The overall goal of this research project is to systematically investigate a number of the possible ways through which presynaptic modulation might influence the effectiveness of local synaptic interactions at the mammalian hippocampal mossy fiber synapse. The potential significance of this research has been dramatically highlighted by the events of this past year, in which several different laboratories conclusively demonstrated, that long-term potentiation (LTP) in the mossy fiber-CA3 synapse involves an enhancement of neurotransmitter release (Bekkers et al., 1990; Malinow and Tsien, 1990; Staubli et al., 1990; Zalutsky and Nicoll, 1990). The LTP of synaptic transmission in the hippocampus is a widely studied model system for understanding the cellular mechanisms of memory and synaptic plasticity. Thus, a definitive link has now been established between mossy fiber synaptic plasticity and the presynaptic modulation of this synaptic input. Specifically, any factor that is capable of enhancing or suppressing the release of mossy fiber transmitters will have a predictable effect on the probability that LTP is maintained in the mossy fiber-CA3 synapse.
A hippocampal subcellular fraction that is highly enriched in large mossy fiber nerve endings was developed by our laboratory to investigate these presynaptic mechanisms for the modulation of synaptic plasticity. The morphological and metabolic properties of this synaptosomal preparation have previously been described, and both glutamic acid and prodynorphin-derived peptides are known to be released from these specialized nerve endings in response to membrane depolarization by calcium-dependent mechanisms. Recently, this laboratory published the first direct biochemical evidence to confirm that a distinct presynaptic acidic amino acid receptor, that is sensitive to \( \text{L}(+) \)-aminophosphonobutyric acid, is capable of suppressing the excitatory mossy fiber synaptic input by inhibiting the release of both glutamic acid and dynorphin peptides. The goal for the first year of this research project was to further characterize the functional properties of this putative autoreceptor and to test several specific hypotheses concerning the presynaptic modulation of concomitantly released glutamic acid and dynorphin peptides. Possible mechanisms for the presynaptic facilitation of neurotransmitter release and their potential involvement in the production and maintenance of mossy fiber LTP have also been investigated.

Significant progress has been made in a number of directions during this past year, despite our relocation to East Carolina University. Within three months of this move, we were able to re-establish our research facilities and resume a full schedule of experimentation. Moreover, several new collaborations have now been established that promise to enhance our experimental capabilities in the immediate future.
PRESYNAPTIC MODULATION OF THE HIPPOCAMPAL MOSSY FIBER SYNAPSE

AFOSR 89-0531

Annual Technical Report

1 Summary

The overall goal of this research project is to systematically investigate a number of the possible ways through which presynaptic modulation might influence the effectiveness of local synaptic interactions at the mammalian hippocampal mossy fiber synapse. The potential significance of this research has been dramatically highlighted by the events of this past year, in which several different laboratories conclusively demonstrated that long-term potentiation (LTP) in the mossy fiber-CA3 synapse involves an enhancement of neurotransmitter release (Bekkers et al., 1990; Malinow and Tsien, 1990; Staubli et al., 1990; Zalutsky and Nicoll, 1990). The LTP of synaptic transmission in the hippocampus is a widely studied model system for understanding the cellular mechanisms of memory and synaptic plasticity. Thus, a definitive link has now been established between mossy fiber synaptic plasticity and the presynaptic modulation of this synaptic input. Specifically, any factor that is capable of enhancing or suppressing the release of mossy fiber transmitters will have a predictable effect on the probability that LTP is maintained in the mossy fiber-CA3 synapse.

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2 Research Objectives

The research objectives for the funding period 15 September 1989 - 14 September 1990 were as follows:

a) Test the hypothesis that the excitatory mossy fiber synaptic input is biochemically complex and may be mediated by the concomitant release of acidic amino acids and multiple opioid peptides.

b) Test the hypothesis that excitatory amino acid autoreceptors mediate the negative feedback regulation of neurotransmitter release from hippocampal mossy fiber nerve endings.

c) Test the hypothesis that neurotransmitter release from mossy fiber terminals is regulated by opioid autoreceptors.

d) Test the hypothesis that the activation of distinct types of voltage-sensitive calcium channels is required for the exocytosis of glutamate and dynorphin peptides.

e) Test the hypothesis that the presynaptic enhancement of mossy fiber synaptic transmission associated with LTP does not require the activation of the protein kinase C (PKC) second messenger system.

f) Test the hypothesis that the liberation of endogenous arachidonic acid from presynaptic membrane phospholipids produces a long-lasting enhancement of neurotransmitter release from mossy fiber nerve endings.

