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UMI
Signaling Cascades Underlying Two Different Forms of LTP in Hippocampus

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A Thesis Submitted in Conformity with Requirements for the Degree of Doctor of Philosophy
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0-612-41219-9
ABSTRACT

Long-term potentiation (LTP) is an activity-dependent strengthening of excitatory synaptic efficacy that is considered to be a cellular model for learning & memory. LTP occurs at many types of synapses throughout the CNS. For example, in the hippocampus two distinct forms of LTP have been identified. These are the NMDAR-dependent LTP at the CA1 synapses and NMDAR-independent LTP at mossy fiber synapses.

NMDAR-dependent LTP is induced by a transient increase in postsynaptic Ca\(^{2+}\). The processes required for the activation of NMDARs are thought to involve several protein kinases. For example, the activities of NMDAR channels are regulated by the tyrosine kinase Src and protein kinase C (PKC). However, the roles of Src kinase and mGluR-coupled PKC signaling pathways in the induction of the CA1 form of LTP have not been determined. I found here that the inhibition of Src kinase in CA1 pyramidal cells blocked LTP induction. Conversely directly activating Src in the postsynaptic neuron enhanced excitatory synaptic responses, occluding LTP induced by tetanus. Src-induced enhancement of AMPAR-mediated synaptic responses required both raised [Ca\(^{2+}\)]\(_i\), and NMDARs. Thus Src activation is necessary and sufficient for inducing LTP, and may act by upregulating NMDARs.

Another protein kinase, PKC, plays a more selective role in LTP. PKC is coupled to a metabotropic glutamate receptor, mGluR5, which is found predominantly in the CA1 postsynaptic neurons. CA1 neurons from mGluR5-deficient mice showed a complete loss of the NMDAR-mediated component of LTP (LTP\(_{NMDA}\)), but normal LTP of the AMPAR-mediated component (LTP\(_{AMPA}\)). Furthermore, the LTP\(_{NMDA}\) deficit in mGluR5 mutant mice could be rescued by stimulating PKC. The results suggest that PKC couples
mGluR5 to NMDAR potentiation during LTP, and this signaling mechanism is distinct from LTP<sub>AMP</sub>.

In mossy fiber-CA3 synapses, however, the induction of LTP arises from the increase in presynaptic glutamate release. The postsynaptic NMDAR plays no role in these events. I show here for the first time that the dietary depletion of bouton Zn<sup>2+</sup> in the hippocampal slice, or with membrane-permeable Zn<sup>2+</sup> chelators, following treatment, leads to deficits in the induction of LTP at Zn<sup>2+</sup>-containing mossy fiber-CA3 synapses. The Zn<sup>2+</sup> effect was selective since no change was found in LTP at two-non-Zn<sup>2+</sup>-containing synapses, including commisural fiber-CA3 and Schaffer collateral-CA1 pathways. The impaired LTP at mossy fiber synapses was rescued by Zn<sup>2+</sup> supplementation. Bath application of the membrane-impermeable Zn<sup>2+</sup> chelator, Ca<sup>2+</sup>EDTA, had no effect on LTP. Our results indicated that presynaptically localized Zn<sup>2+</sup> is specifically required for LTP induction at mossy fiber input into CA3 neurons.

In summary, it is known that the process of LTP induction at the excitatory synapses in the hippocampus is carried out by ligand-gated ion channels and signaling molecules, which are clustered together. Two forms of LTP are induced by different cellular signaling cascades, even though the end result is an increase in synaptic efficacy. I have shown here that each of these two signaling molecules (Src kinases and mGluR-coupled PKC) reveals a distinct target component in LTP. Which of them, if any, are involved in learning and memory remains to be determined.
ACKNOWLEDGMENTS

I would like to express my gratitude to my supervisor Dr John Roder for his guidance, support, and encouragement. I have learned a great deal from his ideas, expertise and insight. I would also like to thank Dr Michael W. Salter for his instruction, and direct supervision on the Src project in this thesis. I am very grateful to Dr Martin J. Wojtowicz for his instruction on LTP recording from hippocampal slice, and to Mr Jonathan Davidow, and Dr Franco Taverna for collaborating on some of the studies described in this thesis.

I wish to acknowledge my supervisor committee members Drs John MacDonald, Martin Wojtowicz, and Michael Salter for their assistance, insights, and critical review of my thesis.

I would like to thank my wife Wei-Hong Tu, my daughter Nancy Lu for their love, encouragement and friendship. I am greatly indebted to my mother and father for their love and support.

