conditions (Bortolotto & Collingridge, 1992). Other studies have indicated that something in addition to postsynaptic calcium entry, possibly glutamate acting at a metabotropic receptor, is necessary for potentiation (Kullmann, Perkel, Manabe & Nicoll, 1992). In area CA3, the suggestion has also been made that a metabotropic receptor may be involved (Ito & Sugiyama, 1991). Nonetheless, the testing of these theories has been difficult because of the lack of a good metabotropic glutamate receptor antagonist. The recent discovery of just such an antagonist, MCPG (Eaton *et al.*, 1993), and its subsequent use (Bashir *et al.*, 1993) seemed to have confirmed the previous theories concerning its role in LTP. In our experiments, however, while we have confirmed that MCPG blocks the action of the selective agonist ACPD, we did not see a block of mossy fiber LTP (nor LTP in area CA1; see Manzoni et al., 1994). MCPG washes out rapidly and its inhibition of the effects of ACPD were relatively weak, which raises the possibility that during a tetanus the MCPG might not effectively prevent the released glutamate from activating the metabotropic receptor. This is unlikely to explain the differences between our results and those of Bashir et al. (1993), however, because the tetanus we used was a fraction of theirs (100Hz for 250ms compared to 100Hz for 1sec). Other experimental differences could still contribute to the different results (including the use of guinea pigs vs. rats, temperatures of 22° vs. 34°, and submerged vs. interface recording chambers); nonetheless, in our system, the metabotropic antagonist MCPG does not block LTP. Our finding also argues against a role for metabotropic receptor-mediated release of calcium from intracellular stores in mossy fiber LTP. Previous studies indicating that preventing the rise in postsynaptic calcium with chelators had no effect on mossy fiber LTP (Zalutsky & Nicoll, 1990; Katsuki *et al.*, 1991; Langdon *et al.*, 1993; but see Williams & Johnston, 1989) are in accord with these newer findings. While these results taken together seem to rule out the postsynaptic

involvement of calcium, they strongly suggest that the entry of calcium into the presynaptic terminal is essential for the induction of mossy fiber LTP.

The dependence of mossy fiber LTP on the entry of calcium into the presynaptic terminal, together with the evidence that the expression of mossy fiber LTP is likely to be presynaptic (Staubli *et al.*, 1990; Zalutsky & Nicoll, 1990; Xiang *et al.*, 1994), leads to a very simple model in which the rise in presynaptic calcium during a tetanus triggers a series of steps within the mossy fiber terminal that result in a long lasting enhancement of evoked release. Another possibility, which cannot be ruled out, is that a transmitter must be released from mossy fiber terminals that then feeds back onto the terminal to initiate LTP. The only known transmitters released from mossy fibers, however, are glutamate and dynorphin and the inability of antagonists of glutamate (see above) or opioid receptors (see chapter 3) to block LTP appears to rule out an essential role for either glutamate or dynorphin. The results of some other groups (Martin, 1983; Derrick *et al.*, 1991; Derrick *et al.*, 1992) that are contrary to ours and do show a block of mossy fiber LTP by naloxone, would be consistent with this second hypothesis.

The role of specific calcium channels in mossy fiber synaptic transmission and long-term potentiation

These results indicating the critical dependence of mossy fiber LTP on presynaptic calcium, along with the results discussed in the previous chapter illustrating an interaction between mossy fiber LTP and the depressant effects of dynorphin, raise the issue of the roles of specific calcium channels at this synapse. Little is known about the role of these channels in normal synaptic transmission at this synapse, therefore we addressed this issue before examining

their relation to mossy fiber LTP. These experiments are only possible because of the detailed pharmacological studies that have identified highly specific antagonists for different members of the voltage sensitive calcium channel family. The L-type calcium channels are sensitive to dihydropyridines such as nifedipine used in our study, while N-type channels are blocked by ω-CgTx (Bean, 1989; Hess, 1990). The more recently discovered P-type calcium channel has been shown to be selectively blocked by ω-Aga-IVA (Mintz *et al.*, 1992; Mintz *et al.*, 1992; Miller, 1993; Mintz & Bean, 1993), although this toxin has not been studied as extensively as ω-CgTx. While these two toxins appear to be selective for their respective channel (Mintz *et al.*, 1992; Mintz *et al.*, 1992), it is possible that they may block as yet uncharacterized types of calcium channels. For simplicity we will refer to nifedipine, ω-CgTx, and ω-Aga-IVA sensitive calcium channels as L-type, N-type, and P-type calcium channels, respectively.

A number of studies using synaptosomal preparations have implicated N-type (Reynolds, Wagner, Snyder, Thayer, Olivera & Miller, 1986) and P-type channels (Turner, Adams & Dunlap, 1992) in transmitter release. Such preparations made from mossy fiber terminals have also been studied and have implicated both L- and N-type channels (Terrian, Dorman & Gannon, 1990), while P-type channel involvement has not yet been addressed. These studies, however, measure release by examining neurotransmitter concentrations after synaptosomes in a test tube have been exposed to high levels of potassium for times of up to some minutes. Clearly the conditions, and potentially the release mechanisms, are significantly different from action potential induced release at a synapse, and, indeed, the results often differ from those obtained from intact synaptic preparations. Notably, synaptic studies of the dependence of release on calcium channels in the nucleus accumbens, cerebellum, spinal cord, and in areas CA1 and CA3 of the hippocampus (Kamiya *et al.*, 1988; Horne & Kemp, 1991;

Takahashi & Momiyama, 1993, and the present findings) have shown that blockers of L-type calcium channels have no effect on synaptic transmission. A number of studies have shown ω-CgTx to affect excitatory transmission with the amount of block they find somewhat variable, although usually not complete (Kamiya *et al.*, 1988; Dutar, Rascol & Lamour, 1989; Horne & Kemp, 1991; Luebke *et al.*, 1993; Takahashi & Momiyama, 1993). Fewer studies have examined the dependence of transmission on P-type calcium channels with the newer toxin ω-Aga-IVA. Those that have, however, find a more effective block of transmission than with ω-CgTx (Luebke *et al.*, 1993; Takahashi & Momiyama, 1993; Yamamoto, Sawada & Ohno-Shosaku, 1994).

Clearly, though, different synapses can be affected differently as evidence has shown that ω-CgTx is a more effective blocker of inhibitory transmission than excitatory (Dutar *et al.*, 1989; Horne & Kemp, 1991). Therefore, we examined the effects of calcium channel blockers in area CA3 of the hippocampus, where we have found that while L-type channels do not play a role in mossy fiber synaptic transmission, both N- and P-type calcium channels do contribute at both mossy fiber and assoc-com synapses. While the effects of ω-CgTx were roughly equivalent at mossy fiber and assoc-com synapses, ω-Aga-IVA was far more effective at mossy fiber synapses where it virtually eliminated transmission, suggesting that the ratio of N- to P-type channels at the two synapses is different. Moreover, mossy fiber transmission was approximately four-fold more sensitive to ω-Aga-IVA than assoc-com transmission, raising the possibility that the calcium channels affected at these two synapses may be different.

In any case, it is clear that mossy fiber synaptic transmission depends on both N- and P-type calcium channels (see also Kamiya *et al.*, 1988; Yamamoto *et al.*, 1994), although more heavily on the P-type. Our data, however, raise an

intriguing question: Why is it that the combined block of mossy fiber synaptic transmission by ω-CgTx and ω-Aga-IVA exceeded 100%? Given the pharmacological data discussed above and our data showing that even with synaptic transmission completely blocked by ω-Aga-IVA an ω-CgTx sensitive calcium component could still be achieved with frequency facilitation (Fig. 21), it is unlikely that the toxins are crossing over to act on the other channel type. A possibility raised by Takahashi and Momiyama (1993) upon facing a similar dilemma in other synapses of the brain is that the greater than 100% combined blockade is due to a power function for the calcium dependence of neurotransmitter release. Their equations were the following:

$$A = (1-a)^m \qquad (1)$$

$$B = (1-b)^m \qquad (2)$$

$$C = c^m \qquad (3)$$

$$a + b + c = 1 \qquad (4)$$

Where m is the power coefficient that dictates the calcium sensitive release; A, B and C represent the fractional response remaining in the presence of ω-Aga-IVA, ω-CgTx and both toxins, respectively; and a, b and c represent a fraction of presynaptic calcium channels sensitive to ω-Aga-IVA, ω-CgTx and neither, respectively.

Applying the same equations to our data we can discard equation (3) because our results show a complete blockade of transmission in the presence of both ω-Aga-IVA and ω-CgTx at both mossy fiber and assoc-com synapses, therefore C = c = 0, and equation (4) then becomes a + b = 1. At the mossy fiber synapse, our data indicate that A = 0.04 and B = 0.25 (Fig. 19). This, then, would be most compatible with m = 3 resulting in a + b = 1.03, which is indeed similar

to the results of Takahashi and Momiyama (1993) and Wu and Saggau (1994) at a number of non-mossy fiber synapses, and agrees well with early estimates of the calcium dependency of release studied at the neuromuscular junction (Dodge & Rahamimoff, 1967). At the assoc-com synapse, however, our data indicate that A = 0.40 and B = 0.47 (Fig. 19). These values would result in an m between 1 and 2 (a + b = 1.13 and 0.68, respectively).

It is possible that the difference in m values simply indicates different dependencies of release on calcium at the two types of synapses. If the calcium power function is actually the same at the two synapses, however, then something else must explain the discrepancy. One possibility is that N- and P-type calcium channels are distributed differently at the two synapses. The high calcium power function we find at mossy fiber synapses is consistent with the N- and P-type both contributing calcium to any given release site at which the dependence of release on calcium is of the high order we found. This would suggest an intermingling of channel types at every release site. If this is indeed the power function for release at the assoc-com synapses as well, then our data showing a more linear dependence could be explained by a segregation of N- and P-type channels to separate release sites. In this case, blocking one type of calcium channel would eliminate one *population* of release sites. Thus, if N-channel and P-channel release sites made up the entire population, then the individual inhibitions by the two antagonists would sum to one as we find, even though release at any given site follows a high calcium power function. If this is the case, an interesting question is whether the N- and P-type channels segregate at different release sites at the same synapse, or whether there are two populations of synapses.

The use of postsynaptic responses as a surrogate measure for presynaptic calcium currents, although necessitated by the difficulty of recording from

presynaptic terminals in this preparation, makes the precise determination of the release characteristics in the terminal impossible. It would seem that calcium imaging techniques might be better suited for such analysis, although at this point the temporal and spatial resolutions of this technique are not sufficient to resolve very localized and rapid transients of calcium (Zucker *et al.*, 1991), an understanding of which is necessary to examine the questions raised above.

The role of specific calcium channels in mossy fiber LTP

The hypothesis raised in the previous chapter, that the expression of mossy fiber LTP was mediated by alterations to the N-type calcium channel, was only made more intriguing by the finding that this LTP depends on presynaptic calcium entry. Therefore, we used the specific inhibitors discussed above to address this issue. We found, however, that mossy fiber LTP could still be evoked even in the presence of ω-CgTx. This LTP generated with N-type calcium channels blocked had an initial decay period that was significantly larger than that seen under control conditions, although the stable LTP was identical. Why this is the case remains a question. Nonetheless, the presence of LTP indicates that induction and expression of mossy fiber LTP can occur in the complete absence of N-type calcium channels, disproving the hypothesis raised. Furthermore, results with ω-CgTx also indicated that the actions of dynorphin at the mossy fiber synapse discussed in the previous chapter are unaltered in the absence of the N-type calcium channel. Indeed, results with specific blockers of L- and P-type calcium channels suggest that dynorphin's actions may be entirely independent of these calcium channels (Castillo, Weisskopf & Nicoll, 1994b). A similar presynaptic inhibition by dynorphin that appears to be independent of

calcium channels has also been demonstrated in the neurohypophysis (Kato *et al.*, 1992).

In the presence of both ω-Aga-IVA and ω-CgTx LTP mossy fiber transmission was always blocked completely, and tetanization never resulted in the appearance of synaptic transmission. The present results with L-, N- and P-type calcium channel blockers, however, indicate that the induction and expression of mossy fiber LTP still occur even in the complete absence of one of these channel types. Furthermore, there was no difference between the effect of N- or P-type calcium channel blockers on potentiated and control responses. These data indicate that the calcium necessary for mossy fiber LTP can enter through either N- or P-type calcium channels, and that the expression of LTP is not the result of an unequal up-regulation of N- and P-type calcium channel activity. The results of the experiments comparing the effects of ω-Aga-IVA or ω-CgTx on tetanized and control responses would still allow for the possibility that LTP results from identical up-regulation of the two types of calcium channel. These results, along with others implicating a presynaptic locus for mossy fiber LTP (Staubli *et al.*, 1990; Zalutsky & Nicoll, 1990; Xiang *et al.*, 1994), favor an induction mechanism in which, during the tetanus, presynaptic calcium rises to a level that triggers a series of events that eventually lead to an increased sensitivity of the release process to calcium. What some of these events triggered by calcium may be is addressed in the following chapter.

CHAPTER FIVE

Cyclic AMP and Mossy Fiber LTP

INTRODUCTION

As discussed in chapter 1, section D, much of the early work on cyclic-AMP (cAMP) was done on non-mammalian and non-neuronal preparations. As is the case for many signal transduction pathways, much early work on cAMP signalling actions within the mammalian central nervous system (CNS) was carried out in the hippocampus. One of the first examples of this were experiments done in the early 1980's by Madison and Nicoll (1982; 1986) that described the action of noradrenaline on pyramidal cells of the CA1 region of the hippocampus.

Depolarizing current pulse injections given to pyramidal cells result in action potential generation with a frequency that decreases dramatically over the course of the current injection, a phenomenon known as accommodation. Madison and Nicoll (1982; 1986) showed that bath application of noradrenaline acting at a beta receptor positively coupled to adenylyl cyclase results in a marked, reversible, reduction of this accommodation. They showed that this reduction in accommodation was the result of a reduction in the slow afterhyperpolarization (AHP), a calcium activated K^+ current inducible by repetitive action potential discharge. Furthermore, they showed that this effect of noradrenaline on the AHP is blocked by inhibitors of adenylyl cyclase and mimicked by forskolin, intracellular cAMP or bath application of its non-hydrolyzable analog 8-bromo-cAMP.

In tissue culture preparations of hippocampal cells cAMP enhances currents through α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) sensitive channels, presumably of the gluR6 variety, since these are known to be enhanced by cAMP (Greengard, Jen, Nairn & Stevens, 1991; Wang, Salter & MacDonald, 1991). It has also been proposed that forskolin in the

presence of 3-isobutyl-1-methylxnthine (IBMX) can cause enhancement of CA1 synaptic responses (Chavez-Noriega & Stevens, 1992). At the mossy fiber synapse, we have found that the initial finding by Hopkins and Johnston (Hopkins & Johnston, 1988) of a forskolin-induced enhancement is quite reproducible.

In the previous chapter, evidence implicated presynaptic calcium entry as critical for inducing mossy fiber long-term potentiation (LTP), but the first hypothesis concerning mechanisms underlying LTP that are engaged by the calcium– that an alteration of a known calcium channel was responsible for the LTP– was disproved. What then are other possible candidates for the intraterminal effects of calcium responsible for LTP? The effects of forskolin at mossy fiber synapses led us to the hypothesis that a cAMP cascade might be involved. In this chapter we demonstrate that the enhancing effect of forskolin is selective for mossy fiber responses over those of associational-commissural (assoc-com) fibers, is presynaptic, and is mimicked by Sp-cAMPS, a non-hydrolyzable membrane permeant analog of cAMP. Furthermore, mossy fiber LTP occludes this forskolin-induced enhancement and is blocked by inhibitors of the cAMP dependent protein kinase (PKA). We propose that cAMP produced by a calcium sensitive adenylyl cyclase is responsible for mossy fiber LTP. These results have previously appeared in press (Weisskopf, Castillo, Zalutsky & Nicoll, 1994).