3 Status of Research

3.1 Test the hypothesis that the excitatory mossy fiber synaptic input is biochemically complex and may be mediated by the concomitant release of acidic amino acids and multiple opioid peptides.

Like other excitatory hippocampal monosynaptic pathways, LTP can be elicited by repetitive stimulation of the mossy fibers. However, unlike these other hippocampal circuits, the induction of LTP in mossy fiber-CA3 synapses is mediated by non-N-methyl-D-aspartate (NMDA) receptors (Harris and Cotman, 1986) and a supersensitivity to glutamate does not follow the removal of the mossy fiber projection to the CA3 pyramidal neurons. Therefore, it has been suggested that an excitatory amino acid other than glutamate may mediate the excitatory mossy fiber synaptic input. We had previously demonstrated that depolarized mossy fiber synaptosomes release both dynorphin B and endogenous glutamate in a Ca\textsuperscript{2+}-dependent manner (Terrian et al., 1988). However, the biochemical identity of the mossy fiber neurotransmitter had not been resolved and aspartate, in particular, was considered to be a legitimate candidate. Therefore, experiments were conducted to determine what relative amounts of prodynorphin-derived peptides and endogenous amino acids are concomitantly released from hippocampal mossy fiber synaptosomes. These experiments demonstrated that, of the eighteen amino acids present in superfusate fractions collected from depolarized mossy fiber synaptosomes, only glutamate was released in a Ca\textsuperscript{2+}-dependent manner. The release of glutamate and aspartate was increased by 360 ± 27% and 54 ± 12% over baseline, respectively. However, the evoked release of glutamate was substantially more Ca\textsuperscript{2+}-dependent (80%) than was the release of aspartate (49%). Depolarization also stimulated the release of the four prodynorphin products examined, in a rank order of dynorphin B >> dynorphin A(1-17) > dynorphin A(1-8) >> dynorphin A(1-13), with dynorphin B efflux increasing by more than five-fold over baseline values. We concluded from these results that the predominant excitatory amino acid in hippocampal mossy fiber synaptic transmission is glutamate and that this synaptic input may be modulated by at least four different products of prodynorphin processing. This work has recently been published (Terrian et al., 1990). Work in our laboratory has continued to address this important issue. We are presently extending our investigations of mossy fiber
transmitter candidates by determining whether methionine-enkephalin, an opioid, and the cholecystokinin octapeptide are also released from isolated mossy fiber nerve endings.

3.2 Test the hypothesis that excitatory amino acid autoreceptors mediate the negative feedback regulation of neurotransmitter release from hippocampal mossy fiber nerve endings.

The presynaptic control of neurotransmitter release by autoreceptors has been extensively studied for monoaminergic and cholinergic neurotransmitters, while the classification of presynaptic glutamatergic receptors and glutamate autoreceptors has received little attention. However, recent studies provide evidence for a distinct subtype of glutamate autoreceptor that is sensitive to the glutamate analogue L(+)aminophosphonobutyric acid (APB) and is associated with specific pathways in certain regions of the brain, including the hippocampal mossy fiber pathway. Early electrophysiological investigations demonstrated that APB suppresses the excitatory mossy fiber synaptic input to hippocampal CA3 pyramidal cells without altering the postsynaptic membrane potential or input resistance, or the response of CA3 cells to exogenously applied glutamate. These results led to the suggestion that APB may act at a presynaptic autoreceptor to inhibit the release of endogenous mossy fiber neurotransmitters.