This work was supported by Michael Smith Doctoral Award from the Medical Research Council of Canada, and the Ontario Neurotrauma Fundation.
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ACPD: 1-amino-cyclopentyl-1,3-dicarboxylate

AMPAR: α-amino-3-hydroxy-5-isoxazolepropionic acid type of glutamate receptor

AP4: L(+)-2-amino-4-phosphonobutyric acid

AP5: D-2-amino-5-phosphonopentanoate

CaM: calmodulin

CaMK-II: Ca^{2+}/calmoduline dependent kinase II

CNS: central nervous system

CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione

CSK: c-terminal Src kinase

EPSCs: excitatory postsynaptic currents

EPSPs: excitatory postsynaptic potentials

fEPSP: field excitatory postsynaptic potential

GluR: ionotropic glutamate receptor

LTD: long-term depression

LTP: Long-term potentiation

LTP_{NMDA}: NMDAR-mediated component of LTP

LTP_{AMPA}: AMPAR-mediated component of LTP

MCPG: (RS)-α-methyl-4-carboxyphenylglycine

mGluR: metabotropic glutamate receptor

NMDAR: N-methyl-D-aspartate type of glutamate receptor

PDBu: phorbol 12,13-dibutyrate

PKA: cAMP-dependent protein kinase A
PKC: protein kinase C
PSD: postsynaptic density
SH2 : Src homology 2
SH3: Src homology 3
Src: tyrosine kinase Src
VSCC: voltage-sensitive Ca^{2+} channel
CHAPTER ONE

GENERAL REVIEW

THE PROPERTIES AND UNDERLYING CAUSES OF TWO DIFFERENT FORMS OF LTP IN HIPPOCAMPUS
1.1 INTRODUCTION
One of the main mechanisms, by which neurons communicate, is synaptic transmission. It was proposed at the turn of the century by Ramon y Cajal (1911), and later formalized by Hebb (1949), that learning occurs and memory is encoded by persistent modification in synaptic efficacy. This attractive idea could not be directly tested, however, because the relevant synapses could not be isolated in behaving (i.e. learning) animals at the time.

In 1973, Bliss and Lomo made the remarkable discovery that communication between neurons across synapses in the rabbit brain undergoes a long-lasting enhancement upon tetanic stimulation (Bliss and Lomo, 1973). This finding was especially exciting because the changes occurred in the hippocampus, a part of the brain known to be important for memory (Lynch et al., 1990; Stevens, 1996., Jeffery, 1997 for reviews), and because the patterns of stimulation included the types of neuronal activity that an animal might conceivably experience during learning (Larson and Lynch, 1989; Lynch and Larson, 1988, for review). The phenomenon was subsequently called long-term potentiation (LTP) and has been intensely investigated ever since. LTP is considered to be a cellular substrate for learning and memory—in part, because it shares certain properties such as associativity, specificity and co-incidence detection.

1.1.A The nature of LTP in hippocampus

The hippocampus is the highest level of the association cortex, and is thought to play a
fundamental role in certain forms of learning and memory. This notion derives primarily from the study of surgical cases in which bilateral removal of the hippocampus, for intractable epilepsy, led to profound and selective loss of memory. The most famous of these cases is the patient called H.M. Following removal of both hippocampi, H.M. lost the ability to learn new information (Scoville and Milner, 1957). Subsequent to these surgical cases, the hypothesis for a role of the hippocampus in memory has been strengthened by studies of human amnestic patients who have selective damage to this area of the brain from a variety of causes, and from patients with Alzheimer’s disease who show a loss of hippocampal neurons (Squire, 1998). Those patients show an early decline in hippocampal associated learning tasks. Because LTP was first described in the hippocampus, and because it has been intensively studied over since, an effort is being made to understand the relationship between the hippocampus and memory and, in particular, the relationship between LTP and memory.

1.1.B The structure of the hippocampus

The hippocampi are buried within the temporal lobe of the brain, as two long, interlocking C-shaped strips. For the sake of convenience (Figure 1.1), the term hippocampus is used to refer to the hippocampus and the dentate gyrus collectively. “Hippocampal formation” is sometimes used as the term to include the subicular complex and the entorhinal cortex. The hippocampus consists of a complex of the three main subfields, the dentate gyrus, CA1 region, and CA3 region (Witter et al., 1989, for review).
Figure 1.1 The location of the hippocampus in brain