RESULTS

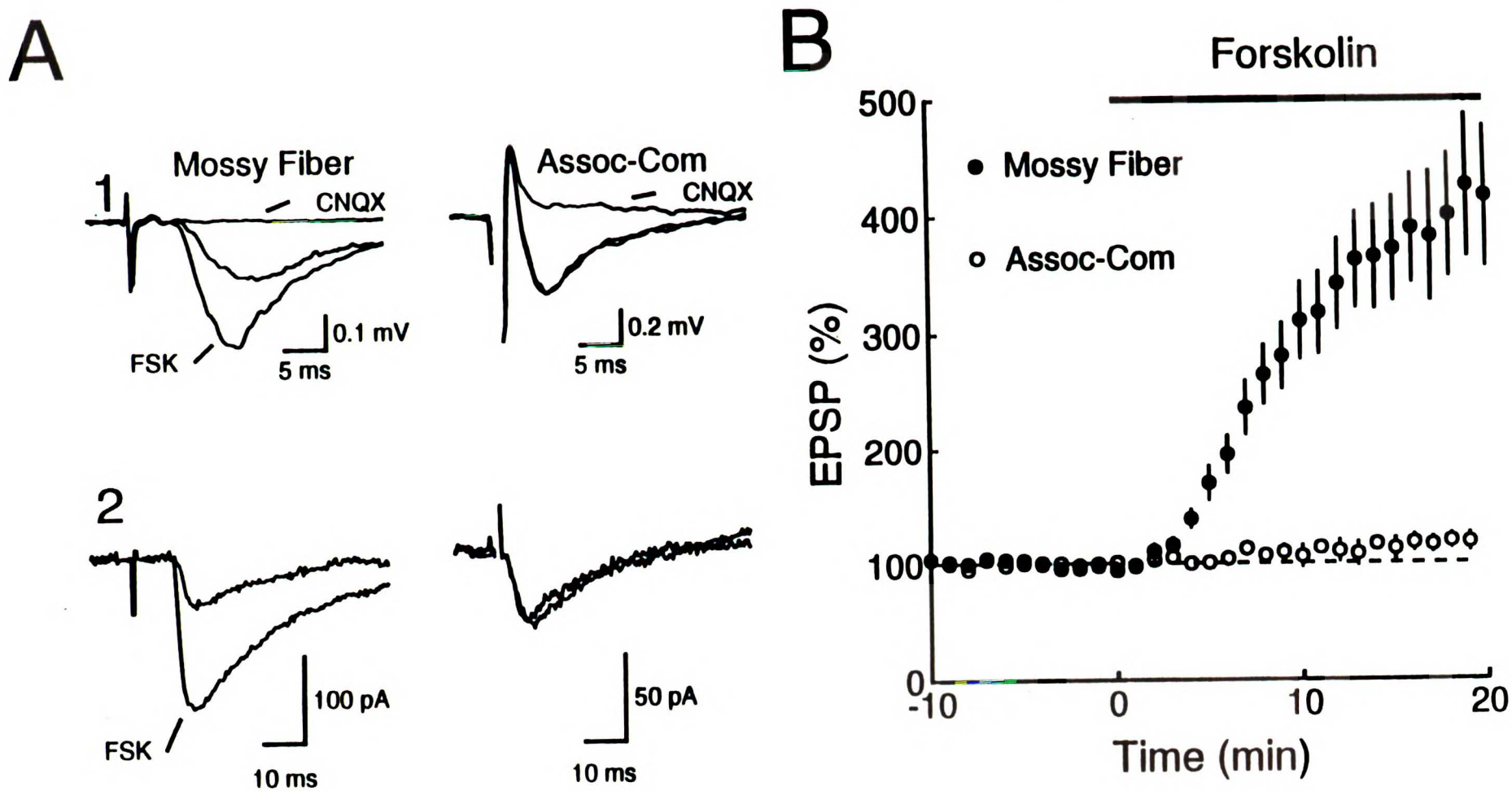
Brief elevation of cAMP produces a long lasting presynaptic enhancement specific to mossy fibers

As demonstrated in figure 24, application of forskolin (1-50 μM), a diterpene that directly activates adenylyl cyclase (Seamon & Daly, 1981), caused a dramatic potentiation of mossy fiber synaptic responses that was seen in every preparation tested (n=74). In contrast, forskolin had little effect on the simultaneously recorded assoc-com responses (Fig. 24). This differential effect of forskolin on the two types of synaptic input could also be seen with whole-cell recording (n = 4) (Fig. 24A2). While this strongly suggests that cAMP mediated actions can enhance mossy fiber synaptic responses, it is well known that there are some cAMP independent effects of forskolin (Laurenza, Sutkowski & Seamon, 1989). Many of the these actions of forskolin, including the blockade of some types of K^+ channels (Hoshi, Garber & Aldrich, 1988), are mimicked by the isomer of forskolin, 1,9-dideoxyforskolin. This isomer, on the other hand, has no effect on adenylyl cyclase (Seamon & Daly, 1981), and, hence, is often used as a control for cAMP independent effects of forskolin. When bath applied, this compound had no effect on mossy fiber responses, while subsequent application of forskolin caused the usual enhancement (Fig. 25A). In addition, Rp-8-CPT-cAMPS (Dostmann, Taylor, Genieser, Jastorff, Døskeland & Øgreid, 1990), an antagonist of PKA, reduced the action of forskolin. A 3 minute application of forskolin (10μM) caused an increase over baseline of 114±15%, while in Rp-8-CPT-cAMPS the increase was only 40±7% (Fig. 25B). We also examined the action of analogs of cAMP that have been shown in other systems to mimic the action of cAMP. The analogs Sp-cAMPS (Figs. 25C and D, 26A) and Sp-8-CPT-

FIGURE 24. Forskolin selectively enhances mossy fiber synaptic responses.

A. Sample superimposed traces from typical experiments using extracellular field recordings (1) and whole cell recordings (2) showing traces before, during 50μM forskolin (FSK) and after the addition of CNQX (20μM). The effects of these compounds are shown for both mossy fiber and associational-commissural (Assoc-com) responses recorded from the same slice in (1) and the same cell in (2). Each trace is the average of 10 and 8 EPSP and EPSC responses, respectively.

C Comparison of the effect of forskolin (50μM; bar) on mossy fiber (filled circles) and assoc-com (open circles) responses in the same slice (n = 7).


A
Mossy Fiber
1
CNQX
0.1 mV
FSK
5 ms
Assoc-Com
CNQX
0.2 mV
5 ms
2
100 pA
FSK
10 ms
50 pA
10 ms
B
Forskolin
Mossy Fiber
Assoc-Com
EPSP (%)
500
400
300
200
100
0
-10
0
10
20
Time (min)

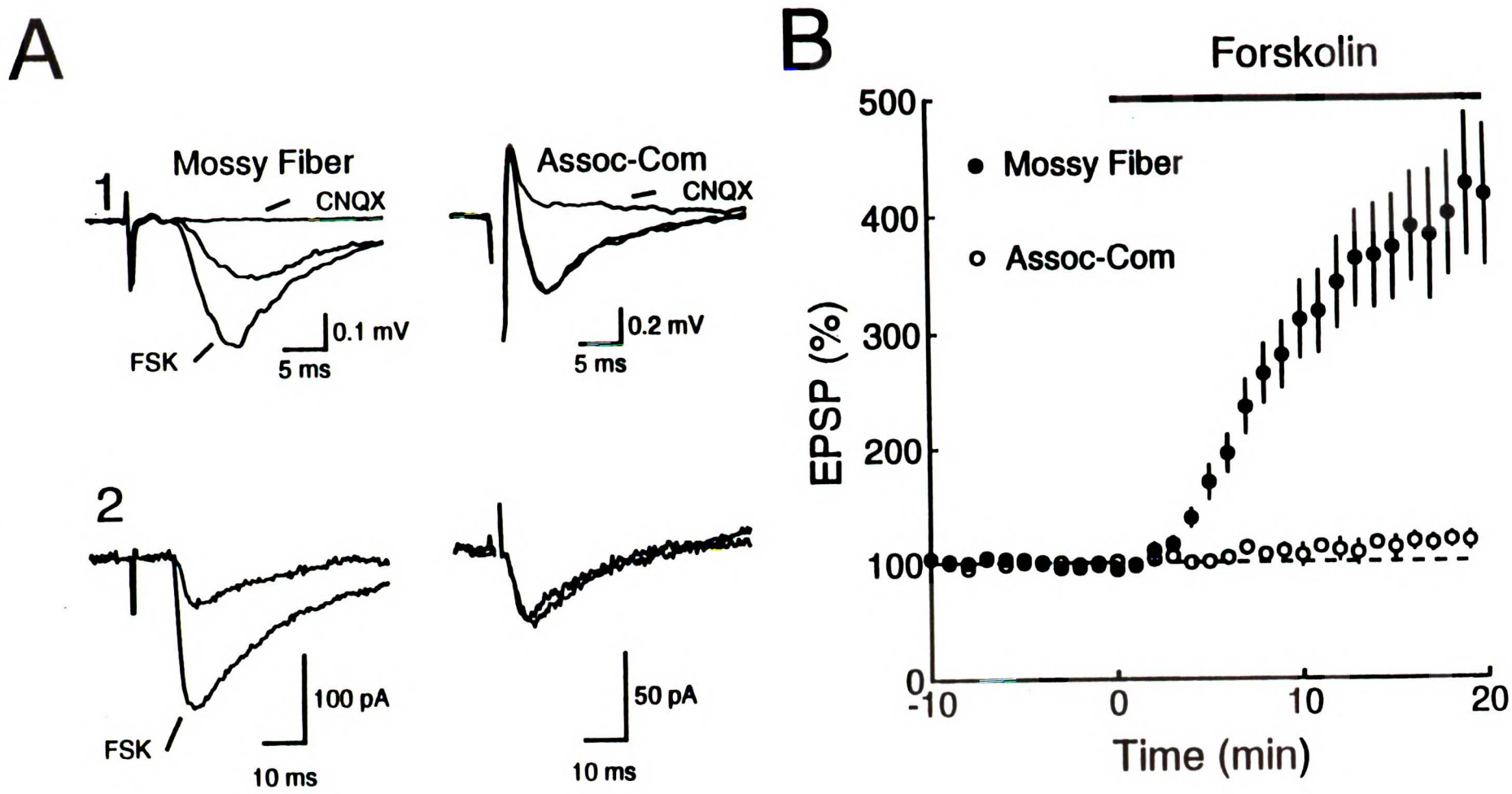
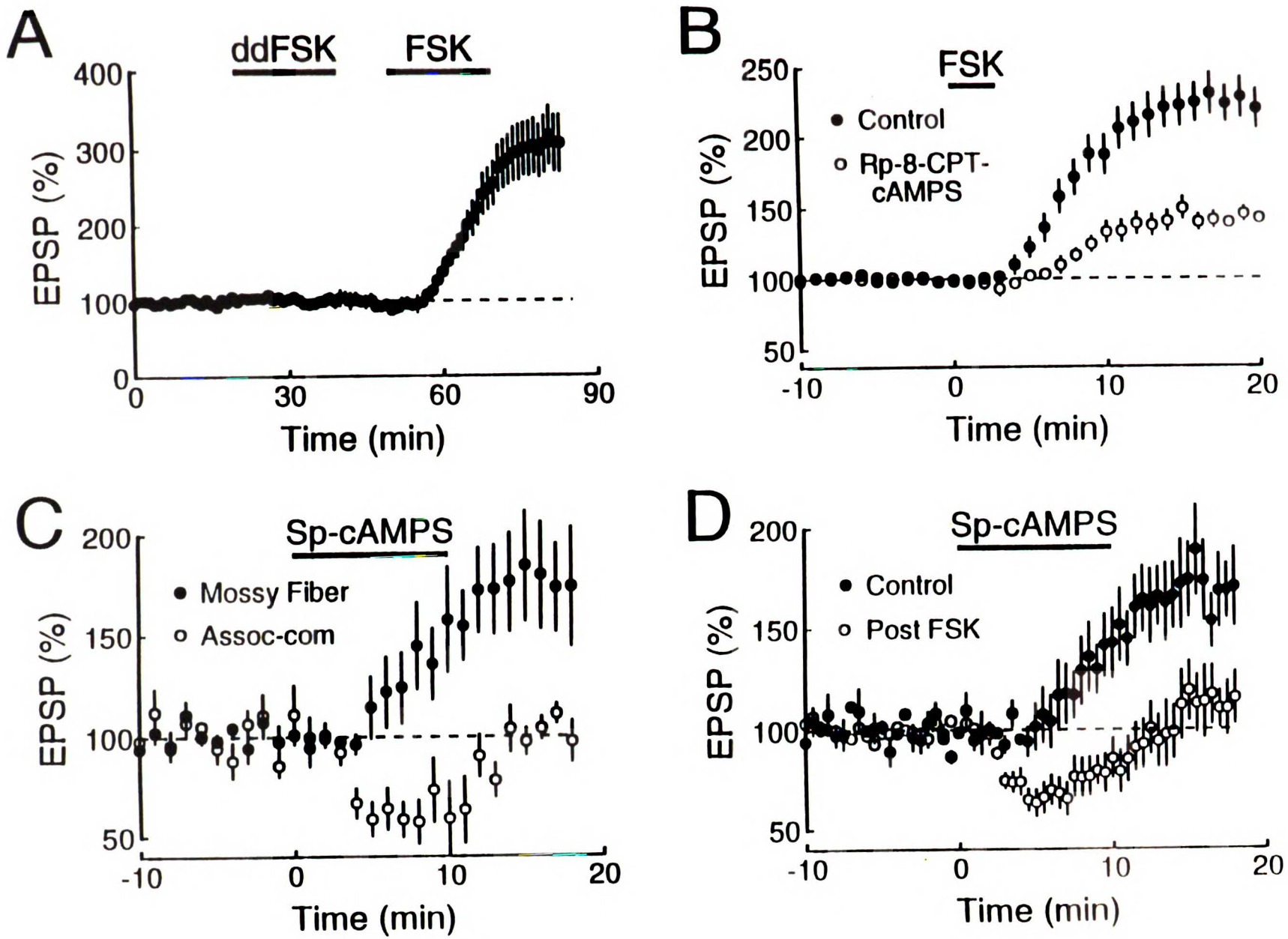

A
Mossy Fiber
Assoc-Com
1
CNQX
CNQX
0.1 mV
5 ms
FSK
0.2 mV
5 ms
2
100 pA
50 pA
FSK
10 ms
10 ms
B
Forskolin
Mossy Fiber
Assoc-Com
EPSP (%)
500
400
300
200
100
0
-10
0
10
20
Time (min)

FIGURE 25. Elevation of cAMP levels is responsible for the selective enhancement of mossy fiber synaptic responses.

A. A 20min application of 50μM 1,9-dideoxyforskolin (ddFSK) had no effect on mossy fiber responses. This was followed 10min later by a 20min application of 10μM forskolin (FSK) which produced enhancement (n=4). **B.** Comparison of the effect of a 3min application of 10μM forskolin (FSK; bar) on control mossy fiber responses (filled circles; n=8), and responses from slices incubated in Rp-8-CPT-cAMPS (open circles; n=7). In the latter case, slices were incubated for at least 2h in 100μM Rp-8-CPT-cAMPS and then transferred to the recording chamber where the concentration was 10-30μM. **C.** Comparison of the effect of Sp-cAMPS on mossy fiber (filled circles) and associational-commissural (Assoc-com; open circles) responses recorded in the same slice (n = 4). As with forskolin, the enhancement is selective for the mossy fiber responses. **D.** Comparison of the effect of Sp-cAMPS (100μM; bar) on control mossy fiber responses (filled circles; n=6), and responses previously enhanced by 50μM forskolin (open circles; n=5). In the latter case the post-forskolin stimulation strength was reduced such that responses were approximately at control levels.

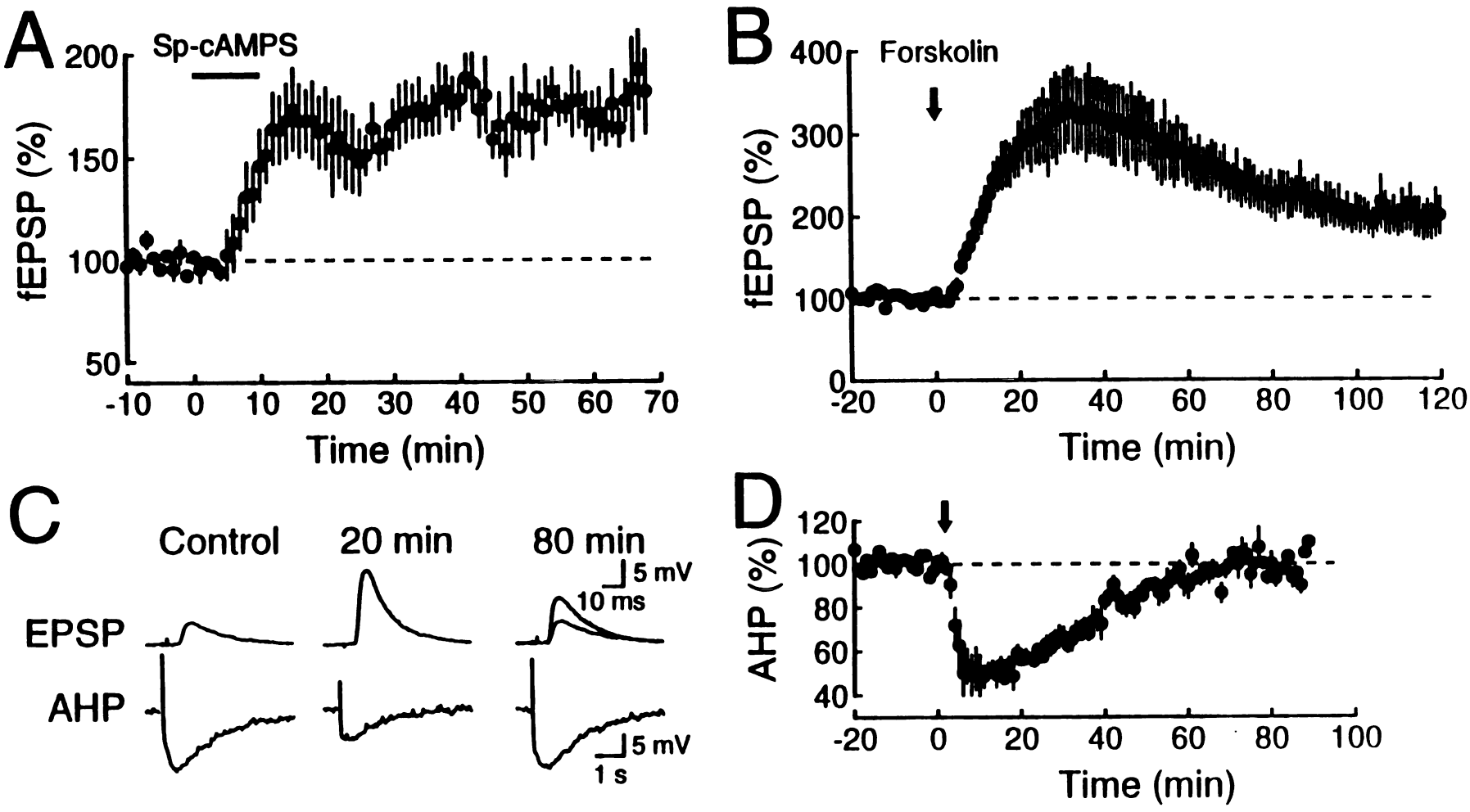

A
ddFSK
FSK
EPSP (%)
400
300
200
100
0
0
30
60
90
Time (min)
B
FSK
Control
Rp-8-CPT-cAMPS
EPSP (%)
250
200
150
100
50
-10
0
10
20
Time (min)
C
Sp-cAMPS
Mossy Fiber
Assoc-com
EPSP (%)
200
150
100
50
-10
0
10
20
Time (min)
D
Sp-cAMPS
Control
Post FSK
EPSP (%)
200
150
100
50
-10
0
10
20
Time (min)

cAMPS (n = 3, data not shown), activators of PKA (Van Haastert, Van Driel, Jastorff, Baraniak, Stec & De Wit, 1984) also reproducibly enhanced mossy fiber responses. As can be seen in figure 25C, the enhancement by Sp-cAMPS is specific for mossy fiber synapses, as is forskolin. If forskolin is acting through production of cAMP to enhance mossy fiber synaptic transmission, then it would be expected that the analog of cAMP, Sp-cAMPS, would be ineffective at enhancing transmission further after forskolin had already caused an enhancement. Indeed, Sp-cAMPS did not produce any enhancement of responses already enhanced by forskolin and only a transient depression was seen (Fig. 25D). A similar depression was seen with the analogs 8-bromo-cAMP (n=7) and dibutyryl cAMP (n=3), but little enhancement. This inhibition, as well as that seen with Sp-cAMPS in the presence of forskolin, may result from an action on adenosine A1 presynaptic receptors, but attempts to unmask an enhancement using A1 antagonists were not possible due to the epileptiform activity induced by the antagonists.