An enhanced efficiency of neurotransmitter release has clearly been shown to be associated with the maintenance of LTP in the mossy fiber-CA3 synapse (Zalutsky and Nicoll, 1990; Staubli et al., 1990). Therefore, any mechanism that is capable of modulating the quantity of neurotransmitter released by the mossy fiber presynaptic terminals during LTP would be expected to influence the expression of this form of synaptic plasticity. In a preliminary study (Gannon and Terrian, 1989), we confirmed the existence of such a potential mechanism by demonstrating that APB suppressed the evoked release of both glutamate and dynorphin from mossy fiber synaptosomes. This finding may be used to suggest that mossy fiber transmitter release is decreased by the glutamate released earlier in time from the same or neighboring release sites, constituting a local negative feedback mechanism for the control of neuronal activity. More recently, we have obtained evidence that suggests this presynaptic action of APB is mediated by an ionotropic subtype of glutamate receptor. In these experiments we observed that low micromolar concentrations of quisqualate inhibited the potassium-evoked release of glutamate and dynorphin A(1-8) from guinea pig hippocampal mossy fiber synaptosomes, as does APB. Our work indicates that the presynaptic inhibition produced by these two receptor agonists is selective, since none of the other "classical" excitatory amino acid agonists tested (kainate, NMDA and AMPA) were able to mimic this effect. Moreover, we have found that the ionotropic quisqualate receptor antagonist 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX) completely blocks the presynaptic inhibition produced by quisqualate. These findings provide the first direct biochemical evidence for an APB/quisqualate-sensitive presynaptic autoreceptor capable of modulating the efficacy of hippocampal mossy fiber synaptic transmission. In contrast, high concentrations of kainate were found to enhance the release of glutamate and dynorphin by a CNQX-sensitive mechanism. These results suggest a bimodal mechanism from the autoregulation of neurotransmitter release from mossy fiber terminals. This work has recently been accepted for publication (Gannon and Terrian, 1990).

It is possible that the positive feedback system activated by kainate could be involved in the neuronal damage produced by beta-N-oxalylamino-L-alanine (Gannon and Terrian, 1989) and domoic acid, both plant-derived amino acids thought to be involved in the etiology of lathyrism and neurovisceral toxic syndrome, respectively. In an additional series of experiments, we have examined the effects of the neurotoxic amino acids BOAA and beta-N-methylamino-L-alanine (BMAA) on the release of both glutamate and dynorphin A(1-8) from hippocampal mossy fiber synaptosomes. Our interest in these amino acids arose from the recent suggestion that BOAA and BMAA exert their neurotoxic effects on the central nervous system by increasing the release of glutamate. We found that the non-NMDA receptor agonist BOAA but not BMAA, an NMDA agonist, enhanced the release of glutamate. Neither of these plant-derived amino acids affected the release of dynorphin A(1-8). These results are consistent with our conclusion that the presynaptic autoreceptor for glutamate is a non-NMDA subtype of receptor. Moreover, previous reports have indicated that the presynaptic kainate subtype of receptor may contribute to the unusual sensitivity of
the mossy fiber-CA3 pathway to epileptic damage and that these receptors may only become fully expressed or active during the reactive synaptogenesis that occurs following hippocampal neuronal damage.

3.3 Test the hypothesis that neurotransmitter release from mossy fiber terminals is regulated by opioid autoreceptors.

A major hypothesis of our original proposal was that opioids serve a dual role in modulating the efficacy of mossy fiber synaptic transmission; direct excitation of CA3 pyramidal cells and presynaptic inhibition of glutamate release. At the time we formalized this hypothesis, there was only indirect evidence for such an opioid autoreceptor. The distribution of kappa opiate binding sites in the hippocampus had been shown to parallel that of APB-sensitive sites, which our previous studies had demonstrated to be localized to the mossy fiber terminals. Therefore, the possibility arises that these kappa binding sites also mediate the presynaptic inhibition of the excitatory mossy fiber synaptic input to the CA3 neurons. Dense kappa binding is found in the mossy fiber terminal field of the guinea pig hippocampus and prodynorphin-derived peptides are relatively selective for kappa opiate binding sites. Moreover, presynaptic kappa receptors appear to depress the release of several transmitter candidates in the brain and it has been reported that both APB and kappa agonists depress mossy fiber-CA3 synaptic transmission without altering the biophysical properties of the postsynaptic membrane.