The hippocampus lies buried in the temporal lobe. Midsagittal view of the brain showing the location of the hippocampus, which consists of two interlocking C-shaped strips of cortex.
**Dentate gyrus.** The dentate gyrus is considered to be the first stage of the intrahippocampal trisynaptic loop. It is the target for the majority of entorhinal afferents which carry sensory information, in multiple modalities, about the external world. The perforant fibers from the lateral entorhinal cortex terminate in the outer one-third, and those from the medial entorhinal cortex terminate in the middle one-third, of the molecular layer, where dendrites of dentate principal cells arborize. Granule cells are the principal cells in the dentate gyrus, which number almost 1 million in the rat and 5 million in the monkey. The relatively small cell bodies of granule cells (8-12 μm in diameter) form a densely packed layer called the granule cell layer, which is 4-8 somata in thickness. The axons of granule cells, called mossy fibers, originate at the opposite pole of the soma and enter the hilus, where they give rise to several local collaterals that largely remain in the hilar region. The main axon of granule cell leaves the hilar region and courses adjacent to the pyramidal cell layer (in stratum lucidum) of the CA3 field, where the axons form the mossy terminals, on the proximal dendrites of pyramidal cells.

**CA3 region.** The CA3 region represents the second stage of the trisynaptic loop. Pyramidal cells of this region are the principal targets of granule cell axons. CA3 pyramidal cells have characteristically large somata arranged in a layer of 40-120 μm thickness. The proximal dendrites bear large complex spines, which form the synaptic complex with the large mossy terminals in a narrow layer called the stratum lucidum. In addition to the mossy fiber projection in stratum lucidum, the commissural and associational collateral input from other CA3 pyramidal cells terminate in the strata radiatum and oriens, respectively.
Chapter one: General Review

**CA1 region.** The CA1 region represents the third and last stage of three intrahippocampal groups of synapses and is the major target of CA3 pyramidal cell axons, the Schaffer collaterals. Pyramidal cells at CA1 region, which have somewhat smaller cell bodies than those in CA3, form a narrow layer (stratum pyramidale) of 50 – 100 µm thickness. The main extrinsic projections of CA1 pyramidal cells are to the subiculum and entorhinal cortex, but other limbic cortical areas, and the olfactory bulb are also among the targets of the CA1 field. The CA1 region should therefore be considered as the major output structure of the hippocampus back to the entorhinal cortex and indirectly to neocortical areas.

1.1.C The excitatory synapses and excitatory amino acid receptors in the hippocampus

Particular classes of neurons and synapses in the hippocampus can be recognized reliably, as shown in Figure 1.2. The main input to the hippocampal formation comes from the entorhinal cortex via the perforant path. The perforant path (PP) fibres synapse onto dentate granule cells and, with minor projections, to CA3 and CA1 pyramidal neurons; (b) the mossy fibres (mf), which arise from dentate granule cells and synapse onto CA3 neurons; and (c) the Schaffer collateral (Sch), which arise from CA3 neurons and synapse onto CA1 neurons.

All of these excitatory synapses use glutamate as their neurotransmitter. Glutamate is known to bind to both ionotropic receptors (iGluRs) and metabotropic
A. Three particular groups of synapses in hippocampus. 1) The perforant fiber (PP) projects onto dentate granule cells. 2) The mossy fiber (mf) arises from granule cells and projects onto CA3 pyramidal cells. 3) The Schaffer collateral (Sch), arise from CA3 neurons, and projects to CA1 neurons. B All of three groups of synapses use glutamate as their transmitter. Glutamate binds to NMDARs, AMPARs, and mGluRs.
receptors (mGluRs) (Hollmann and Heinemann, 1994; Nakanishi, 1994, for review). iGluRs can be further classified into two major types, called \( \alpha \)-amino-3-hydroxy-5-methyl-4-isozazolepropionate (AMPA) and N-methyl-D-aspartatic acid (NMDA) receptors (AMPARs and NMDARs, respectively). Increased release of glutamate from excitatory pre-synaptic terminals would increase the post-synaptic currents generated by both the NMDARs and the AMPARs if the membrane potential was depolarized, and this has been confirmed experimentally. Thus, paired pulse facilitation (Muller and Lynch, 1988) and frequency facilitation (Muller et al., 1989), as well as post-tetanic potentiation (Kauer et al., 1988), and all transient forms of synaptic facilitation that involve increased glutamate release, cause large increases in the responses generated by both classes of receptors. Moreover, under physiological conditions the excitatory postsynaptic potentials (EPSPs) are mediated by the AMPARs, but the NMDARs contribute little to transmission because their associated ion channels are blocked by Mg\(^{2+}\).

mGluRs comprise a large family of receptors coupled to second messenger systems via G-proteins. At least eight mGluR subtypes have been cloned to date (Pin and Duoisin, 1995., Conn and Pin, 1997, for reviews). Based on sequence similarity, coupling to different second messenger systems and pharmacological properties, mGluRs can be grouped into three classes. Class I mGluRs (subtype 1 and 5) are coupled to phospholipase C (PLC), which catalyzes the breakdown of membrane phospholipids to diacylglycerol (DAG) and inositol trisphosphates (IP3). Class II (subtypes 2,3) and class III (subtype 4,6,7,8) negatively couple to adenylate cyclase and regulate cAMP levels.