The enhancement evoked by manipulations that elevated cAMP was long lasting. Thus, following Sp-cAMPS application (3 - 10 min) responses remained stably potentiated for the duration of the experiment (Fig. 26A). In addition, after a 3 min application of forskolin the enhancement remained elevated for over 2 hours (Fig. 26B). Since it is possible that forskolin washes out slowly from the slice, we have monitored its effects on the calcium-activated slow AHP recorded in pyramidal cells, which has previously been shown to be a target of cAMP (Madison & Nicoll, 1986). The action of forskolin on the AHP, in contrast to the mossy fiber response, completely reverses within an hour of a 3 min application (Fig. 26C and D). These results are consistent with the model that a brief elevation of cAMP triggers a persistent enhancement in synaptic transmission that does not depend on the continued presence of cAMP.

FIGURE 26. Cyclic AMP effects are long lasting.

A. Effect of a 3-10 min application of Sp-cAMPS (1 mM; bar) (n = 10) on mossy fiber responses. **B.** Effect of a 3 min application of forskolin (50 μM; arrow) (n = 6). **C.** Sample records of intracellular recordings obtained with sharp electrodes in the same CA3 pyramidal cell. Each trace represents the average of 6 and 3 EPSP and AHP responses, respectively. **D.** Time course of AHP amplitude recorded from CA3 pyramidal cells (n = 5). Forskolin was bath applied at the same concentration and for the same duration as in **B.**


A
Sp-cAMPS
200
150
100
50
fEPSP (%)
-10
0
10
20
30
40
50
60
70
Time (min)
B
Forskolin
400
300
200
100
0
fEPSP (%)
-20
0
20
40
60
80
100
120
Time (min)
C
Control
20 min
80 min
5 mV
10 ms
EPSP
AHP
5 mV
1 s
D
120
100
80
60
40
AHP (%)
-20
0
20
40
60
80
100
Time (min)

The fact that the enhancement by forskolin was seen even in whole cell recordings, during which the membrane potential was clamped at -60 to -75 mV, indicates that forskolin's actions are independent of postsynaptic depolarization. This result, however, does not rule out the possibility that the action of forskolin depends on some other consequence of synaptic activity. We found, however, that if forskolin was applied in the absence of stimulation, responses were nonetheless already enhanced when stimulation was resumed. This was seen both in cases where a second set of mossy fibers were stimulated continuously in order to monitor normal enhancement, and in cases where no fibers received any stimulation. If another factor, such as presynaptic calcium entry, had been necessary for the forskolin-induced enhancement, then one would have expected to see the responses at baseline levels upon resumption of stimulation and then increase with the normal time course. This was clearly not the case, as we found the action of forskolin to be entirely independent of any synaptic activity.

Previous experiments showing that disruption of postsynaptic processes (Zalutsky & Nicoll, 1990) or synaptic transmission (chapter 4) has no effect on mossy fiber LTP helped demonstrate that mossy fiber LTP is presynaptic. For a membrane permeant compound such as forskolin, such a distinction regarding the site of action cannot be made. It has been shown in a number of systems that elevating cAMP can enhance the release of transmitter (Kuba & Kumamoto, 1986; Schacher *et al.*, 1988; Scholz & Byrne, 1988; Dixon & Atwood, 1989; Chavez-Noriega & Stevens, 1994). On the other hand, it has been shown that CA3 pyramidal cells, the postsynaptic component at the mossy fiber synapse, express mRNA for the GluR 6 subtype of glutamate receptor (Egebjerg, Bettler, Hermans-Borgmeyer & Heinemann, 1991), which has been shown to be up-regulated by cAMP dependent phosphorylation (Raymond, Blackstone & Huganir, 1993;

Wang, Taverna, Huang, MacDonald & Hampson, 1993). Therefore, it was clearly critical to address specifically the site of action of forskolin.

In a first set of experiments a double barrel pipette was positioned in *stratum (s.) lucidum* where glutamate was released from one of the barrels and the responses to the local action of glutamate and to mossy fiber stimulation were recorded from the other barrel and compared. In these experiments we used glutamate, rather than a non-N-methyl-D-aspartate (NMDA) agonist, because glutamate is rapidly removed by uptake, thus localizing its action. In addition, responses to glutamate similar in size to the synaptic response were used in order to activate only glutamate receptors in *s. lucidum* close to the recorded synaptic response. During the enhancing action of forskolin on the mossy fiber responses (Fig. 27A1) there was no change in the response evoked by the iontophoretic pulse of glutamate (Fig. 27A2). The results from four experiments are graphed in figure 27B, and are consistent with the enhancing action of forskolin being presynaptic.

In a second set of experiments, we monitored paired pulse facilitation (PPF) during the action of forskolin. When a synapse is activated twice at short intervals (40 ms in our experiments) the second response is typically larger due to a facilitation of transmitter release, and manipulations that increase transmitter release have been shown to strongly decrease the magnitude of PPF (Katz & Miledi, 1968; Mallart & Martin, 1968; Creager *et al.*, 1980; Zucker, 1989; Manabe *et al.*, 1993). During the action of forskolin there was a clear decrease in the degree of PPF. An example of the effect of forskolin is shown in figure 28A1 and the results from a number of experiments are plotted in figure 28B. Forskolin decreased PPF to 61±9% (n = 7) of baseline values. When using extracellular field recording techniques, however, it is necessary to change the stimulus strength after a drug induced alteration in the response size so that the

FIGURE 27. Forskolin enhances mossy fiber synaptic responses, but not responses to iontophoretic glutamate.

A. Sample traces showing (1) mossy fiber-CA3 synaptic responses (Evoked) and (2) responses to iontophoretically applied glutamate (Glutamate; solid bars; 200mM in H_2O, pH 8, 600nA, 100ms, no retaining current) before (Control) and after (Forskolin) the addition of 50μM forskolin to the bath. All traces are the average of 5-15 responses, and the response remaining in the presence of 40μM CNQX and 50μM D-APV have been subtracted. **B.** Comparison of the effect of 50μM forskolin (solid bar) on evoked responses (filled circles) and responses to iontophoretically applied glutamate (open circles; 400-600nA, 50-100ms, no retaining current) recorded from a double-barelled pipette in *s. lucidum*.

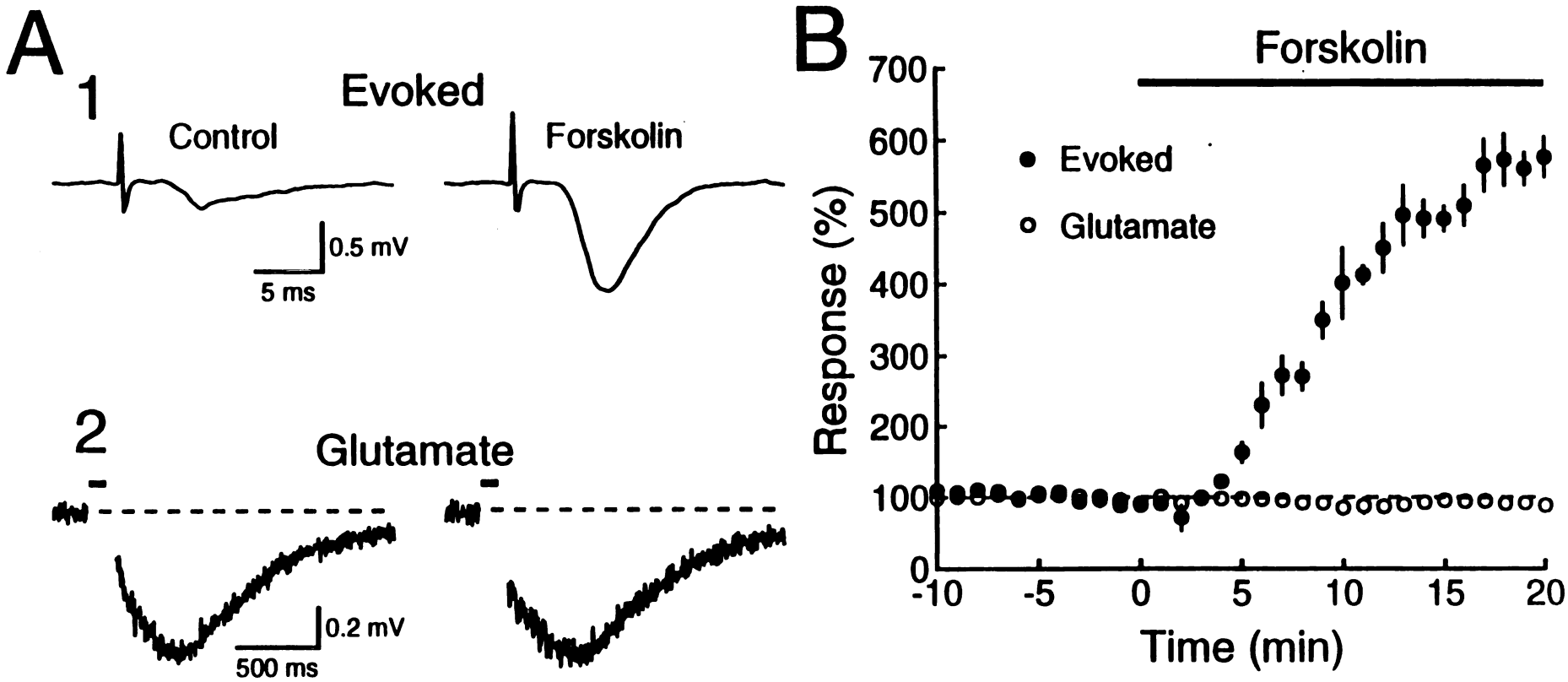
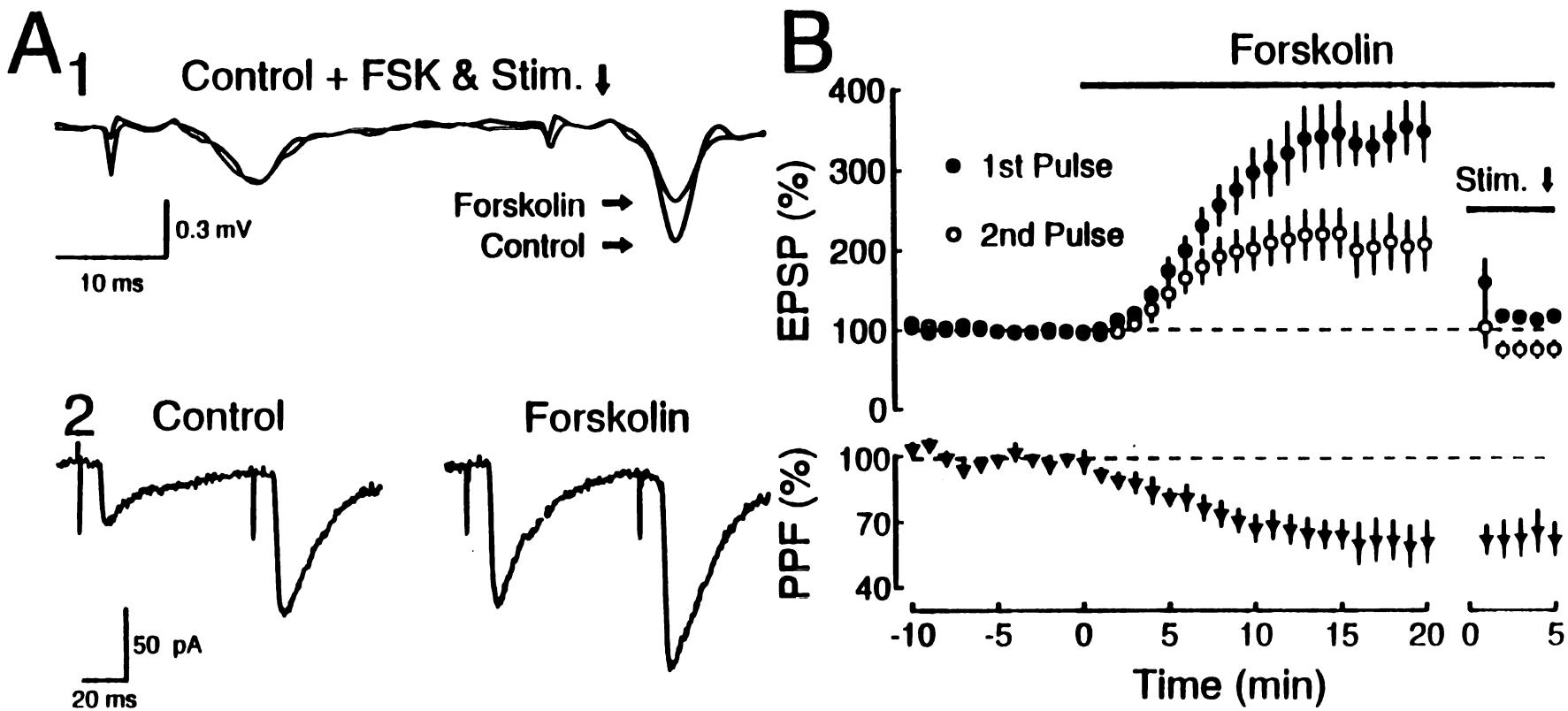

A
1
Evoked
Control
Forskolin
0.5 mV
5 ms
2
Glutamate
0.2 mV
500 ms
B
Forskolin
Evoked
Glutamate
Response (%)
700
600
500
400
300
200
100
0
-10
-5
0
5
10
15
20
Time (min)

FIGURE 28. Paired pulse facilitation is reduced by forskolin.

A. (1) Sample extracellular mossy fiber responses to paired pulses delivered 40 ms apart recorded before the addition of 50μM forskolin to the bath (Control). Superimposed on this is the response after the addition of forskolin and the subsequent reduction of the stimulus strength such that the response to the first of the two pulses was similar to that in control conditions (FSK & Stim. ↓). The response to the second pulse is less after the addition of forskolin (Forskolin) than before (Control). (2) Sample MF responses recorded using the whole cell recording mode both before (Control) and after (Forskolin) the addition of 50μM forskolin to the bath. All traces are the average of 5-10 responses. **B.** The top panel shows the average of 7 experiments as in **A1** comparing the effect of 50μM forskolin (long solid bar) on the response to the first of paired pulses given 40 ms apart (filled circles) and to the second pulse (open circles) both before and after the reduction in stimulus strength (short solid bar; Stim. ↓) such that the responses to the first pulse were similar to those in control conditions. The bottom panel shows the concurrent changes in the paired pulse facilitation ratio (2nd pulse/1st pulse; normalized to pre-forskolin levels).


A1
Control + FSK & Stim.
0.3 mV
10 ms
Forskolin
Control
2
Control
Forskolin
50 pA
20 ms
B
Forskolin
400
300
200
100
0
EPSP (%)
1st Pulse
2nd Pulse
Stim.
100
70
40
PPF (%)
-10
-5
0
5
10
15
20
0
5
Time (min)

post-drug response to the first pulse is similar to control levels. This is done in order to avoid possible non-linear effects of the altered response size. As shown in figure 28B, this control did not affect the decrease in PPF by forskolin (to 62±8%). In addition, the decrease in PPF could also clearly be seen with excitatory postsynaptic currents (EPSCs) recorded in the whole cell mode (48±12% of control) (n = 4) (Fig. 28A2).

The whole cell recording also permitted an analysis of the coefficient of variation (CV) of EPSCs before and after forskolin application. The analysis of the term $1/CV^2$, which is equal to the square of the mean response size divided by the variance around this mean (M^2/σ^2), has been used previously to examine the locus of synaptic modifications (Malinow & Tsien, 1990; Manabe *et al.*, 1993; Kullmann, 1994). Assuming a binomial distribution of release probability, this term depends only on the presynaptic variables n and p, where n is the number of release sites and p is the probability of release ($M^2/\sigma^2 = np/(1-p)$; see Malinow & Tsien, 1990; Korn & Faber, 1991). Thus, for a presynaptic change (i.e., n or p), $1/CV^2$ will change, and it will do so in proportion to the change in the mean response size, M (M=npX, where X is a term representing the unchanged postsynaptic responsiveness). In forskolin $1/CV^2$ increased (289±90%) and this was similar to the increase in the mean EPSC amplitude (296±31%, n = 4). While this suggests a presynaptic enhancement, it should be noted that the analysis depends on the assumption of binomial statistics of release, which is an unknown at this synapse, and thus the results should be interpreted with caution (Korn & Faber, 1991).