During the past year we have initiated an investigation of this hypothesis by testing the effects of various different opioid receptor agonists and antagonists on calcium availability and the release of glutamate and dynorphin B from mossy fiber synaptosomes. The selective kappa opioid agonists U-50,488H and U-62,066E were tested for their ability to modulate the depolarization-induced rise of cytosolic free calcium in, and transmitter release from, guinea pig mossy fiber terminals. Both agonists dose-dependently inhibited the evoked release of glutamate and dynorphin B, while also limiting the rise in cytosolic calcium that resulted from membrane depolarization. The presynaptic inhibitory effects of U-50,488H were attenuated by the selective kappa opioid antagonist nor-binaltorphimine (nor-BNI), but were insensitive to naloxone and the delta opioid antagonist ICI 174,864. Agonists selective for two other subtypes of opioid receptors (i.e., delta and mu) were also tested and found to have no effect. This is the first experimental evidence to confirm the presence of a presynaptic kappa opioid receptor in the hippocampal mossy fiber-CA3 synapse and to describe the nature of its influence on transmitter release. Our results suggest that endogenous dynorphin peptides may interact with this kappa receptor to autoregulate the excitatory mossy fiber synaptic input. The results of these experiments are described in a manuscript that has recently been submitted for publication (Gannon and Terrian, Brain Res., submitted).

3.4 Test the hypothesis that the activation of distinct types of voltage-sensitive calcium channels is required for the exocytosis of glutamate and dynorphin peptides.

It has been hypothesized that calcium entry via L-type channels may be required for the release of neuropeptides from neurons in the central nervous system. The N-type calcium channel, on the other hand, is thought to be primarily involved in the release of other neurotransmitters. We have tested this hypothesis by examining the differential effects of various classes of calcium channel antagonists on the concomitant release of glutamate and dynorphin A(1-8) from mossy fiber synaptosomes. This work was initiated at the USAF School of Aerospace Medicine and concluded here, at the East Carolina University School of Medicine. Briefly, we found that presynaptic N-type calcium channels make the most substantial contribution to the calcium influx required for the exocytosis of dynorphin A(1-8) from hippocampal mossy fiber nerve endings (Terrian et al., 1989b), but that the release of glutamate may be mediated by multiple types of calcium channels or a channel that is insensitive to the available pharmacological compounds (Terrian et al., in press).
3.5 Test the hypothesis that the presynaptic enhancement of mossy fiber synaptic transmission associated with LTP does not require the activation of the protein kinase C (PKC) second messenger system.

It has been reported that unesterified free fatty acids increase the probability that LTP in the hippocampal dentate gyrus will be maintained following subthreshold stimulation. Several different investigators have proposed that the mechanism by which the free fatty acids enhance plasticity involves the activation of PKC and subsequent phosphorylation of the growth-associated phosphoprotein GAP-43 in presynaptic terminals. Such a mechanism would explain how the release of glutamate remains elevated for a prolonged period following the induction of LTP. However, three indirect experimental findings argue against a similar mechanism being involved in the maintenance of mossy fiber LTP: a) autoradiographic visualization of phorbol ester binding sites in the hippocampus reveals little or no PKC in the mossy fiber terminal zone, b) electrophysiological studies found no synergistic interaction between phorbol esters and tetanic stimulation of the mossy fiber pathway and c) it was demonstrated that the hippocampal granule cells that give rise to the mossy fiber axons do not express the GAP-43 phosphoprotein mRNA. Together, these results suggest that an alternative strategy for the maintenance of mossy fiber LTP may have been adapted for the storage of neuronal information and leads to the prediction that isolated hippocampal mossy fiber nerve endings contain little, if any, endogenous PKC activity and that, unlike other hippocampal nerve endings, the release of glutamate would be insensitive to the presence of phorbol esters. In collaboration with Dr. Kirk Ways, East Carolina University School of Medicine, we have tested these hypotheses. Our results argue convincingly against the suggestion that PKC is not involved in the regulation of glutamate release from hippocampal mossy fiber nerve endings. We have found that the active isomers of two different phorbol esters produce a dose-dependent increase in the depolarization-induced release of endogenous glutamate from mossy fiber synaptosomes, while the inactive 4-α-phorbol ester is without effect. The phorbol ester-induced facilitation of glutamate release is blocked by the protein kinase inhibitor, staurosporine, and is associated with a dose-dependent increase in the availability of cytosolic free calcium. We have determined that PKC activity, measured using the histone binding assay, in isolated mossy fiber synaptosomes is roughly equivalent to that measured in a conventional hippocampal synaptosomal preparation. Western blot analysis, using monoclonal antibodies raised against synthetic polypeptide sequences unique to four of the PKC subspecies, also show that the mossy fiber nerve endings contain the PKC-α, β, γ and ε subspecies. We are currently examining the endogenous phosphoproteins that serve as substrates for PKC in the mossy fiber nerve endings. Finally, we have collaborated with Dr. Jacqueline McGinty to localize the various PKC subspecies in hippocampal tissue using immunocytochemical techniques. The results of these experiments provide the first demonstration that PKC-ε is localized to the mossy fiber terminal field. Therefore, we have concluded that PKC-ε represents at least one of the PKC isoforms that are present in the hippocampal presynaptic terminals and that the activation of the compliment of kinases that are present is sufficient to augment the excitatory mossy fiber synaptic input. The results of these studies have recently been submitted for publication (Terrian et al., Hippocampus, submitted).