1.1.D Definitions of LTP in Hippocampus
LTP is defined as a persistent enhancement of excitatory synaptic efficacy. Short-term forms of plasticity such as facilitation and post-tetanic potentiation are considered a different class of phenomena because they last on the order of milliseconds to minutes, compared with LTP, which persists an hour or more. It is generally assumed that the mechanisms underlying LTP are divided in two distinct phases: the induction and the expression phase (Nicoll and Malenka, 1995, for review). The induction phase is that which occurs during and shortly after the high-frequency stimulation used to initiate LTP. It consists of all the steps and mechanisms that lead to the long-lasting changes that are associated with LTP. The expression phase is that period following the induction in which the change in synaptic efficacy is maintained. The perturbation of processes involved in the induction phase will either eliminate the initial enhancement of the synaptic responses or prevent the transformation from induction to expression. In the latter condition, a high frequency stimulation produces only a short term potentiation (STP), but does not produce LTP.

LTP described at all these three groups of synapses in hippocampus is superficially similar (ie, the end result is an increase in synaptic efficacy). However, the blockade of NMDARs prevents the induction of LTP in both the CA1 region and dentate gyrus, and is without effect on mossy fiber LTP. LTP in hippocampus thus can be generally categorized into two forms, those that require the activation of NMDARs for their induction and those that do not. These have been named NMDAR-dependent and NMDAR-independent forms of LTP (Malenka and Nicoll 1993, for review).

The following discussion will be on the molecular aspects of cellular events in these two distinct forms of LTP. First, I will discuss how the intracellular signaling
molecules regulate the NMDAR channels, and how those molecules are related to the induction of LTP. In this part, the roles of the protein-protein interaction, PKC, tyrosine kinase and mGluRs in LTP induction will be described. Second, I will address how AMPARs are modified during the expression of LTP. The polarized debate on a pre-versus post-synaptic locus of LTP expression, at CA1 synapse, will be described. Finally, I will briefly discuss the fundamental cellular mechanisms in the processes of NMDAR-independent LTP at mossy fiber synapses.

1.2 NMDAR activation and modifications during LTP induction

NMDARs are often co-localized with AMPARs at excitatory synapses. A striking feature of the NMDAR channel is the high permeability of Ca$^{2+}$ relative to Na$^+$, and the regulation of the channel function by Mg$^{2+}$ in a voltage-dependent manner. The voltage dependence of the channel regulation arises from the fact that neuronal depolarization allows the Mg$^{2+}$-block to be relieved. In the open state the NMDAR allows the entry of Ca$^{2+}$, which triggers a cascade of biochemical events resulting in synaptic change (McBain and Mayer, 1994, for review). Since the induction of LTP is completely blocked by the NMDAR antagonist, AP5, or the NMDAR channel blocker, MK-801, the prevailing hypothesis is that LTP induction requires, in at least one of its forms, the activation of NMDARs (Bliss and Collingridge, 1993, for review).

1.2.A NMDAR channels
The cloning of the first NMDAR subunit gene (named NMDAR1 or NR-1) was reported in 1991 by Moriyoshi et al, and eight NR-1 subunit variants are derived from this single gene (Sugihara et al., 1992). Several additional subunit genes have been isolated with low-stringency hybridization screening and PCR using probes derived from NR-1 sequences. These have been named NR-2. There are four NR-2 subunit genes, including NR-2A, NR-2B, NR-2C, NR-2D. (Ikeda et al., 1992; Kutsuwada et al., 1992).