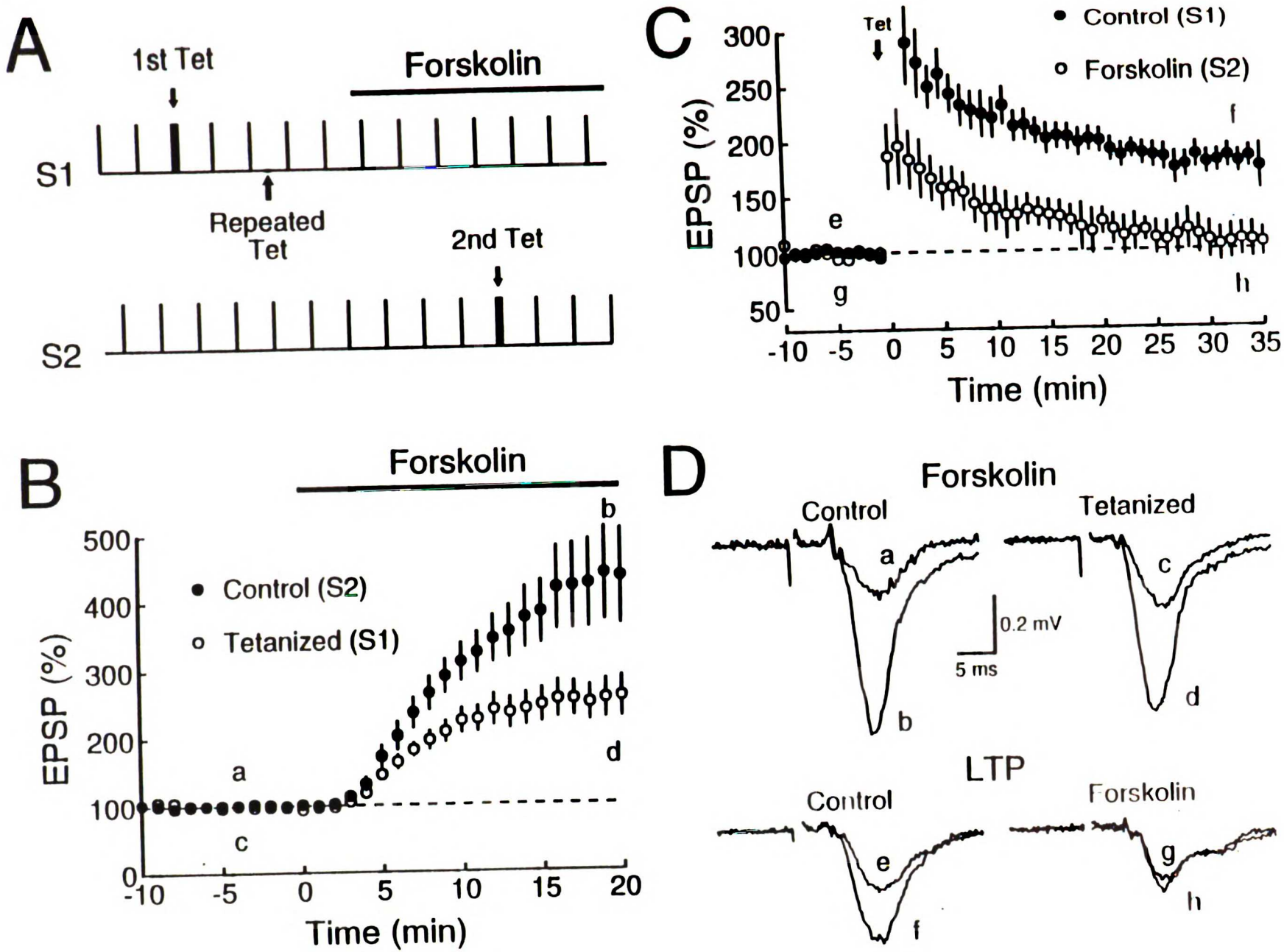
The weight of the above results suggest that the enhancement of synaptic transmission caused by forskolin is due to an enhancement of transmitter release. A trivial explanation for this presynaptic enhancement would be that forskolin enhances the excitability of the dentate granule cells. This possibility can be

ruled out for two reasons: (1) input-output curves generated before and after CNQX and then in both CNQX and forskolin revealed that forskolin had no effect on the fiber volley, however increased stimulus strength levels that gave synaptic responses similar to those seen after forskolin application in the absence of CNQX did increase the fiber volley (n = 3); (2) norepinephrine, which causes a cAMP-dependent increase in granule cell excitability (Haas & Rose, 1987), did not enhance mossy fiber synaptic responses (n = 5).

Forskolin-induced enhancement and mossy fiber LTP occlude

How might this cAMP-dependent enhancement of transmitter release normally be activated? First, we tested a number of agonists that activate receptors positively coupled to adenylyl cyclase. These agonists, which were all tested in at least 3 slices, included isoproterenol (100 μM) to activate beta adrenergic receptors, 5-HT (100 μM) to activate 5-HT4 receptors, DPMA (100 nM) to activate adenosine A2 receptors (Bridges, Bruns, Ortwine, Priebe, Szotek & Trivedi, 1988), dimaprit (100 μM) to activate histamine H2 receptors (Parsons, Owen, Ganellin & Durant, 1977) and PACAP-27 (1 μM) to activate vasoactive intestinal peptide (VIP) and type 1 PACAP receptors. PACAP-27 was used because it has been shown that type 1 PACAP receptor mRNA is expressed at high levels in dentate granule cells (Hashimoto, Ishihara, Shigemoto, Mori & Nagata, 1993). None of these agonists enhanced mossy fiber responses, however, presumably because mossy fiber terminals lack receptors for these agonists. Second, since the cAMP-induced enhancement and mossy fiber LTP both appear to be long lasting presynaptic phenomena, we reasoned that the two may be related and that tetanization engages the cAMP cascade. If this is indeed the case, and these two processes share a common step, one would expect that

FIGURE 29. Forskolin-mediated enhancement and mossy fiber LTP occlude. **A.** Schematic diagram of the experimental protocol. Two independent mossy fiber-CA3 synaptic inputs were monitored (S1 and S2). After responses were stable for at least 10 min a single tetanus (100 Hz, 250 ms) was given to one input (S1; 1st Tet). Once responses had again stabilized, repeated tetani were given to S1 (4x 100 Hz for 1 s, separated by 20 s) to attempt to maximize LTP. After responses had stabilized, the stimulus strength of S1 was reduced such that the two inputs gave similar responses. After a 10 min stable baseline period, 50 μM forskolin (solid bar) was added to the bath. After the responses had stabilized, the stimulus strength of S2 was reduced such that the response to S2 was similar to that to S1 prior to the first tetanus. After a 10 min stable baseline period, a single tetanus (100 Hz, 250 ms) was given to S2 (2nd Tet). **B.** Comparison of the effect of 50 μM forskolin (solid bar) on control (filled circles) and previously tetanized (open circles) responses (n = 12). **C.** Comparison of the effect of a tetanus given at time 0 on control responses (filled circles) and responses previously enhanced by forskolin (open circles; n = 5). In a first series of experiments we only compared the effect of forskolin on a naive pathway and a pathway which had received the repeated tetani that were used to maximize LTP. Thus we examined the differential effect of forskolin in 12 slices while the effect of forskolin on single tetanus LTP was compared in only 5 slices. For these 5 slices the effect of forskolin at 15-20 min was significantly different between the two pathways ($P<0.02$). **D.** Sample traces from a single experiment as described in **A**. The enhancement by forskolin is greater on control responses than those previously tetanized (compare a to b with c to d). The effect of a tetanus is greater on control responses than those previously enhanced by forskolin (compare e to f with g to h). All traces are the average of 5-10 responses. The letters refer to the times at which the responses were taken as shown in **B** and **C**.


A
1st Tet
Forskolin
S1
Repeated Tet
2nd Tet
S2
B
Forskolin
Control (S2)
Tetanized (S1)
EPSP (%)
Time (min)
C
Tet
Control (S1)
Forskolin (S2)
EPSP (%)
Time (min)
D
Forskolin
Control
Tetanized
0.2 mV
5 ms
LTP
Control
Forskolin

maximal activation of one process would occlude the other. The general design of the experiment to test this hypothesis is shown schematically in figure 29A. This involved two independent mossy fiber pathways so that in the same slice the action of forskolin could be compared on a control pathway (S2; 408±56%) and a pathway expressing LTP (S1; 251±28%) (Fig. 29B). In addition, within the same experiment, LTP in a control pathway (S1; 176±16%) could be compared to LTP evoked in the presence of forskolin (S2; 111±14%) (Fig. 29C). Traces from a typical experiment are shown in figure 29D: comparison of the increase from response a to response b with the increase from c to d demonstates the effect of forskolin on control and previously tetanized responses, respectively (note that initially c is larger than a); comparison of the increase from response e to response f with the change from g to h demonstates the effect of a tetanus on control and forskolin-enhanced responses, respectively. Forskolin completely occluded LTP and the action of forskolin was reduced after LTP ($P<0.01$, $n = 12$).

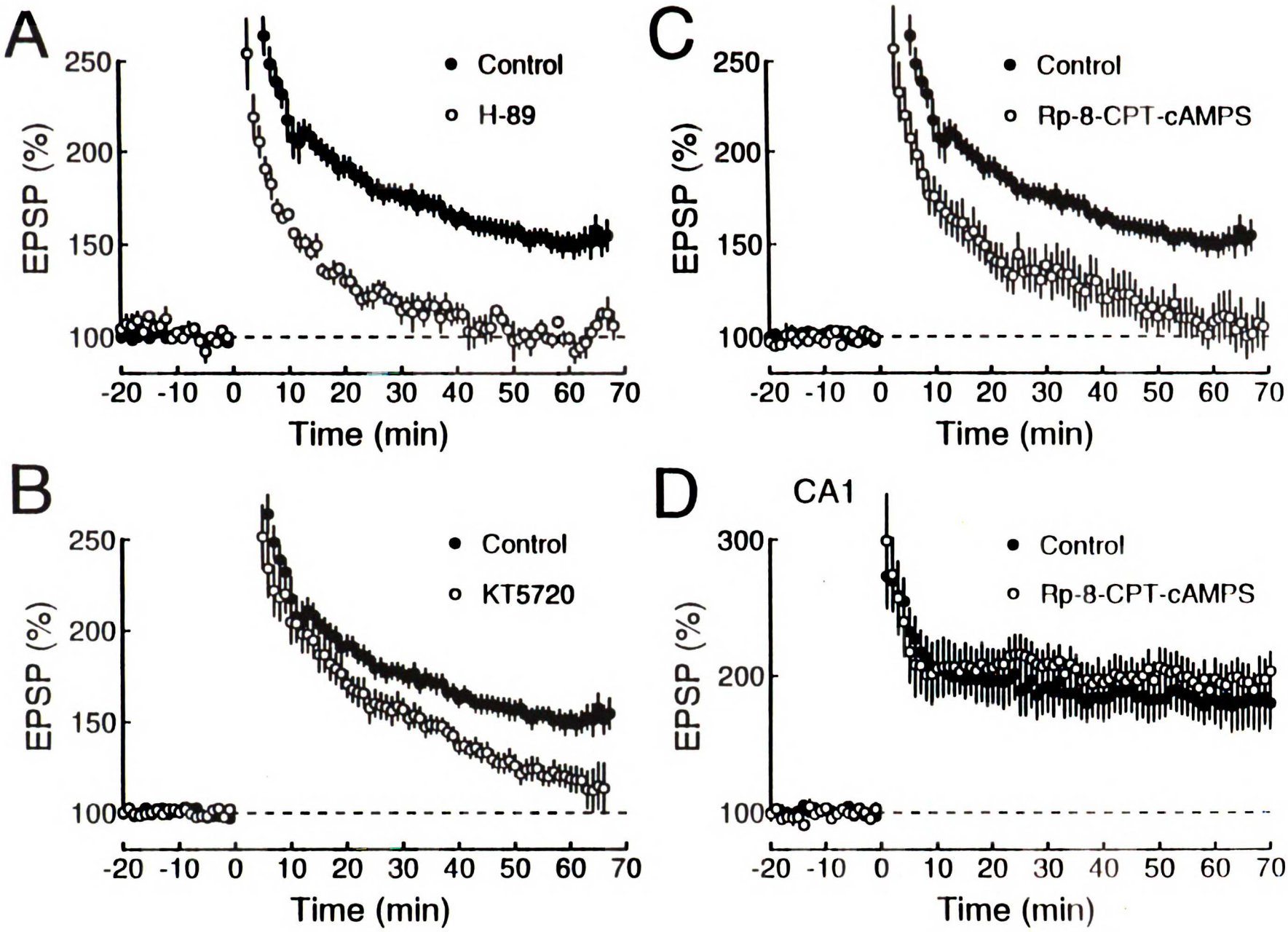
Mossy fiber LTP is blocked by cAMP cascade inhibitors

These experiments clearly indicate that forskolin and LTP occlude one another and suggest that they share a common process. This could occur in one of two possible ways. Either the cAMP cascade is an independent, parallel process that converges on some step in the LTP pathway, or the cascade is positioned in series with the LTP pathway and is an essential step in the expression of LTP. One way to distinguish between these two alternatives is to determine whether or not blockade of the cAMP cascade interrupts LTP. Two distinct classes of PKA inhibitors were tested on LTP. H-89 ($n = 6$) and KT 5720 ($n=7$), at concentrations of 10 and 1μM, respectively, are selective blockers of the catalytic site (Kase, Iwahashi, Nakanish, Matsuda, Yamada, Takahashi,

Murakata, Sato & M., 1987; Chijiwa, Mishima, Hagiwara, Sano, Hayashi, T., K., Toshioka & Hidaka, 1990; Cabell & Audesirk, 1993; Wieprecht, Wieder & Geilen, 1994). At these selective concentrations, both H-89 (Fig. 30A) and KT 5720 (Fig. 30B) antagonize LTP and neither affects baseline transmission. In experiments with the regulatory site blocker, Rp-cAMPS, we found it to be completely ineffective: superfused at 100uM it did not block LTP (n=10), even when preincubated at 1mM for over 2 hours (7 of 10), nor did it reduce forskolin's effects (n=5, 4 preincubated at 1mM). The more membrane permeant and potent analog, Rp-8-CPT-cAMPS (Dostmann *et al.*, 1990), however, did inhibit mossy fiber LTP (Fig. 30C). This compound also had a depressant effect on baseline responses, although this cannot explain the inhibition of LTP since (i) nearly complete block of responses by ω-Aga-IVA did not inhibit LTP (Chapter 4, Fig. 22), and (ii) the closely related compound Rp-cAMPS caused a similar depression of baseline transmission, but did not inhibit LTP. Furthermore, Rp-8-CPT-cAMPS also depressed baseline transmission in area CA1, but had no effect on the NMDA-dependent LTP generated there (Fig. 30D). This confirms the selectivity of Rp-8-CPT-cAMPS, but contradicts one report claiming that Rp-cAMPS, which we found to be an ineffective antagonist, completely blocks CA1 LTP (Musgrave *et al.*, 1993). Other reports agree with ours, however, claiming that CA1 LTP measured at one hour is unaffected by PKA antagonists (Matthies & Reymann, 1993) or only inhibited slightly, and this only with stronger tetani than we used (Frey *et al.*, 1993; Huang & Kandel, 1994).

FIGURE 30. Inhibition of mossy fiber LTP by protein kinase A inhibitors.

A. 10 μM H-89 (open circles; n = 6) and **B.** 1 μM KT 5720 (open circles; n = 7), concentrations that selectively inhibit the catalytic site of PKA inhibit LTP at the mossy fiber synapse. The slices were incubated in the drug for at least 2h before being transferred to the recording chamber where the drug was present at the same concentration. **C.** The regulatory site inhibitor, Rp-8-CPT-cAMPS (open circles; n = 8), also antagonized mossy fiber LTP. These slices were incubated for at least 2 h in 100 μM Rp-8-CPT-cAMPS before being transferred to the recording chamber where the concentration was 10-30 μM. For all three cases the slices remained in the inhibitors for the duration of the experiment. LTP in control conditions (filled circles; n = 23) obtained during the same period as, and interleaved with, the experimental cases is superimposed on each panel. LTP was induced by a 100 Hz tetanus for 250 ms. **D.** Rp-8-CPT-cAMPS applied in the same manner as in C has no effect on NMDA receptor-dependent LTP in the CA1 region of the hippocampus recorded for 70 min. Control slices (filled circles; n = 9) were interleaved with experimental slices (open circles; n = 7).


A
250
200
150
100
EPSP (%)
Control
H-89
-20 -10 0 10 20 30 40 50 60 70
Time (min)
C
250
200
150
100
EPSP (%)
Control
Rp-8-CPT-cAMPS
-20 -10 0 10 20 30 40 50 60 70
Time (min)
B
250
200
150
100
EPSP (%)
Control
KT5720
-20 -10 0 10 20 30 40 50 60 70
Time (min)
D
CA1
300
200
100
EPSP (%)
Control
Rp-8-CPT-cAMPS
-20 -10 0 10 20 30 40 50 60 70
Time (min)

DISCUSSION

These experiments were designed to examine the role of cAMP-dependent processes in mossy fiber synaptic transmission. The initial finding, that forskolin strongly and reproducibly enhances mossy fiber synaptic transmission, suggested that cAMP may play some role endogenously. Forskolin, however, aside from being a potent activator of adenylyl cyclase, has other potential actions that are independent of cAMP (Laurenza *et al.*, 1989). One such action is to block some types of potassium channels (Hoshi *et al.*, 1988), which could conceivably be the cause of enhanced mossy fiber transmission as a result of prolonging the action potential duration in the mossy fiber terminal. The fact that forskolin's effects were absent from assoc-com responses onto the same CA3 pyramidal cells is suggestive that an action on K^+ channels is not the underlying cause, however it is far from proof. Much stronger evidence comes from the isomer of forskolin, 1,9-dideoxyforskolin, that is inactive at adenylyl cyclase, but shares forskolin's actions at voltage sensitive ion channels and is commonly used to distinguish between the two effects of forskolin. Consistent with a role for cAMP, we found that 1,9-dideoxyforskolin did not enhance mossy fiber responses. We further implicated cAMP in this enhancement by demonstrating that analogs of cAMP that activate PKA also enhance mossy fiber responses, while the PKA inhibitor, Rp-8-CPT-cAMPS, dramatically reduces the effects of forskolin.