3.6 Test the hypothesis that the liberation of endogenous arachidonic acid from presynaptic membrane phospholipids produces a long-lasting enhancement of neurotransmitter release from mossy fiber nerve endings.

It has been suggested that the induction of LTP in the dentate gyrus may be associated with the liberation of arachidonic acid from postsynaptic phospholipids and that this arachidonate may then function as a retrograde messenger to enhance the release of glutamate and ensure the maintenance of LTP. However, it has not previously been possible to directly test this hypothesis since such an investigation requires the use of nerve endings isolated from a synapse that has been shown to express this form of synaptic plasticity, such as the hippocampal mossy fiber synaptosomal preparation. We found ourselves in a relatively unique position, therefore, to be able to directly examine the relationship between the metabolism of arachidonic acid and the release of endogenous glutamate from a population of presynaptic terminals that express LTP. These investigations were conducted in collaboration with Dr. Robert V. Dorman at Kent State University.
We found that the activation of endogenous phospholipase A_2 and the addition of unesterified arachidonic acid stimulated the release of endogenous glutamate from hippocampal mossy fiber synaptosomes. Moreover, these treatments also significantly enhanced glutamate release upon subsequent exposure of the nerve endings to depolarizing conditions. Based on these results, we have proposed that the liberation of arachidonic acid from presynaptic phospholipids may be involved in the mechanisms of LTP in the mossy fiber-CA3 synapse. These observations have recently been published (Freeman et al., 1990).
4 Publications

4.1 Full papers and review articles


4.2 Abstracts


5 Professional Personnel Associated With the Research Project

David M. Terrian, Ph.D. - Principal Investigator
Robert L. Gannon, Ph.D. - Co-investigator
Debbie A. Zetts, B.S. - Research Technician
Meena H. Patel, B.S. - Research Technician
Teresa A. Conner-Kerr - Graduate Student
Thomas H. Privette - Graduate Student
John F. Rhodes, Jr. - Medical Student
6 Interactions

10/29/89-11/02/89 Society for Neuroscience, Phoenix, Arizona.


3/19/90 NIH Neurobiology B-II Study Section, Washington, DC.

5/9/90 Space Biomedical Peer Review Panel to NASA, Washington, DC.

5/16/90 North Carolina Chapter Meeting of the Society for Neuroscience, Durham, North Carolina.

6/20/90 Seminar for the Department of Pharmacology, East Carolina University School of Medicine, Greenville, North Carolina.

7/27/90-7/30/90 Dr. Robert V. Dorman visit to ECU for planning collaborative research, Greenville, North Carolina.


8/27/90 Chairman, Neuroscience Peer Review Panel to NASA, Washington, DC.

7 New Discoveries, Inventions, or Patent Applications

None.

9 References