**Heteromultimeric NMDARs.** NMDARs are formed by assembling the NR-1 subunit with any one of four NR-2 subunits. The NR-2 subunits appear to modulate receptor channel kinetics in different ways (Hollmann and Heinemann, 1994; Nakanishi and Masu, 1994). NR-1 is expressed by the majority of central neurons throughout all developmental stages, whereas the NR-2 subunits are expressed in distinct spatial and temporal patterns. Prenatal NMDARs contain NR-2B or NR-2D, whereas NR-2A and NR-2C are expressed only after birth, the NR-2A is expressed predominantly in the forebrain and NR-2C mainly in cerebellar granule cells (Watanabe et al., 1993; Monyer et al., 1994). The NR-1 subunit carries in its putative channel forming M2 segment the determinant for the high Ca²⁺ permeability, the NR-2 subunits carry in the homologous position the determinant for the voltage-dependent Mg²⁺ block. These determinants are asparagine residues.

**Physiological relevance of NMDARs.** Engineered mouse mutants which lack particular receptor subunits have revealed that the absence of NR-1 expression leads to perinatal death in the absence of obvious morphological brain abnormalities (Forrest et al., 1994; Li et al., 1994). This indicates the importance of the NMDARs in brain physiology. Perinatal lethality was also found for NR-2B mutant mice (Kutsuwada et al.,
1996), but not NR-2D mutant mice (Ikeda et al., 1995), which suggests that during CNS development, the NR-1/NR-2B subtype is the more important of the two. Mice lacking the postnatally expressed NR-2A and NR-2C subtypes were viable. NR-2A deficient mice had impaired LTP, whereas normal LTP was revealed in NR-2C mutant mice (Sakimura et al., 1995; Ebralidze et al., 1996; Kadotani et al., 1996).

1.2.B NMDAR clustering with PSD molecules

**PSD-95/NMDAR clustering.** Effective transmission of neuronal impulses requires the precise localization and concentration of neurotransmitter receptors on the receiving, postsynaptic neurons. At glutamate excitatory synapses, neurotransmission clearly depends on the AMPARs, NMDARs and signaling molecules being concentrated at appropriate postsynaptic sites where they can respond to presynaptically released glutamate. Some insight into the mechanisms that assemble these components has been revealed from studies of PSD-95 (for a recent review, see Kennedy, 1997). PSD-95 was initially identified as a component of postsynaptic densities (PSDs) and subsequently shown to bind to the intracellular C termini of NMDARs and shaker K^+ channels (Kornau, et al., 1997., Sheng, 1997). The C-terminus of these target proteins end with the sequence –E-S/T-D-V* that mediates their interactions with the PDZ domains of PSD-95. The function of the interaction is receptor clustering. When co-transfected with NMDAR subunits, PSD-95 forms large clusters together with the interacting channels. These PSD-95/NMDAR clusters are not found when the proteins are expressed alone or when the C-terminal PDZ-binding motifs are mutated (Kim et al., 1995).
NMDAR/PSD-95 clustering may serve as a cellular signaling transducer. The C-terminal residues of the NR-2 subunits bind to members of the PSD-95/SAP90 family of PDZ domain-containing proteins (Niethammer et al., 1996; Kornau et al., 1997). These proteins interact with synaptic NMDARs and appear to link the receptors to intracellular signaling pathways by providing multivalent binding sites. As indicated by genetic evidence in Drosophila and C.elegans, interfering with the interaction of receptors and channels with their cognate PDZ-domain proteins leads to disruption of cellular signaling pathways (Kornau et al., 1997; Tsunoda et al., 1997). A photoreceptor neuron in Drosophila attains high sensitivity by having an exceptionally large number of receptor molecules on its surface. To couple this large number of receptor molecules to the downstream transduction complexes rapidly and efficiently would require a diffusible coupling molecule, a G-protein. In this model, each G-protein would need to sample only a small number of receptors in the membrane (an ultramicrodomain of signaling) and report their activity to the downstream transduction complexes. The findings in Tsunoda's group (Tsunoda et al., 1997) of a quantum bump may represent the coordinated activation of a few hundred light-activated ion channels in response to the activation of a single rhodopsin molecule. The organization of InaD (inactivation no-after potential protein) complexes into a supramolecular complex, either through PDZ-PDZ domain interactions or PDZ-cytoskeletal interactions within a microvillus, could represent the structural basis of a quantum bump, ensuring both reliability and coordinated signaling.

The evidence that NR-2 C-termini organize signaling cascades derives from their interaction with neuronal nitric oxide syntheses (nNOS) (Brenman et al., 1996). Both
NMDAR subunits and nNOS can interact with the same PSD-95 molecule, forming a ternary complex. This occurs via a PDZ-PDZ interaction in which the second PDZ domain of PSD-95 binds a single PDZ domain in nNOS. In the cultured neurons, nNOS is activated by Ca$^{2+}$ coming through NMDARs, while Ca$^{2+}$ entry through other channels is not as effective (Brennan et al., 1996). PSD-95 binding to both the NMDAR and nNOS may provide a scaffold to link nNOS selectively to the NMDAR Ca$^{2+}$ source.