If this cAMP mediated enhancement is indicative of an endogenous phenomenon, the simplest method by which it could be brought about endogenously would probably be activation of a cyclase by a receptor mediated system. After testing numerous transmitters that in other systems are positively coupled to adenylyl cyclase, however, we were unable to find one that produced

such an enhancement, most likely due to a lack of the appropriate receptors at this synapse. Failing this, several lines of evidence suggested that the cAMP system may underlie LTP: (i) our demonstration that the forskolin-induced enhancement is presynaptic, (ii) that it occludes mossy fiber LTP, and (iii) that specifically within the mossy fiber system there are cyclases activated by calcium (Tang & Gilman, 1992; Cali, Zwaagstra, Mons, Cooper & Krupinski, 1994), which we showed is necessary presynaptically for mossy fiber LTP (chapter 4). The way to prove that the cAMP cascade underlies mossy fiber LTP is to demonstrate a block of LTP by inhibitors of the cAMP cascade. Our initial experiments using the PKA inhibitor Rp-cAMPS, which had been shown to be effective in some systems (Frey *et al.*, 1993; Matthies & Reymann, 1993; Musgrave *et al.*, 1993), were unfruitful.. Rp-cAMPS did not reduce LTP, nor did it reduce the forskolin-induced enhancement. The more potent and membrane permeable inhibitor, Rp-8-CPT-cAMPS, however, did prove effective as it inhibited both LTP and the effects of forskolin. Further experiments confirmed this as other blockers, both of PKA and adenylyl cyclase, also proved effective at inhibiting LTP.

The present results are remarkably similar to previous results obtained at a number of synapses discussed in chapter 1, section D. In the peripheral autonomic nervous system , tetanization of preganglionic fibers results in a presynaptic form of LTP (Briggs, Brown & McAffee, 1985; Briggs & McAfee, 1988; Minota *et al.*, 1991) and manipulations that increase cAMP also potentiate these synapses (Kuba & Kumamoto, 1986). Furthermore, a similar series of findings have been made at the crustacean neuromuscular junction (Baxter *et al.*, 1985; Wojtowicz & Atwood, 1988; Dixon & Atwood, 1989) where it is possible to inject inhibitors of the cAMP cascade directly into the presynaptic axon and block the long lasting potentiation (Dixon & Atwood, 1989). One difference in this form of potentiation is that it does not require the entry of calcium into the

presynaptic terminal (Wojtowicz & Atwood, 1988), contrary to mossy fiber LTP (Ito & Sugiyama, 1991 and chapter 4). In addition, while there are marked differences in the mechanisms underlying mossy fiber LTP and NMDA receptor-dependent LTP at CA1 synapses, the proposed involvement of cAMP in a late phase of NMDA receptor-dependent LTP (Frey *et al.*, 1993; Matthies & Reymann, 1993; Huang & Kandel, 1994), suggests that similarities may also exist.

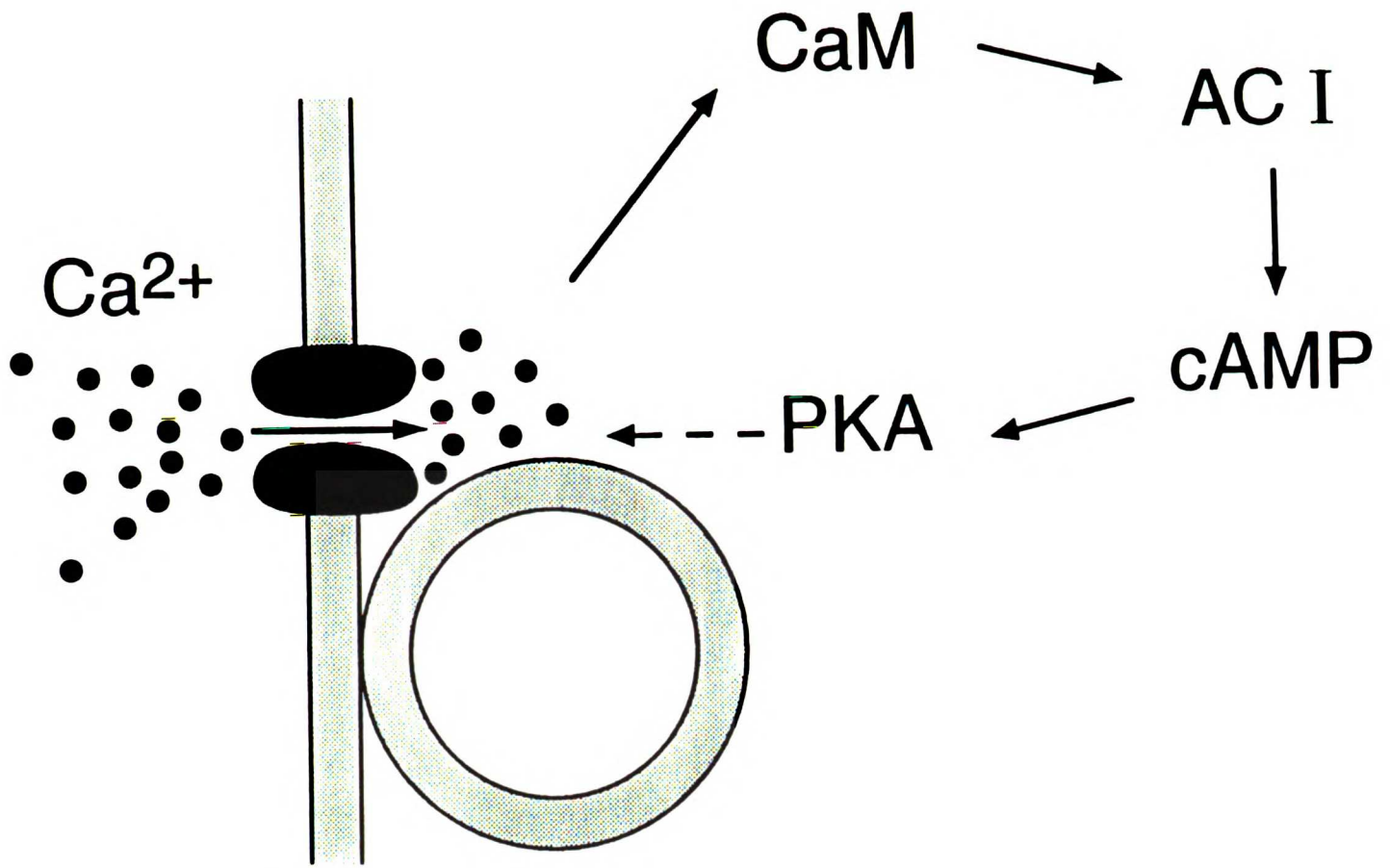
Our results also have features in common with other behavioral models of learning and memory. As discussed in chapter 1, section D, the process of sensitization and classical conditioning of the sensory fibers in the abdominal ganglion of Aplysia involves changes in presynaptic cAMP (Byrne *et al.*, 1993; Hawkins *et al.*, 1993). A completely different approach to studying learning and memory has been taken using Drosophila genetics, and here as well, a striking connection with the present results has been found. In this system, the genetic alterations leading to many learning impaired mutants, including *rutabaga* and *dunce*, have been isolated to a calcium sensitive adenylyl cyclase and PKA (Dudai, 1988).

In summary this study suggests that a brief elevation of cAMP in mossy fiber terminals causes a large and persistent increase in evoked glutamate release. The observation that the action of forskolin and mossy fiber LTP occlude each other and that blockers of PKA block mossy fiber LTP indicate that PKA activation is an essential step in mossy fiber LTP. Furthermore, it has recently been reported that mRNA for both adenylyl cyclase I (Xia, Refsdal, Merchant, Dorsa & Storm, 1991; Glatt & Snyder, 1993) and adenylyl cyclase VIII (Cali *et al.*, 1994), subtypes of cyclase that are activated by calcium via calmodulin (Tang *et al.*, 1991; Cali *et al.*, 1994) and as such specifically implicated in learning and memory (Dudai, 1988; Byrne *et al.*, 1993; Hawkins *et al.*, 1993), are highly expressed in dentate granule cells, which give rise to the mossy fibers. The high

concentration of forskolin binding sites present in the mossy fiber terminals (Worley, Baraban, De Souza & Snyder, 1986) is consistent with the cyclases being situated there. In addition, it has been estimated with fura-2 imaging that stimulation of the mossy fibers of 100Hz for 25 pulses (our tetanus protocol) raises calcium levels to approximately 300nM in the terminals (Regehr & Tank, 1991; Regehr, Delaney & Tank, 1994). These are levels of calcium that give almost peak activation of adenylyl cyclase I at physiological levels of calmodulin (Wu, Wong & Storm, 1993) which is similar to the activation of adenylyl cyclase VIII (Cali *et al.*, 1994). The approximate correlation of these values should perhaps be taken with a grain of salt given that the gradient of calcium concentration, from its point of entry to the average deeper within the terminal, may well vary enormously and not be reflected in the fura-2 measurements. Therefore, the precise location of the enzyme with respect to the site of calcium entry is an unknown that is of critical importance. Nonetheless, the correlations are quite suggestive.

Given all these results, we propose the following hypothesis for mossy fiber LTP, as summarized in figure 31: a tetanus-stimulated entry of calcium into the terminal, an essential step in mossy fiber LTP (chapter 4), complexes with calmodulin to activate a calcium/calmodulin sensitive adenylyl cyclase (e.g. type I, VIII, or both) producing a transient rise in cAMP, which, via the activation of PKA enhances the calcium triggered release process, resulting in a persistent enhancement of evoked glutamate release.

FIGURE 31. Schematic diagram of proposed mechanism for mossy fiber LTP. The entry of Ca^{2+} into the presynaptic terminal via Ca^{2+} channels is responsible for the release of glutamate from synaptic vesicles. In addition, it is proposed that the tetanus induced increase of Ca^{2+} above a threshold binds to calmodulin (CaM), which in turn stimulates adenylyl cyclase I (AC I; and/or AC VIII) thereby generating cAMP and activating protein kinase A (PKA). PKA (dashed arrow) either causes a long lasting increase in Ca^{2+} channel activity or a long lasting increase in the sensitivity of the release process to Ca^{2+}.


CaM
AC I
Ca2+
cAMP
PKA

CHAPTER SIX

CONCLUSION

Synaptic transmission at the mossy fiber-CA3 synapse

Aside from the classical fast neurotransmission mediated by glutamate at the mossy fiber synapse, high frequency stimulation of mossy fibers leads to the release of dynorphin which we have found to have synaptic actions that last many minutes. Our experiments demonstrating this endogenous peptidergic action within the nervous system (CNS) have shown that the conditions for release of dynorphin at the mossy fiber synapse and its time course of action are similar to those found at some peripheral synapses. While we do not know the extent to which dynorphin can diffuse to mediate its action, it must work in a paracrine manner since we monitor the effects on a separate set of mossy fibers from the ones releasing the dynorphin. The synaptically released dynorphin acts on presynaptic kappa 1 receptors of these neighboring mossy fiber terminals to depress mossy fiber transmission, although it does not do this by depressing presynaptic N-type calcium channel current as might be suggested by results in some other systems. The overall effect then is to increase the signal to noise ratio of a high frequency input by creating a surround inhibition. Furthermore, we have demonstrated that, under the proper conditions, dynorphin can block long-term potentiation (LTP) in the same fibers from which it is released. Thus the inhibition by dynorphin is capable of increasing the threshold for inducing mossy fiber LTP.

The LTP at the mossy fiber synapse is quite distinct from all other forms described at synapses within the hippocampus and indeed the whole CNS, but it does resemble that seen at some phylogenetically older, more simple nervous systems and some peripheral synapses. Most notably, it is independent of N-methyl-D-aspartate (NMDA) receptors (Harris & Cotman, 1986) and entirely presynaptic. We have found that it is dependent on calcium, as are the other

forms of LTP, but at the mossy fiber synapse the critical calcium is presynaptic. While normal mossy fiber synaptic transmission depends more heavily on calcium entry through presynaptic P-type calcium channels than N-type, the path of entry of the calcium necessary to trigger LTP appears irrelevant. We have also shown that at least one of the underlying mechanisms of mossy fiber LTP involves activation of the cyclic-AMP (cAMP) dependent protein kinase (PKA). Taken together with the specific localization of message for the calcium sensitive adenylyl cyclase type I in dentate granule cells (Xia *et al.*, 1991; Glatt & Snyder, 1993), this leads to our hypothesis (Fig. 31) that a large bolus of presynaptic calcium during high frequency activation of the mossy fibers activates adenylyl cyclasetype I to produce a rise in cAMP that leads to enhanced glutamate release through activation of PKA.

These presynaptic events that we have elucidated at the mossy fiber synapse further stress the importance of understanding release mechanisms at nerve terminals. Indeed, a deeper understanding of the events described herein would be greatly aided by more precise knowledge of the steps involved in neurotransmitter release.

Implications for models of hippocampal learning and memory processing

The physiological differences we have found between the mossy fiber synapse and those of area CA1 or the recurrent associational-commissural (assoc-com) system suggest that different computational functions are being performed at mossy fiber synapses compared with the latter two. Since the theories put forward by David Marr (1971) much attention has focused on area CA3 of the hippocampus as an auto-association memory matrix (McNaughton & Nadel, 1990; Rolls, 1990; Willshaw & Buckingham, 1990). The CA3 region may serve to

associate the occurrence of disparate sensory events and form a memory of the concurrent occurrence of two or more. For example, if you eat a burrito for lunch with a friend, the sensations accompanying the burrito and those accompanying your friend are associated with each other within area CA3 and a memory is formed of that burrito lunch with your friend. This type of association is hypothesized to be carried out by the associative LTP found at the recurrent CA3 synapses (assoc-com). The input to this system, however, is carried by the mossy fibers, and the experiments described in this thesis demonstrate clear differences between the mossy fiber synapse and those of the assoc-com fibers or area CA1. It is interesting to speculate as to how the physiology of the mossy fiber-CA3 synapse might fit in with the models of memory mentioned above. In particular, if area CA3 does serve as an auto-association matrix, what role could the effects of synaptically released dynorphin have, and what is the significance of an entirely presynaptic form of LTP at the mossy fiber synapse?

As discussed in chapter 3, dynorphin acts to produce a form of lateral inhibition. High frequency mossy fiber (input) activity results in release of dynorphin that depresses transmission from neighboring mossy fibers. In other systems lateral inhibition enhances discrimination such as with two-point discrimination in the somatosensory system or enhanced edge detection in the visual system. While physiologically quite distinct, the lateral inhibition at the mossy fiber synapse might serve a like function and enhance the ability to discriminate distinct experiential inputs that will then be associated to form memories.

The presynaptic LTP at mossy fiber synapses is a way that biology accomplishes synaptic enhancement that is distinct from that seen in area CA1; it may even be a phylogenetically older form since mossy fiber LTP resembles enhancement seen in invertebrates. While they both accomplish enhancement,

however, there is one significant functional difference between the two types of LTP which most likely underlies very different and intriguing computational aspects of mossy fiber synapses compared to those of area CA1 or assoc-com fibers: the absence of associativity or the related phenomenon cooperativity (see chapter 1, section C). Indeed, the theory dealing with the auto-associative model for area CA3, has concentrated very little on the connection from the input fibers. These synapses are essentially treated as unmaleable "detonator" synapses that serve only to initiate activity in the system. The aspects of transmission at the mossy fiber synapse examined in this thesis, including LTP itself, strongly suggest that the functions performed by this synapse are not nearly so banal as their treatment as simple detonator synapses would suggest. The potential computational power of the modulatory capabilities at the mossy fiber synapse merit close theoretical scrutiny.

The study of the hippocampal brain slice has contributed greatly to the present day understanding of CNS synaptic physiology. The majority of this work has been done on the Schaffer collateral-CA1 synapse, and the progress made has been an important and useful tool in examining function and information processing of not only the hippocampus, but many other regions of the brain as well. The insight gained into synaptic processes at the very different mossy fiber synapse will hopefully also extend beyond the hippocampus and aid in our understanding of the whole brain.

REFERENCES

Abrams, T. W., Karl, K. A., & Kandel, E. R. (1991). Biochemical studies of stimulus convergence during classical conditioning in Aplysia: Dual regulation of adenylate cyclase by Ca2+/calmodulin and transmitter. *Journal of Neuroscience.* **11,** 2655-2665.

Adamson, P., Xiang, J.-Z., Mantzourides, T., Brammer, M. J., & Campbell, I. C. (1989). Presynaptic α-adrenoceptor and κ-opiate receptor occupancy promotes closure of neuronal (N-type) calcium channels. *European Journal of Pharmacology.* **174,** 63-70.

Akil, H., Watson, S. J., Young, E., Lewis, M. E., Khachaturian, H., & Walker, J. M. (1984). Endogenous opioids: biology and function. *Annual Review of Neuroscience.* **7,** 223-255.

Alzheimer, C., & Ten Bruggencate, G. (1990). Nonopioid actions of the κ-opioid receptor agonists, U 50488 and U 69693, on electrophysiological properties of hippocampal CA3 neurons in vitro. *Journal of Pharmacology and Experimental Therapeutics.* **255,** 900-905.

Amaral, D. G., & Witter, M. P. (1989). The three dimensional organization of the hippocampal formation: a review of anatomical data. *Neuroscience.* **31,** 571-591.

Andersen, P., Sundberg, S. H., Sveen, O., & Wigström, H. (1977). Specific long-lasting potentiation of synaptic transmission in hippocampal slices. *Nature.* **266,** 736-737.

Aosaki, T., & Kasai, H. (1989). Characterization of two kinds of high-voltage-activated Ca-channel currents in chick sensory neurons: differential sensitivity to dihydropyridines and ω-conotoxin GVIA. *Pflügers Archiv.* **414**, 150-156.

Augustine, G. J., Charlton, M. P., & Smith, S. J. (1987). Calcium action in synaptic transmitter release. *Annual Review of Neuroscience.* **10**, 633-93.

Basbaum, A. I., & Fields, H. L. (1984). Endogenous pain control systems: Brainstem spinal pathways and endorphin circuitry. *Annual Review of Neuroscience.* **7**, 309-338.