Many neuronal populations lack nNOS and in these cells NMDAR may link different signaling molecules via PSD-95. Indeed, a recently identified ras-GTPase activating protein, p135 SynGAP (Chen et al., 1998; Kim et al., 1998), interacts with all three PDZ domains of PSD-95 and colocalizes at synapses with NMDARs and PSD-95. A critical source of regulation is suggested by the phosphorylation of SynGAP by CaMK-II, and the inhibition of rasGAP activity in isolated PSDs by active CaMK-II (Chen et al., 1998). CaMK-II is an abundant PSD protein that is activated by Ca$^{2+}$ through NMDARs. By analogy to nNOS, the interaction of SynGAP with PSD-95 may link its regulation to NMDAR-gated Ca$^{2+}$ pathways. Another example of such an interaction is the reduction of NMDAR activity by direct binding of calmodulin to NR-1 subunits, which inactivates NMDARs (Ehlers et al., 1996).

**NMDARs/clustering molecules are required for LTP induction.** The C-terminal domains comprise approximately one-third of the NR-2 subunits. The phosphoserines and phospho-tyrosines, as well as proline-rich sequences present in these regions, may provide the sites for adapter proteins or signal transduction components (Burbelo and Hall, 1995; Birge et al., 1996). To evaluate the functional role for the cytoplasmic domains of NMDARs, the C-terminal tails of the NR-2A, NR-2B, or NR-2C subunits
were deleted by genetic targeting in mice, rendering these proteins unable to interact with PSD-95 or related proteins (Sprengel et al., 1998). The phenotypes of these mice closely resemble those of the corresponding gene knockout null mutations, indicating that the C-termini are critically involved in NMDAR function. Mice lacking the C-terminal tail of NR-2A had deficits in LTP in CA1 and in spatial learning. Though receptor localization was not explored in detail, synaptic NMDA currents were normal in these mice. Therefore, the deficit in LTP is downstream of NMDAR activation, suggesting C-terminal truncation of NR-2 subunits may prevent the physical coupling of cellular components from the point of Ca\(^{2+}\) entry (Bading et al., 1993; Bito et al., 1997). Physical coupling of the NMDAR channel to a Ca\(^{2+}\) signaling machinery may well be essential, because Ca\(^{2+}\) ions entering the channel diffuse rapidly (Klingauf and Neher, 1997) and, hence, amplitude of Ca\(^{2+}\) transients is at the intracellular face of the channel.

1.2.C NMDAR is regulated by PKC

The C-terminal loops of NMDARs include potential phosphorylation sites for a number of kinases. There is also accumulating evidence that NMDA receptor function can be modulated by channel phosphorylation. For example, activators of protein kinase C (PKC) potentiate NMDAR function in CNS neurons, and recently it has been shown that the NR1 subunit of the NMDAR is directly phosphorylated by PKC in neuronal cell cultures (Suen et al., 1998., Leonard and Hell, 1997).

PKC\(\gamma\) is the dominant PKC isozyme in the hippocampus. PKC is a serine/threonine kinase that is the major receptor for phorbol esters which activate the kinase in vitro by mimicking DAG (Soderling, 1990, for review). PKC is a large family
of proteins with closely related structures, but with distinct enzymatic characteristics. The PKC family is classified into classical (cPKC), novel (nPKC) and atypical (aPKC) isoforms. The first group, the classical or cPKC, consists of four isoenzymes (α, βI, βII, and γ), that emerged from the initial screening by hydroxyapatite chromatography. It is suggested that the different isoforms of PKC respond differently to various combinations of Ca$^{2+}$, DAG and/or phospholipid thus producing different patterns of activation with respect to duration, intracellular location and substrate specificity. Indeed, cellular distribution and intracellular localization of the members of the PKC family is very different. Immunohistochemical studies using subtype-specific antibodies revealed that PKCγ isoform is expressed abundantly in the hippocampus, and may be restricted to the PSD (Van der Zee et al., 1997 for review). The βII isoform is the forebrain in striatal neurons. PKCα is distributed unevenly in the brain where it has a presynaptic localization. Among the other isoforms, PKCe is expressed mainly in spinal cord and primary sensory neurons.

**PKC is required for LTP induction.** Recent data with gene targeting mice suggested that an important role for PKC in the mechanism of LTP. LTP could not be induced with high frequency stimulation in mice lacking PKCγ, although, LTP could readily be induced after the induction of LTD with low frequency stimulation (Abeliovich et al., 1993).