Bashir, Z. I., Bortolotto, Z. A., Davies, C. H., Berretta, N., Irving, A. J., Seal, A. J., Henley, J. M., Jane, D. F., Watkins, J. C., & Collingridge, G. L. (1993). Induction of LTP in the hippocampus needs synaptic activation of glutamate metabotropic receptors. *Nature.* **363**, 347-350.

Baskys, A., & Malenka, R. C. (1991). Agonists at metabotropic glutamate receptors presynaptically inhibit EPSCs in neonatal rat hippocampus. *Journal of Physiology.* **444**, 687-701.

Baxter, D. A., Bittner, G. D., & Brown, T. H. (1985). Quantal mechanism of long-term synaptic potentiation. *Proceedings of the National Academy of Sciences, USA.* **82**, 5978-5981.

Bean, B. (1989). Neurotransmitter inhibition of neuronal calcium currents by changes in channel voltage dependence. *Nature.* **340**, 153-156.

Bean, B. P. (1989). Classes of calcium channels in vertebrate cells. **51**, 367-384.

Blackstad, T. W., Brink, K., Hem, J., & Jeune, B. (1970). Distribution of hippocampal mossy fibers in the rat. An experimental study with silver impregnation methods. *Journal of Comparative Neurology*. **138**, 433-450.

Bliss, T. V. P., & Collingridge, G. L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. *Nature*. **361**, 31-39.

Bliss, T. V. P., & Gardner-Medwin, A. R. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the unanaesthetized rabbit following stimulation of the perforant path. *Journal of Physiology*. **232**, 357-374.

Bliss, T. V. P., & Lømo, T. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *Journal of Physiology*. **232**, 331-356.

Böhme, G. A., Bon, C., Stutzmann, J. M., Doble, A., & Blanchard, J. C. (1991). Possible involvement of nitric oxide in long-term potentiation. *European Journal of Pharmacology*. **199**, 379-381.

Bortolotto, Z. A., & Collingridge, G. L. (1992). Activation of glutamate metabotropic receptors induces long-term potentiation. *European Journal of Pharmacology*. **214**, 297-298.

Bozarth, M. A., & Wise, R. A. (1984). Anatomically distinct opiate receptor fields mediate reward and physical dependence. *Science*. **224**, 516-517.

Bradler, J. E., & Barrionuevo, G. (1990). Heterosynaptic correlates of long-term potentiation induction in hippocampal CA3 neurons. *Neuroscience*. **35**, 265-271.

Bridges, A. J., Bruns, R. F., Ortwine, D. F., Priebe, S. R., Szotek, D. L., & Trivedi, B. K. (1988). N6-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)ethyl]adenosine and its uronamide derivatives. Novel adenosine agonists with both high affinity and high selectivity for the adenosine A2 receptor. *Journal of Medicinal Chemistry*. **31**, 1282-1285.

Briggs, C. A., Brown, T. H., & McAffee, D. A. (1985). Neurophysiology and pharmacology of long-term potentiation in the rat sympathetic ganglion. *Journal of Physiology*. **359**, 503-521.

Briggs, C. A., & McAfee, D. A. (1988). Long-term potentiation at nicotinic synapses in the rat superior cervical ganglion. *Journal of Physiology*. **404**, 129-144.

Brownstein, M. J. (1993). A brief history of opiates, opioid peptides, and opioid receptors. *Proceedings of the National Academy of Sciences USA*. **90**, 5391-5393.

Byrne, J. H., Zwartjes, R., Homayouni, R., Critz, S. D., & Eskin, A. (1993). Roles of second messenger pathways in neuronal plasticity and in learning and memory. Insights gained from Aplysia. In *Advances in Second Messenger and Phosphoprotein Research* ed. Shenolikar, S., & Nairn, A. C., pp. 47-108. Raven Press, New York.

Cabell, L., & Audesirk, G. (1993). Effects of selective inhibition of protein kinase C, cyclic AMP-dependent protein kinase, and Ca(2+)-calmodulin-dependent protein kinase on neurite development in cultured rat hippocampal neurons. *International Journal of Developmental Neuroscience.* **11,** 357-368.

Cali, J. J., Zwaagstra, J. C., Mons, N., Cooper, D. M., & Krupinski, J. (1994). Type VIII adenylyl cyclase. A Ca2+/calmodulin-stimulated enzyme expressed in discrete regions of rat brain. *Journal of Biological Chemistry.* **269,** 12190-12195.

Castellucci, V. F., Blumenfeld, H., Goelet, P., & Kandel, E. R. (1989). Inhibitor of protein synthesis blocks long-term behavioral sensitization in the isolated gill-withdrawal reflex of Aplysia. *Journal of Neurobiology.* **20,** 1-9.

Castellucci, V. F., Nairn, A., Greengard, P., Schwartz, J. H., & Kandel, E. R. (1982). Inhibitor of adenosine 3':5'-monophosphate-dependent protein kinase blocks presynaptic facilitation in Aplysia. *Journal of Neuroscience.* **2,** 1673-1681.

Castillo, P. E., Weisskopf, M. G., & Nicoll, R. A. (1994a). The role of Ca^{2+} channels in hippocampal mossy fiber synaptic transmission and long-term potentiation. *Neuron.* **12,** 261-269.

Castillo, P. E., Weisskopf, M. G., & Nicoll, R. A. (1994b). Mechanisms of action of dynorphin at mossy fiber synapses. *Society for Neuroscience Abstract.* **20,** 60.

Caudle, R. M., & Chavkin, C. (1990). MU-opioid receptor activation reduces inhibitory postsynaptic potentials in hippocampal CA3 pyramidal cells of rat and guinea pig. *Journal of Pharmacology and Experimental Therapeutics*. **252**, 1361-1369.

Chattarji, S., Stanton, P. K., & Sejnowski, T. J. (1989). Commissural synapses, but not mossy fiber synapses, in hippocampal field CA3 exhibit associative long-term potentiation and depression. *Brain Research*. **495**, 145-150.

Chavez-Noriega, L. E., & Stevens, C. F. (1994). Increased transmitter release at excitatory synapses produced by direct activation of adenylate cyclase in rat hippocampal slices. *Journal of Neuroscience*. **14**, 310-317.

Chavez-Noriega, L. E., & Stevens, C. F. (1992). Modulation of synaptic efficacy in field CA1 of the rat hippocampus by forskolin. *Brain Research*. **574**, 85-92.

Chavkin, C., James, I. J., & Goldstein, A. (1982). Dynorphin is a specific endogenous ligand of the κ opioid receptor. *Science*. **215**, 413-415.

Cherubini, E., & North, R. A. (1985). Mu and kappa opioids inhibit transmitter release by different mechanisms. *Proceedings of the national academy of sciences USA*. **82**, 1860-1863.

Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., T., I., K., N., Toshioka, T., & Hidaka, H. (1990). Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-

bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. *Journal of Biological Chemistry.* **265,** 5267-5272.

Claiborne, B. J., Amaral, D. G., & Cowan, W. M. (1986). A light and electron microscopic analysis of the mossy fibers of the rat dentate gyrus. *Journal of Comparative Neurology.* **246,** 435-458.

Collier, T. J., & Routtenberg, A. (1984). Selective impairment of declarative memory following stimulation of dentate gyrus granule cells: a naloxone-sensitive effect. *Brain Research.* **310,** 384-387.

Collingridge, G. L., Kehl, S. J., & McLennan, H. (1983). Excitatory amino acids in synaptic transmission in the Schaffer collateral-commissural pathway of the rat hippocampus. *Journal of Physiology.* **334,** 33-46.

Cox, B. M. (1982). Endogenous opioid peptides: A guide to structure and terminology. *Life Sciences.* **31,** 1645-1658.

Cox, D. H., & Dunlap, K. (1992). Pharmacological discrimination of N-type from L-type calcium current and its selective modulation by transmitters. *Journal of Neuroscience.* **12,** 906-914.

Crawford, I. L., & Connor, J. D. (1973). Localization and release of glutamic acid in relation to the hippocampal mossy fibre pathway. *Nature.* **244,** 442-443.

Creager, R., Dunwiddie, T., & Lynch, G. (1980). Paired-pulse and frequency facilitation in the CA1 region of the *in vitro* rat hippocampus. *Journal of Physiology*. **299**, 409-424.

Dash, P. K., Hochner, B., & Kandel, E. R. (1990). Injection of the cAMP-responsive element into the nucleus of Aplysia sensory neurons blocks long-term facilitation. *Nature*. **345**, 718-721.

Decker, M. W., & McGaugh, J. L. (1991). The role of interactions between the cholinergic system and other neuromodulatory systems in learning and memory. *Synapse*. **7**, 151-168.

Derrick, B. E., Rodriguez, S. B., Lieberman, D. N., & Martinez, J. L. J. (1992). Mu opioid receptors are associated with the induction of hippocampal mossy fiber long-term potentiation. *Journal of Pharmacology and Experimental Therapeutics*. **263**, 725-733.

Derrick, B. E., Weinberger, S. B., & Martinez, J. L. J. (1991). Opioid receptors are involved in an NMDA receptor-independent mechanism of LTP induction at hippocampal mossy fiber-CA3 synapses. *Brain Research Bulletin*. **27**, 219-223.

Dickenson, A. H. (1991). Mechanisms of the analgesic actions of opiates and opioids. *British Medical Bulletin*. **47**, 690-702.

Dixon, D., & Atwood, L. (1989). Adenylate cyclase system is essential for long-term facilitation at the crayfish neuromuscular junction. *Journal of Neuroscience*. **9**, 4246-4252.

Dodge, F. A. J., & Rahamimoff, R. (1967). Co-operative action of calcium ions in transmitter release at the neuromuscular junction. *Journal of Physiology.* **193,** 419-432.

Dostmann, W. R. G., Taylor, S. S., Genieser, H.-G., Jastorff, B., Døskeland, S. O., & Øgreid, D. (1990). Probing the cyclic nucleotide binding sites of cAMP-dependent protein kinases I and II with analogs of adenosine 3',5'-cyclic phosphorothioates. *Journal of Biological Chemistry.* **265,** 10484-10491.

Dudai, Y. (1988). Neurogenetic dissection of learning and short-term memory in Drosophila. *Annual Review of Neuroscience.* **11,** 537-563.

Duggan, A. W., & Hendry, I. A. (1986). Laminar localization of the sites of release of immunoreactive substance P in the dorsal horn with antibody-coated electrodes. *Neuroscience Letters.* **68,** 134-140.

Duggan, A. W., Hope, P. J., Jarrott, B., Schaible, H. G., & Fleetwood-Walker, S. M. (1990). Release, spread and persistence of immunoreactive neurokinin A in the dorsal horn of the cat following noxious cutaneous stimulation. Studies with antibody microprobes. *Neuroscience.* **35,** 195-202.

Duggan, A. W., & North, R. A. (1984). Electrophysiology of opioids. *Pharmacological Reviews.* **35,** 219-281.

Duggan, A. W., Schaible, H. G., Hope, P. J., & Lang, C. W. (1992). Effect of peptidase inhibition on the pattern of intraspinally released immunoreactive substance P detected with antibody microprobes. *Brain Research.* **579,** 261-269.

Dutar, P., Rascol, O., & Lamour, Y. (1989). ω-Conotoxin GVIA blocks synaptic transmission in the CA1 field of the hippocampus. *European Journal of Pharmacology.* **174,** 261-266.

Eaton, S. A., Jane, D. E., Jones, P. L. S. J., Porter, R. H. P., Pook, P. C. K., Sunter, D. C., Vdvarhelyi, P. M., Roberts, P. J., Salt, T. E., & Watkins, J. C. (1993). Competitive antagonism at metabotropic glutamate receptors by (S)-4-carboxyphenylglycine and (RS)-α-methyl-4-carboxyphenylglycine. *European Journal of Pharmacology.* **244,** 195-197.

Eccles, J. C. (1964). *The Physiology of Synapses..* Springer-Verlag, Berlin. .

Egebjerg, J., Bettler, B., Hermans-Borgmeyer, I., & Heinemann, S. (1991). Cloning of a cDNA for a glutamate receptor subunit activated by kainate but not AMPA. *Nature.* **351,** 745-748.

Fields, H. L. (1987). *Pain..* McGraw-Hill, Inc., .

Fox, A. P., Nowycky, M. C., & Tsien, R. W. (1987). Kinetic and pharmacological properties distinguishing three types of calcium currents in chick sensory neurones. *Journal of Physiology.* **394,** 149-172.

Frey, U., Huang, Y.-Y., & Kandel, E. R. (1993). Effects of cAMP simulate a late stage of LTP in hippocampal CA1 neurons. *Science.* **260,** 1661-1664.

Frey, U., Krug, M., Reymann, K. G., & Matthies, H. (1988). Anisomycin, an inhibitor of protein synthesis, blocks late phases of LTP phenomena in the hippocampal CA1 region in vitro. *Brain Research.* **452,** 57-65.

Gaarskjaer, F. B. (1986). The organization and development of the hippocampal mossy fiber system. *Brain Research Reviews.* **11,** 335-357.

Gaarskjaer, F. B. (1978). Organization of the mossy fiber system of the rat studied in extended hippocampi. I Terminal area related to number of granule and pyramidal cells. *Journal of Comparative Neurology.* **178,** 49-72.

Gaarskjaer, F. B. (1978). Organization of the mossy fiber system of the rat studied in extended hippocampi. II Experimental analysis of fiber distribution with silver impregnation methods. *Journal of Comparative Neurology.* **178,** 73-88.

Gall, C., Brecha, N., Karten, H. J., & Chang, K. (1981). Localization of enkephalin-like immunoreactivity to identified axonal and neuronal populations of the rat hippocampus. *Journal of Comparative Neurology.* **198,** 335-350.

Gallagher, M. (1988). Behavioral significance of opioid peptides in relation to hippocampal function. In *Opioids in the hippocampus* ed. McGinty, J. F., & Friedman, D. P., pp. 118-132. NIDA Res. Monograph, .

Gannon, R. L., & Terrian, D. M. (1991). U-50,488H inhibits dynorphin and glutamate release from guinea pig hippocampal mossy fiber terminals. *Brain Research.* **548,** 242-247.

Gauchy, C., Desban, M., Krebs, M. O., Glowinski, J., & Kemel, M. L. (1991). Role of dynorphin-containing neurons in the presynaptic inhibitory control of the acetylcholine-evoked release of dopamine in the striosomes and the matrix of the cat caudate nucleus. *Neuroscience.* **41,** 449-458.

Gillan, M. G. C., & Kosterlitz, H. W. (1982). Spectrum of the μ-, δ- and κ-binding sites in homogenates of rat brain. *British Journal of Pharmacology.* **77,** 461-469.

Glatt, C. E., & Snyder, S. H. (1993). Cloning and expression of an adenylyl cyclase localized to the corpus striatum. *Nature.* **361,** 536-538.

Goldstein, A. (1987). Binding selectivity profiles for ligands of multiple receptor types: focus on opioid receptors. *Trends in Pharmacological Sciences.* **8,** 456-459.

Goldstein, A. (1976). Opioid peptides (endorphins) in pituitary and brain. *Science.* **193,** 1081-1086.

Greengard, P., Jen, J., Nairn, A. C., & Stevens, C. F. (1991). Enhancement of the glutamate response by cAMP-dependent protein kinase in hippocampal neurons. *Science.* **253,** 1135-1138.

Gribkoff, V. K., & Lum-Ragan, J. T. (1992). Evidence for nitric oxide synthase inhibitor-sensitive and insensitive hippocampal synaptic potentiation. *Journal of Neurophysiology*. **68**, 639-642.

Gross, R. A., & Macdonald, R. L. (1987). Dynorphin A selectively reduces a large transient (N-type calcium current of mouse dorsal root ganglion neurons in cell culture. *Proceedings of the National Academy of Sciences USA*. **84**, 5469-5473.

Grudt, T. J., & Williams, J. T. (1993). Kappa-opioid receptors also increase potassium conductance. *Proceedings of the national academy of sciences*. **90**, 11429-11432.

Gustafsson, B., & Wigström, H. (1986). Hippocampal long-lasting potentiation produced by pairing single volleys and brief conditioning tetani evoked in separate afferents. *Journal of Neuroscience*. **6**, 1575-1582.

Haas, H. L., & Rose, G. M. (1987). Noradrenaline blocks potassium conductance in rat dentate granule cells *in vitro*. *Neuroscience Letters*. **78**, 171-174.

Haley, J. E., Malen, P. L., & Chapman, P. F. (1993). Nitric oxide synthase inhibitors block long-term potentiation induced by weak but not strong tetanic stimulation at physiological brain temperatures in rat hippocampal slices. *Neuroscience Letters*. **160**, 85-88.

Haley, J. E., Wilcox, G. L., & Chapman, P. F. (1992). The role of nitric oxide in hippocampal long-term potentiation. *Neuron*. **8**, 211-216.

Hamlyn, L. H. (1961). Electron microscopy of mossy fibre endings in Ammon's Horn. *Nature.* **190,** 645-646.

Harris, E. W., & Cotman, C. W. (1986). Long-term potentiation of guinea-pig mossy fiber responses is not blocked by N-methyl- D-aspartate antagonists. *Neuroscience Letters.* **70,** 132-137.

Hashimoto, H., Ishihara, T., Shigemoto, R., Mori, K., & Nagata, S. (1993). Molecular cloning and tissue distribution of a receptor for pituitary adenylate cyclase-activating polypeptide. *Neuron.* **11,** 333-342.

Hawkins, R. D., Kandel, E. R., & Siegelbaum, S. A. (1993). Learning to modulate transmitter release: Themes and variations in synaptic plasticity. *Annual Review of Neuroscience.* **16,** 625-665.

Hebb, D. O. (1949). *The organization of behavior..* John Wiley and Sons, New York. .

Herkenham, M. (1987). Mismatches between neurotransmitter and receptor localizations in brain: Observations and implications. *Neuroscience.* **23,** 1-38.

Hescheler, J., Rosenthal, W., Trautwein, W., & Schultz, G. (1987). The GTP-binding protein, G_O, regulates neuronal calcium channels. *Nature.* **325,** 445-447.

Hess, P. (1990). Calcium channels in vertebrate cells. *Annual Review of Neuroscience.* **13,** 1337-1356.

Hirata, K., Sawada, S., & Yamamoto, C. (1991). Enhancement of transmitter release accompanying long-term potentiation in synapses between mossy fibers and CA3 neurons in hippocampus. *Neuroscience Letters*. **123**, 73-86.

Hökfelt, T. (1991). Neuropeptides in Perspective: the last ten years. *Neuron*. **7**, 867-879.

Hopkins, W. F., & Johnston, D. (1988). Noradrenergic enhancement of long-term potentiation at mossy fiber synapses in the hippocampus. *Journal of Neurophysiology*. **59**, 667-687.

Horne, A. L., & Kemp, J. A. (1991). The effect of ω-conotoxin GVIA on synaptic transmission within the nucleus accumbens and hippocampus of the rat in vitro. *British Journal of Pharmacology*. **103**, 1733-1739.

Hoshi, T., Garber, S. S., & Aldrich, R. W. (1988). Effect of forskolin on voltage-gated K+ channels is independent of adenylate cyclase activation. *Science*. **240**, 1652-1655.

Huang, Y.-Y., & Kandel, E. R. (1994). Recruitment of long-lasting and protein kinase A-dependent long-term potentiation in the CA1 region of hippocampus requires repeated tetanization. *Learning & Memory*. **1**, 74-82.

Hughes, J., Smith, T. W., Kosterlitz, H. W., Fothergill, L. A., Morgan, B. A., & Morris, H. R. (1975). Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature*. **258**, 577-579.

Isaacson, J. S., & Nicoll, R. A. (1990). Excitatory amino acid receptors underlying the associational/commissural and mossy fiber synaptic currents of CA3 pyramidal neurons. *Society for Neuroscience Abstract.* **20,** 468.

Ishizuka, N., Weber, J., & Amaral, D. G. (1990). Organization of intrahippocampal projections originating from CA3 pyramidal cells in the rat. *Journal of Comparative Neurology.* **295,** 580-623.

Ito, I., & Sugiyama, H. (1991). Roles of glutamate receptors in long-term potentiation at hippocampal mossy fiber synapses. *NeuroReport.* **2,** 333-336.

Iwama, T., Ishihara, K., Satoh, M., & Takagi, H. (1986). Different effects of dynorphin A on in vitro guinea pig hippocampal CA3 pyramidal cells with various degrees of paired-pulse facilitation. *Neuroscience Letters.* **63,** 190-194.

Jaffe, D., & Johnston, D. (1990). Induction of long-term potentiation at hippocampal mossy-fiber synapses follows a Hebbian rule. *Journal of Neurophysiology.* **64,** 948-960.

Jaffe, J. H. (1990). Drug addiction and drug abuse. In *Goodman and Gilman's The Pharmacological Basis of Therapeutics* ed. Gilman, A. G., Goodman, T. W., & Murad, F., pp. 522-573. Pergamon, New York.

Jan, L. Y., & Jan, Y. N. (1982). Peptidergic transmission in sympathetic ganglia of the frog. *Journal of Physiology.* **327,** 219-246.

Jan, Y. N., & Jan, L. Y. (1983). A LHRH-like peptidergic neurotransmitter capable of action at a distance in autonomic ganglia. *Trends in Neurosciences.* **6,** 320-325.

Jessell, T. M., & Iversen, L. L. (1977). Opiate analgesics inhibit substance P release from rat trigeminal nucleus. *Nature.* **268,** 549-551.

Johnston, D., Williams, S., Jaffe, D., & Gray, R. (1992). NMDA-receptor independent long-term potentiation. *Annual Review of Physiology.* **54,** 489-505.

Kamiya, H., Sawada, S., & Yamamoto, C. (1988). Synthetic ω-conotoxin blocks synaptic transmission in the hippocampus in vitro. *Neuroscience Letters.* **91,** 84-88.

Kandel, E. R., & Schwartz, J. H. (1982). Molecular biology of learning: Modulation of transmitter release. *Science.* **218,** 433-443.

Kase, H., Iwahashi, K., Nakanish, i. S., Matsuda, Y., Yamada, K., Takahashi, M., Murakata, C., Sato, A., & M., K. (1987). K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. *Biochemical and Biophysical Research Communications.* **142,** 436-440.

Kato, M., Chapman, C., & Bickness, R. J. (1992). Activation of κ-opioid receptors inhibits depolarisation-evoked exocytosis but not the rise in intracellular Ca^{2+} in secretory nerve terminals of the neurohypophysis. *Brain Research.* **574,** 138-146.

Katsuki, H., Kaneko, S., Tajima, A., & Satoh, M. (1991). Separate mechanisms of long-term potentiation in two input systems to CA3 pyramidal neurons of rat hippocampal slices as revealed by the whole-cell patch-clamp technique. *Neuroscience Research.* **12,** 393-402.

Katz, B., & Miledi, R. (1968). The role of calcium in neuromuscular facilitation. *Journal of Physiology.* **195,** 481-492.

Kauer, J. A., & Nicoll, R. A. (1988). An APV-resistant non-associative form of long-term potentiation in the rat hippocampus. In *Synaptic Plasticity in the Hippocampus* ed. Haas, H. L., & Buzsaki, G., pp. 65-66. Springer, Berlin.

Kemp, J. A., Foster, A. C., Leeson, P. D., Priestley, T., Tridgett, R., Iversen, L. L., & Woodruff, G. N. (1988). 7-Chlorokynurenic acid is a selective antagonist at the glycine modulatory site of the N-methyl-D-aspartate receptor complex. *Proceedings of the National Academy of Sciences, U.S.A.* **85,** 6547-6550.

Klein, M. (1994). Synaptic augmentation by 5-HT at rested aplysia sensorimotor synapses: independence of action potential duration. *Neuron.* **13,** 159-166.

Kohonen, T., Lehtiö, P., Rovamo, J., Hyvärinen, J., Bry, K., & Vainio, L. (1977). A principle of neural associative memory. *Neuroscience.* **2,** 1065-1076.

Kohonen, T., Oja, E., & Lehtiö, P. (1981). Storage and processing of information in distributed associative memory systems. In *Parallel models of associative memory* ed. Hinton, G. E., & Anderson, J. A., pp. 105-143. Lawrence Erlbaum Associates, Inc., Hillsdale, N.J.

Konishi, S., Tsunoo, A., & Otsuka, M. (1981). Enkephalin as a transmitter for presynaptic inhibition in sympathetic ganglia. *Nature*. **294**, 80-82.

Koob, G. F. (1992). Drugs of abuse: anatomy, pharmacology and function of reward pathways. *Trends in Pharmacological Sciences*. **13**, 177-184.

Korn, H., & Faber, D. S. (1991). Quantal analysis and synaptic efficacy in the CNS. *Trends in Neurosciences*. **14**, 439-445.

Koyano, K., Kuba, K., & Minota, S. (1985). Long-term potentiation of transmitter release induced by repetitive presynaptic activities in bull-frog sympathetic ganglia. *Journal of Physiology*. **359**, 219-233.

Kuba, K., & Kumamoto, E. (1986). Long-term potentiation of transmitter release induced by adrenaline in bull-frog sympathetic ganglia. *Journal of Physiology*. **374**, 515-530.

Kullmann, D. M. (1994). Amplitude fluctuations of dual-component EPSCs in hippocampal pyramidal cells: implications for long-term potentiation. *Neuron*. **12**, 1111-1120.

Kullmann, D. M., Perkel, D. J., Manabe, T., & Nicoll, R. A. (1992). Ca^{2+} entry via postsynaptic voltage-sensitive Ca^{2+} channels can transiently potentiate excitatory synaptic transmission in the hippocampus. *Neuron*. **9**, 1175-1183.

Kupferman, I. (1991). Functional studies of cotransmission. *Physiological Reviews*. **71**, 683-732.

Langdon, R. B., Johnson, J. W., & Barrionuevo, G. (1993). Asynchrony of mossy fibre inputs and excitatory postsynaptic currents in rat hippocampus. *Journal of Physiology*. **472**, 157-176.

Langdon, R. B., Johnson, J. W., & Barrionuevo, G. (1993). Long-term potentiation of low-amplitude EPSCs elicited by mossy fiber stimulation in rat hippocampus. **19**, (in press).

Laurenza, A., Sutkowski, E. M., & Seamon, K. B. (1989). Forskolin: A specific stimulator of adenylyl cyclase or a diterpene with multiple sites of action? *Trends in Pharmacological Sciences*. **10**, 442-447.

Lee, K. S. (1983). Cooperativity among afferents for the induction of potentiation in the CA1 region of the hippocampus. *Journal of Neuroscience*. **3**, 1369-1372.

Lipkin, D., Cook, W. H., & Markham, R. (1959). Adenosine-3':5'-phosphoric acid: A proof of structure. *Journal of the American Chemical Society*. **81**, 6198-6203.

Llinás, R., Sugimori, M., Hillman, D. E., & Cherksey, B. (1992). Distribution and functional significance of the P-type, voltage-dependent Ca^{2+} channels in the mammalian central nervous system. *Trends in Neuroscience*. **15**, 351-355.

Luebke, J. I., Dunlap, K., & Turner, T. J. (1993). Multiple calcium channel types control glutamatergic synaptic transmission in the hippocampus. *Neuron*. **11**, 895-902.

Lundberg, J. M., & Hokfelt, T. (1983). Coexistence of peptides and classical neurotransmitters. *Trends in Neurosciences*. **6**, 325-333.

Lupica, C. R., & Dunwiddie, T. V. (1991). Differential effects of mu- and delta-receptor selective opioid agonists on feedforward and feedback GABAergic inhibition in hippocampal brain slices. *Synapse*. **8**, 237-248.

Lynch, G., Larson, J., Kelso, S., Barrionuevo, G., & Schottler, F. (1983). Intracellular injections of EGTA block induction of hippocampal long-term potentiation. *Nature*. **305**, 719-721.

Macdonald, R. L., & Werz, M. A. (1986). Dynorphin A decreases voltage-dependent calcium conductance of mouse dorsal root ganglion neurones. *Journal of Physiology*. **377**, 237-249.

Mackey, S. L., Glanzman, D. L., Small, S. A., Dyke, A. M., Kandel, E. R., & Hawkins, R. D. (1987). Tail shock produces inhibition as well as sensitization of the siphon-withdrawal reflex of Aplysia: possible behavioral role for presynaptic inhibition mediated by the peptide Phe-Met-Arg-Phe-NH2. *Proceedings of the National Academy of Sciences USA*. **84**, 8730-8734.

Madison, D. V. (1983). The actions of norepinephrine in the hippocampus. University of California, San Francisco. Ph.D.

Madison, D. V., Malenka, R. C., & Nicoll, R. A. (1991). Mechanisms underlying long-term potentiation of synaptic transmission. *Annual Review of Neuroscience.* **14,** 379-397.

Madison, D. V., & Nicoll, R. A. (1986). Cyclic adenosine 3',5'-monophosphate mediates β-receptor actions of noradrenaline in rat hippocampal pyramidal cells. *Journal of Physiology.* **372,** 245-259.

Madison, D. V., & Nicoll, R. A. (1982). Noradrenaline blocks accommodation of pyramidal cell discharge in the hippocampus. *Nature.* **299,** 636-638.

Malenka, R., Lancaster, B., & Zucker, R. S. (1992). Temporal limits on the rise in postsynaptic calcium required for long-term potentiation. *Neuron.* **9,** 121-128.

Malenka, R. C., Kauer, J. A., Perkel, D. J., Kelly, P. T., Nicoll, R. A., & Waxham, M. N. (1989). An essential role for postsynaptic calmodulin and protein kinase activity in long-term potentiation. *Nature.* **340,** 554-557.

Malenka, R. C., Kauer, J. A., Zucker, R. J., & Nicoll, R. A. (1988). Postsynaptic calcium is sufficient for potentiation of hippocampal synaptic transmission. *Science.* **242,** 81-84.

Malinow, R., Schulman, H., & Tsien, R. W. (1989). Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP. *Science.* **245,** 862-866.

Malinow, R., & Tsien, R. W. (1990). Presynaptic enhancement shown by whole-cell recordings of long-term potentiation in hippocampal slices. *Nature.* **346,** 177-180.

Mallart, A., & Martin, A. R. (1968). The relation between quantum content and facilitation at the neuromuscular junction of the frog. *Journal of Physiology.* **196,** 593-604.

Manabe, T., Wyllie, D. J. A., Perkel, D. J., & Nicoll, R. A. (1993). Modulation of synaptic transmission and long-term potentiation: effects on paired pulse facilitation and EPSC variance in the CA1 region of the hippocampus. *Journal of Neurophysiology.* **70,** 1451.

Manzoni, O. J., Weisskopf, M. G., & Nicoll, R. A. (1994). MCPG antagonizes metabotropic glutamate receptors but not long-term potentiation in the hippocampus. *European Journal of Pharmacology.* **6,** 1050-1054.

Marr, D. (1971). Simple memory: a theory for archicortex. *Philosophical Transactions of the Royal Society (London) B.* **262,** 23-81.

Martin, M. R. (1983). Naloxone and long-term potentiation of hippocampal CA3 field potentials in vitro. *Neuropeptides.* **4,** 45-50.

Matthies, H., & Reymann, K. G. (1993). Protein kinase A inhibitors prevent the maintenance of hippocampal long-term potentiation. *NeuroReport.* **4,** 712-714.

McDaniel, K. L., Mundy, W. R., & Tilson, H. A. (1990). Microinjection of dynorphin into the hippocampus impairs spatial learning in rats. *Pharmacology, Biochemistry and Behavior.* **35**, 429-435.

McFadzean, I. (1988). The ionic mechanism underlying opioid actions. *Neuropeptides.* **11**, 173-180.

McFadzean, I., Lacey, M. G., Hill, R. G., & Henderson, G. (1987). κ-Opioid receptor activation depresses excitatory synaptic input to rat locus coeruleus neurones in vitro. *Neuroscience.* **20**, 231-239.

McGinty, J. F., Henriksen, S. J., Goldstein, A., Terenius, L., & Bloom, F. E. (1983). Dynorphin is contained within hippocampal mossy fibers: immunochemical alterations after kainic administration and colchicine-induced neurotoxicity. *Proceedings of the National Academy of Sciences USA.* **80**, 589-593.

McLean, S., Rothman, R. B., Jacobson, A. E., Rice, K. C., & Herkenham, M. (1987). Distribution of opiate receptor subtypes and enkephalin and dynorphin immunoreactivity in the hippocampus of squirrel, guinea pig, rat, and hamster. *Journal of Comparative Neurology.* **255**, 497-510.

McNaughton, B. L., & Nadel, L. (1990). Hebb-Marr networks and the neurobiological representation of action in space. In *Neuroscience and connectionist theory* ed. Gluck, M. A., & Rumelhart, D. E., pp. 1-64. Lawrence Erlbaum Associates, Inc., Hillsdale, N.J.

Miles, R., & Wong, R. K. S. (1987). Inhibitory control of local excitatory circuits in the guinea-pig hippocampus. *Journal of Physiology.* **388,** 611-629.

Miller, R. J. (1993). A tale of two toxins. *Current Biology.* **3,** 481-483.

Minota, S., Kumamoto, E., Kitakoga, O., & Kuba, K. (1991). Long-term potentiation induced by a sustained rise in the intraterminal Ca2+ in bull-frog sympathetic ganglia. *Journal of Physiology.* **435,** 421-438.

Mintz, I. M., Adams, M. E., & Bean, B. P. (1992). P-type calcium channels in rat central and peripheral neurons. *Neuron.* **9,** 85-95.

Mintz, I. M., & Bean, B. P. (1993). Voltage-dependence of P-type calcium channel block by synthetic ω-AGA-IVA. *Society for Neuroscience Abstract.* 1478.

Mintz, I. M., Venema, V. J., Swiderek, K., Lee, T., Bean, B. P., & Adams, M. E. (1992). P-type calcium channels blocked by the spider toxin ω-Aga-IVA. *Nature.* **355,** 827-829.

Monaghan, D. T., & Cotman, C. W. (1985). Distribution of N-methyl-D-aspartate-sensitive L-[^{3}H] glutamate-binding sites in rat brain. *Journal of Neuroscience.* **5,** 2909-2919.

Montarolo, P. G., Goelet, P., Castellucci, V. F., Morgan, J., Kandel, E. R., & Schacher, S. (1986). A critical period for macromolecular synthesis in long-term heterosynaptic facilitation in Aplysia. *Science.* **234,** 1249-1254.

Mosberg, H. I., Hurst, R., Hruby, V. J., Gee, K., Yamamura, H. I., Galligan, J. J., & Burks, T. F. (1983). Bis-penicillamine enkephalins possess highly improved specificity toward δ opioid receptors. *Proceedings of the National Academy of Sciences USA*. **80**, 5871-5874.

Mudge, A. W., Leeman, S. E., & Fischbach, G. D. (1979). Enkephalin inhibits release of substance P from sensory neurons in culture and decreases action potential duration. *Proceedings of the National Academy of Sciences USA*. **76**, 526-530.

Musgrave, M. A., Ballyk, B. A., & Goh, J. W. (1993). Coactivation of metabotropic and NMDA receptors is required for LTP induction. *NeuroReport*. **4**, 712-714.

Nadel, L. (1991). The hippocampus and space revisited. *Hippocampus*. **1**, 221-229.

Nakanishi, S. (1992). Molecular diversity of glutamate receptors and implications for brain function. *Science*. **258**, 597-603.

Neumaier, J. F., Mailheau, S., & Chavkin, C. (1988). Opioid receptor-mediated responses in the dentate gyrus and CA1 region of the rat hippocampus. *Journal of Pharmacology and Experimental Therapeutics*. **244**, 564-570.

Nicoll, R. A., & Alger, B. E. (1981). A simple chamber for recording from submerged brain slices. *Journal of Neuroscience Methods*. **4**, 153-156.

Nicoll, R. A., Siggins, G. R., Ling, N., Bloom, F. E., & Guillemin, R. (1977). Neuronal actions of endorphins and enkephalins among brain region: A comparative microiontophoretic study. *Proceedings of the National Academy of Sciences, U.S.A.* **74**, 2584-2588.