Another approach to study the role of PKC in LTP was to examine the effect of bath application of membrane permeable kinase blockers on the induction of LTP. However, these studies did not distinguish between pre- and postsynaptic PKC or its different isoforms. Among the substances tested were H-7, sphingosine, polymyxin B,
which all appeared to suppress LTP (Malinow et al., 1988; 1989). These results indicate that PKC activity plays an important role in LTP and are consistent with data showing the translocation of PKC activity following tetanic stimulation, as measured by enzymatic assay or PKC immuoreactivity (Van der Zee, 1997).

The limitation associated with this pharmacological approach is the fact that the inhibitors used are not completely selective. A more specific demonstration for the involvement of postsynaptic PKC activity in LTP was found by injecting kinase inhibitors into the postsynaptic cell. The specific PKC inhibitor peptide, PKC(19-31), was used. PKC(19-31) is a peptide fragment representing the pseudosubstrate region of the regulatory domain of PKC. Intracellular injection of PKC(19-31) prevented the induction of LTP in the cell which was recorded, but not in the neighboring cells which were also electrophysiologically monitored by means of field potential recordings. The control pathway expressed normal LTP (Malinow et al., 1989; Wang and Kelly, 1995).

**PKC may act via NMDAR phosphorylation for LTP induction.** A possible postsynaptic target for PKC modulation was suggested by Ben-Ari et al (1992), who proposed that PKC modulation of NMDA currents was of significance for the induction of LTP. Indeed, there is evidence suggesting that NMDARs can be regulated by PKC mediated phosphorylation. For example the potentiation of NMDA currents in Xenopus oocytes by mGluRs is mediated by PKC (Ben-Ari et al., 1992) and PKC activation reduces the Mg^{2+} block which required to transform short-term potentiation (STP) into LTP (Colley et al., 1989., Asztely et al., 1990). It is important to compare the mechanisms underlying these two forms of synaptic enhancement. Studies by Malenka (1991), and by Asztely et al (1991) have shown that STP and LTP have similar inductive
mechanisms. Thus, STP as well as LTP was blocked by NMDAR antagonists and by Ca\(^{2+}\) chelators. Furthermore, procedures which augmented the initial current and Ca\(^{2+}\) influx through the NMDA channel (i.e. elevation of [Ca\(^{2+}\)]\(_{e}\), stronger tetanic stimulation or increased intracellular depolarization) transformed STP to LTP. The key element to this mechanism appear to be [Ca\(^{2+}\)]\(_{i}\), which in turn depends on the level of activation of NMDARs. Therefore, the effects of PKC activators or inhibitors could be due to their interference with NMDA signalling during LTP induction.

1.2.D **NMDAR is regulated by the tyrosine kinase family**

NMDAR phosphorylation on tyrosine residues has attracted wide attention as a cellular mechanism for the regulation of synaptic activity. Initially identified in association with oncogene products and certain growth factor receptors, tyrosine phosphorylation was considered to be primarily important for cell growth, proliferation, and differentiation (review in Hunter, 1996). It soon became apparent, however, that in the mature CNS there are high levels of protein tyrosine kinases, and that many are expressed in neurons, which are post-mitotic cells after development is complete. Over the past several years, it has become apparent that one function of protein tyrosine kinase is to regulate the activity of ion channels in neurons (Wang et al., 1994., Fadool et al., 1997., Cataldi et al., 1996., Moss et al., 1995., Lev et al., 1995). Analysis of isolated synaptic fractions, including PSDs, synaptic membranes and synaptic vesicles, demonstrated that several synaptic proteins are phosphorylated on tyrosine residues and further that these synaptic organelles are associated with intrinsic tyrosine kinases (Gurd and Bissoon, 1985., Ellis
et al., 1988., Hirano et al., 1988., Pang et al., 1988., Barnekow et al., 1990). While these earlier studies suggested that tyrosine phosphorylation was involved in synaptic function it is only in the last few years that progress has been made in identifying specific consequences of tyrosine phosphorylation for synaptic receptors.

**Tyrosine kinase.** Tyrosine kinase falls into two structurally distinct categories: (i) membrane-spanning receptor tyrosine kinases that transduce signals from growth and neurotrophic factors and (ii) nonreceptor tyrosine kinases associated with the cytoplasmic side of the plasma membrane. For example, receptor tyrosine kinases are actually the receptors (trk A, B, and C) of neurotrophins. Neurotrophins that are ligands for receptor tyrosine kinases can also modify synaptic function. For example, neurotrophins can enhances excitatory transmission in a number of systems. The neurotrophin brain-derived neurotrophic factor (BDNF) also regulates hippocampal LTP (Kang and Schuman, 1995). The receptor for BDNF, the tyrosine kinase TrkB, is found in isolated PSDs (Thoenen, 1995) where it can stimulate phosphorylation of the NMDAR.