Nicoll, R. A., Wyllie, D. J. A., Manabe, T., & Perkel, D. J. (1993). Current physiological models for long-term potentiation in the CA1 region of the hippocampus. In *Dahlem Conference on Cellular and Molecular Mechanisms Underlying Higher Neural Functions* ed. Selverston, A. I., & Ascher, P., pp. Wiley, Chichester.

Nock, B., Giordano, L., Cicero, T., & O'Connor, L. (1990). Affinity of drugs and peptides for U-69,593-sensitive and -insensitive kappa opiate binding sites: the U-69,593-insensitive site appears to be the beta endorphin-sepcific epsilon receptor. *Journal of Pharmacology and Experimental Therapeutics.* **254**, 412-419.

North, R. A. (1986). Opioid receptor types and membrane ion channels. *Trends in Neurosciences.* 114-117.

Nowak, L., Bregestovski, P., Ascher, P., Herbet, A., & Prochiantz, A. (1984). Magnesium gates glutamate-activated channels in mouse central neurones. *Nature.* **307**, 462-465.

O'Dell, T. J., Hawkins, R. D., Kandel, E. R., & Arancio, O. (1991). Tests of the roles of two diffusible substances in long-term potentiation: Evidence for nitric oxide as a possible early retrograde messenger. *Proceedings of the National Academy of Sciences, USA.* 88, 11285-11289.

O'Dell, T. J., Kandel, E. R., & Grant, S. G. N. (1991). Long-term potentiation in the hippocampus is blocked by tyrosine kinase inhibitors. *Nature.* **353,** 558-560.

Parsons, M. E., Owen, D. A., Ganellin, C. R., & Durant, G. J. (1977). Dimaprit-(S-[3-(N,N-dimethylamino)prophyl]isothiourea) - a highly specific histamine H2 -receptor agonist. Part 1. Pharmacology. *Agents and Actions.* **7,** 31-37.

Paton, W. D. M. (1958). Central and synaptic transmission in the nervous system (pharmacological aspects). *Annual Review of Physiology.* **20,** 431-470.

Perkel, D. J., Petrozzino, J. J., Nicoll, R. A., & Connor, J. A. (1993). The role of Ca2+ entry via synaptically activated NMDA receptors in the induction of long-term potentiation. *Neuron.* **11,** 817-823.

Pert, C. B., & Snyder, S. H. (1973). Opiate receptor: Demonstration in nervous tissue. *Science.* **179,** 1011-1014.

Pinnock, R. D. (1992). Activation of κ-opioid receptors depresses electrically evoked excitaotry possynaptic potentials on 5-HT-sensitive neurones in the rat dorsal raphé nucleus in vitro. *Brain Research.* **583,** 237-246.

Plummer, M. R., Logothetis, D. E., & Hess, P. (1989). Elementary properties and pharmacological sensitivities of calcium channels in mammalian peripheral neurons. *Neuron.* **2,** 1453-1463.

Raymond, L. A., Blackstone, C. E., & Huganir, R. L. (1993). Phosphorylation and modulation of recombinant GluR6 glutamate receptors by cAMP-dependent protein kinase. *Nature.* **361,** 637-641.

Regehr, W. G., Delaney, K. R., & Tank, D. W. (1994). The role of presynaptic calcium in short-term enhancement at the hippocampal mossy fiber synapse. *Journal of Neuroscience.* **14,** 523-537.

Regehr, W. G., & Tank, D. W. (1991). The maintenance of LTP at hippocampal mossy fiber synapses is independent of sustained presynaptic calcium. *Neuron.* **7,** 451-459.

Reisine, T., & Bell, G. I. (1993). Molecular biology of opioid receptors. *Trends in Neurosciences.* **16,** 506-510.

Reynolds, I., Wagner, J., Snyder, S., Thayer, S., Olivera, B., & Miller, R. (1986). Brain voltage-sensitive calcium channel subtypes differentiated by ω-conotoxin fraction GVIA. *Proceedings of the National Academy of Sciences.* **83,** 8804-8807.

Rolls, E. T. (1990). Theoretical and neurophysiological analysis of the functions of the primate hippocampus in memory. *Cold Spring Harbor Symposia on Quantitative Biology.* **55,** 995-1006.

Sah, P., & Nicoll, R. A. (1991). Mechanisms underlying potentiation of synaptic transmission in rat anterior cingulate cortex in vitro. *Journal of Physiology.* **433,** 615-630.

Sawada, S., & Yamamoto, C. (1984). Fast and slow depolarizing potentials induced by short pulses of kainic acid in hippocampal neurons. *Brain Research.* **324,** 279-287.

Schacher, S., Castellucci, V. F., & Kandel, E. R. (1988). cAMP evokes long-term facilitation in Aplysia sensory neurons that requires new protein synthesis. *Science.* **240,** 1667-1669.

Schoepp, D. D., & Conn, P. J. (1993). Metabotropic glutamate receptors in brain function and pathology. *Trends in Pharmacological Sciences.* **14,** 13-20.

Scholz, K. P., & Byrne, J. H. (1988). Intracellular injection of cAMP induces long-term reduction of neuronal K^+ currents. *Science.* **240,** 1664-1666.

Schroeder, J. E., Fischbach, P. S., Zheng, D., & McCleskey, E. W. (1991). Activation of mu opioid receptors inhibits transient high- and low-threshold Ca2+ currents, but spares a sustained current. *Neuron.* **6,** 13-20.

Schuman, E. M., & Madison, D. V. (1994). Locally distributed synaptic potentiation in the hippocampus. *Science.* **263,** 532-536.

Schuman, E. M., & Madison, D. V. (1991). A requirement for the intercellular messenger nitric oxide in long-term potentiation. *Science.* **254,** 1503-1506.

Seamon, K. B., & Daly, J. W. (1981). Forskolin: A unique diterpene activator of cyclic AMP-generating systems. *Journal of Cyclic Nucleotide Research.* **7,** 201-224.

Seward, E., Hammond, C., & Henderson, G. (1991). Mu-opioid-receptor-mediated inhibition of the N-type calcium-channel current. *Proceedings of the Royal Society of London (B).* **244,** 129-135.

Shimahara, T., & Icard-Liepkalns, C. (1987). Activation of enkephalin receptors reduces calcium conductance in neuroblastoma cells. *Brain Research.* **415,** 357-361.

Shuster, M. J., Camardo, J. S., Siegelbaum, S. A., & Kandel, E. R. (1985). Cyclic AMP-dependent protein kinase closes the serotonin-sensitive K+ channels of Aplysia sensory neurons. *Nature.* **313,** 392-395.

Siegelbaum, S. A., Camardo, J. S., & Kandel, E. R. (1982). Serotonin and cAMP close single K channels in Aplysia sensory neurons. *Nature.* **299,** 413-.

Simon, E. J., Hiller, J. M., & Edelman, I. (1973). Stereospecific binding of the potent narcotic analgesic [^{3}H]etorphine to rat-brain homogenate. *Proceedings of the National Academy of Sciences USA.* **70,** 1947-1949.

Smith, S. J., & Augustine, G. J. (1988). Calcium ions, active zones and synaptic transmitter release. *Trends in Neurosciences.* **11,** 458-464.

Squire, L. R. (1992). Memory and the hippocampus: a synthesis from findings with rats, monkeys, and humans. *Psychological Review.* **99,** 195-231.

Starke, K. (1977). Regulation of noradrenaline release by presynaptic receptor systems. *Reviews of Physiology Biochemistry and Pharmacology.* **77,** 1-124.

Staubli, U., Larson, J., & Lynch, G. (1990). Mossy fiber potentiation and long-term potentiation involve different expression mechanisms. *Synapse.* **5,** 333-335.

Storm-Mathisen, J., Leknes, A. K., Bore, A. T., Vaaland, J. L., Edminson, P., Haug, F. M. S., & Ottersen, O. P. (1983). First visualization of glutamate and GABA in neurones by immunocytochemistry. *Nature.* **301,** 517-520.

Sutherland, E. W. (1972). Studies on the mechanism of hormone action. *Science.* **177,** 401-408.

Takahashi, T., & Momiyama, A. (1993). Different types of calcium channels mediate central synaptic transmission. *Nature.* **366,** 156-158.

Takemori, A. E., Ho, B. Y., Naeseth, J. S., & Portoghese, P. S. (1988). Nor-binaltorphimine, a highly selective kappa-opioid antagonist in analgesic and receptor binding assays. *Journal of Pharmacology and Experimental Therapeutics.* **246,** 255-258.

Tang, W.-J., & Gilman, A. G. (1992). Adenylyl cyclases. *Cell.* **70,** 869-872.

Tang, W.-J., Krupinski, J., & Gilman, A. G. (1991). Expression and characterization of calmodulin-activated (type I) adenylylcyclase. *Journal of Biological Chemistry.* **266,** 8595-8603.

Terenius, L. (1973). Stereospecific interaction between narcotic analgesics and a synaptic plasma membrane fraction of rat cerebral cortex. *Acta Pharmacologica et Toxicolica*. **32**, 317-320.

Terrian, D. M., Dorman, R. V., & Gannon, R. L. (1990). Characterization of the presynaptic calcium channels invovled in glutamate exocytosis from rat hippocampal mossy fiber synaptosomes. *Neuroscience Letters*. **119**, 211-214.

Terrian, D. M., Johnston, D., Claiborne, B. J., Ansah-Yiadom, R., Strittmatter, W. J., & Rea, M. A. (1988). Glutamate and dynorphin release from a subcellular fraction enriched in hippocampal mossy fiber synaptosomes. *Brain Research Bulletin*. **21**, 343-351.

Teyler, T. J., & DiScenna, P. (1986). The hippocampal memory indexing theory. *Behavioral Neuroscience*. **100**, 147-154.

Tsien, R. W., Ellinor, P. T., & Horne, W. A. (1991). Molecular diversity of voltage-dependent Ca^{2+} channels. **12**, 349-354.

Turner, T. J., Adams, M. E., & Dunlap, K. (1992). Calcium channels coupled to glutamate release identified by ω-Aga-IVA. *Science*. **258**, 310-313.

Van Haastert, P. J. M., Van Driel, R., Jastorff, B., Baraniak, J., Stec, W. J., & De Wit, R. J. W. (1984). Competitive cAMP antagonists for cAMP-receptor proteins. *Journal of Biological Chemistry*. **259**, 10020-10024.

Wagner, J. J., Caudle, R. M., & Chavkin, C. (1992). K-Opioids decrease excitatory transmission in the dentate gyrus of the guinea pig hippocampus. *Journal of Neuroscience*. **12**, 132-141.

Wagner, J. J., Evans, C. J., & Chavkin, C. (1991). Focal stimulation of the mossy fibers releases endogenous dynorphins that bind κ_1-opioid receptors in guinea pig hippocampus. *Journal of Neurochemistry*. **57**, 333-343.

Wang, L.-Y., Salter, M. W., & MacDonald, J. F. (1991). Regulation of kainate receptors by cAMP-dependent protein kinase and phosphatases. *Science*. **253**, 1132-1135.

Wang, L. Y., Taverna, F. A., Huang, X. P., MacDonald, J. F., & Hampson, D. R. (1993). Phosphorylation and modulation of a kainate receptor (GluR6) by cAMP-dependent protein kinase. *Science*. **259**, 1173-1175.

Watkins, J. C., Krogsgaard-Larsen, P., & Honoré, T. (1990). Structure—activity relationships in the development of excitatory amino acid receptor agonists and competitive antagonists. *Trends in Pharmacological Sciences*. **11**, 25-33.

Weisskopf, M. G., Castillo, P. E., Zalutsky, R. A., & Nicoll, R. A. (1994). Mediation of hippocampal mossy fiber long-term potentiation by cyclic AMP. *Science*. **265**, 1878-1882.

Weisskopf, M. G., Zalutsky, R. A., & Nicoll, R. A. (1993). The opioid peptide dynorphin mediates heterosynaptic depression of hippocampal mossy fiber synapses and modulates long-term potentiation. *Nature*. **362**, 423-427.

Werz, M. A., & Macdonald, R. L. (1984). Dynorphin reduces calcium-dependent action potential duration by decreasing voltage-dependent calcium conductance. *Neuroscience Letters*. **46**, 185-190.

Werz, M. A., & Macdonald, R. L. (1983). Opioid peptides selective for mu- and delta-opiate receptors reduce calcium-dependent action potential duration by increasing potassium conductance. *Neuroscience Letters*. **42**, 173-178.

Wieprecht, M., Wieder, T., & Geilen, C. C. (1994). N-[2-bromocinnamyl(amino)ethyl]-5-isoquinolinesulphonamide (H-89) inhibits incorporation of choline into phosphatidylcholine via inhibition of choline kinase and has no effect on the phosphorylation of CTP:phosphocholine cytidylyltransferase. *Biochemical Journal*. **297**, 241-247.

Williams, J. H., Li, Y. G., Nayak, A., Errington, M. L., Murphy, K. P., & Bliss, T. V. (1993). The suppression of long-term potentiation in rat hippocampus by inhibitors of nitric oxide synthase is temperature and age dependent. *Neuron*. **11**, 877-884.

Williams, J. T., Egan, T. M., & North, R. A. (1982). Enkephalin opens potassium channels on mammalian central neurones. *Nature*. **299**, 74-77.

Williams, S., & Johnston, D. (1989). Long-term potentiation of hippocampal mossy fiber synapses is blocked by postsynaptic injection of calcium chelators. *Neuron*. **3**, 583-588.

Willshaw, D. J., & Buckingham, J. T. (1990). An assessment of Marr's theory of the hippocampus as a temporary memory store. *Philosophical Transactions of the Royal Society of London B*. **329**, 205-215.

Wise, R. A. (1989). Opiate reward: sites and substrates. *Neuroscience & Biobehavioral Reviews*. **13**, 129-133.

Wojtowicz, J. M., & Atwood, H. L. (1988). Presynaptic long-term facilitation at the crayfish neuromuscular junction: Voltage-dependent and ion-dependent phases. *Journal of Neuroscience*. **8**, 4667-4674.

Wojtowicz, J. M., Parnas, I., Parnas, H., & Atwood, H. L. (1988). Long-term facilitation of synaptic transmission demonstrated with macro-patch recording at the crayfish neuromuscular junction. *Neuroscience Letters*. **90**, 152-158.

Worley, P. F., Baraban, J. M., De Souza, E. B., & Snyder, S. H. (1986). Mapping second messenger systems in the brain: Differential localizations of adenylate cyclase and protein kinase C. *Proceedings of the National Academy of Sciences USA*. **83**, 4053-4057.

Wu, L. G., & Saggau, P. (1994). Pharmacological identification of two types of presynaptic voltage-dependent calcium channels at CA3-CA1 synapses of the hippocampus. *Journal of Neuroscience*. **14**, 5613-5622.

Wu, Z., Wong, S. T., & Storm, D. R. (1993). Modification of the calcium and calmodulin sensitivity of the type I adenylyl cyclase by mutagenesis of its calmodulin binding domain. *Journal of Biological Chemistry*. **268**, 23766-23768.

Xia, Z., Refsdal, C. D., Merchant, K. M., Dorsa, D. M., & Storm, D. R. (1991). Distribution of mRNA for the calmodulin-sensitive adenylate cyclase in rat brain: Expression in areas associated with learning and memory. *Neuron.* **6**, 431-443.

Xiang, J.-Z., Adamson, P., Brammer, M. J., & Campbell, I. C. (1990). The κ-opiate agaonist U50,488H decreases the entry of ^{45}Ca into rat cortical synaptosomes by inhibiting N- but not L-type calcium channels. *Neuropharmacology.* **29**, 439-444.

Xiang, Z., Greenwood, A. C., Kairiss, E. W., & Brown, T. H. (1994). Quantal mechanism of long-term potentiation in hippocampal mossy-fiber synapses. *Journal of Neurophysiology.* **71**, 2552-2556.

Yamamoto, C., Sawada, S., & Kamiya, H. (1992). Enhancement of postsynaptic responsiveness during long-term potentiation of mossy fiber synapses in guinea pig hippocampus. *Neurosciences Letters.* **138**, 111-114.

Yamamoto, C., Sawada, S., & Ohno-Shosaku, T. (1994). Suppression of hippocampal synaptic transmission by the spider toxin omega-agatoxin-IV-A. *Brain Research.* **634**, 349-352.

Zalutsky, R. A., & Nicoll, R. A. (1990). Comparison of two forms of long-term potentiation in single hippocampal neurons. *Science.* **248**, 1619-1624.

Zalutsky, R. A., & Nicoll, R. A. (1992). Mossy fiber long-term potentiation shows specificity but no apparent cooperativity. *Neuroscience Letters.* **138**, 193-197.

Zhuo, M., Small, S. A., Kandel, E. R., & Hawkins, R. D. (1993). Nitric oxide and carbon monoxide produce activity-dependent long-term synaptic enhancement in hippocampus. *Science.* **260,** 1946-1950.

Zieglgansberger, W., French, E. D., Siggins, G. R., & Bloom, F. E. (1979). Opioid peptides may excite hippocampal pyramidal neurons by inhibiting interneurons. *Science.* **205,** 415-417.

Zucker, R. S. (1989). Short-term synaptic plasticity. *Annual Review of Neuroscience.* **12,** 13-31.

Zucker, R. S., Delaney, K. R., Mulkey, R., & Tank, D. W. (1991). Presynaptic calcium in transmitter release and posttetanic potentiation. *Annals of the New York Academy of Sciences.* **635,** 191-207.

Zucker, R. S., & Landò, L. (1986). Mechanism of transmitter release: Voltage hypothesis and calcium hypothesis. *Science.* **231,** 574-579.

Zukin, R., Eghbali, M., Olive, D., Unterwald, E., & Tempel, A. (1988). Characterization and visualization of rat and guinea pig κ opioid receptors: evidence for κ_1 and κ_2 opioid receptors. *Proceedings of the National Academy of Sciences USA.* **85,** 4061-4065.