**Nonreceptor tyrosine kinases: the mechanisms for regulation of Src kinase activity.** The nonreceptor tyrosine kinases belonging to the Src family are key players in signal transduction and much effort has gone into understanding their physiological role as well as their regulation (Brown and Cooper, 1996., Pawson and Scott, 1997, for review). The Src family consists of nine members— Src, Fyn, Yes, Fgr, Lyn, Hck, Lck, Blk, and Yrk— which share the same domain structure. The N-terminal, unique domain contains a myristylation site and frequently a palmitoylation site. It is followed by the regulatory SH3 and SH2 domains, a catalytic domain that is bilobed and has its active site wedged between the two lobes, and a C-terminal regulatory tail that contains the hallmark
regulatory tyrosine residue (Tyr527) in Src (Figure 1.3). Kinase activity is reduced when the latter is phosphorylated and bound to the SH2 domain. The SH2 and SH3 domains bind phosphotyrosyl and proline-rich peptides, respectively. Through these interactions, they participate in intra-and intermolecular regulation of kinase activity, as well as localization and substrate recognition. Several studies demonstrated that the tyrosine kinase activity of Src can be stimulated by the binding of target proteins containing proline-rich sequences to the SH3 domain (Brown and Cooper, 1996). Src activity can be also stimulated by binding of phosphotyrosine-containing sequences to the SH2 domain or by dephosphorylation of pTyr527 (Figure 1.3). For example, a short phosphotyrosine-containing peptide, EPQ(pY)EEIP, binding the Src SH2 domain with high affinity (Kd = 40 nM) (Liu et al., 1993). This high affinity peptide was able to displace Src phosphorylated tail from SH2 domain, and increased the ability of c-Src to autophosphorylate, as well as phosphorylate, the exogenous substrate enolase by three-fold.

The kinase, SH2, SH3 and C-terminal domains are highly homologous in all members of the src family. The structure varsity in the Src family is in the so-called unique domain, a region of low sequence conservation near the N-terminus. It has been suggested that the unique domain may simply be a spacer in the molecule (Superti-Furga et al., 1995). Subsequently, the Src unique domains have been found to be crucial for directing the kinase and permitting the interaction with particular substrates (Vonakis, et al., 1997., Carrera, et al., 1995). Thus, even though it is outside the catalytic region, the unique domain may allow the function of Src kinases to be specificity. The main isoform of Src expressed by all cell types is c-Src. While, it was also discovered many years ago
Figure 1.3 The structure and intramolecular interaction-mediated autoinhibition of Src

Inactive conformation  Active conformation

SH3- and SH2-mediated autoinhibition of Src. The Src kinase is maintained in an inactive state by means of intramolecular interactions mediated by the SH3 and SH2 domains. The SH3 domain binds to a proline-rich sequence in the SH2-kinase linker and the SH2 domain binds to pTyr527 in the C-terminal tail of Src, thus maintaining the enzyme in an inactive state (Closed). The tyrosine activity of Src can be stimulated by binding of target proteins containing proline-rich sequences to the SH3 domain, or by dephosphorylation of pTyr527. Autoinhibition is released in open conformation enabling kinase activation.
that neurons express a high-activity neuronal isoforms of Src called n-Src (Brugge et al., 1985., Martinez et al., 1987). One isoform of n-Src was found to contain a spliced cassette of six amino acids inserted in the SH3 domain of c-Src (Martinez et al., 1987). Some other isoforms of n-Src have been found, more recently, that a distinct cassette may be inserted alone or in tandem in the SH3 domain (Pyper et al., 1990).

Tyrosine kinase regulates NMDAR channel function. Src is expressed throughout the CNS with high levels of expression in the cerebral cortex, hippocampus, midbrain, and spinal cord (Sugrue et al., 1990., Ross et al., 1988). Src is expressed in both pre- and post-synaptic neurons. The postsynaptic Src is particularly relevant with respect to the regulation of NMDAR activity, as both Src and NMDARs have been found physically associated in the PSD (Atsumi et al., 1993., Cudmore et al., 1991., Yu et al., 1997). Each of the NMDA receptor subunits contains sites for phosphorylation by different protein kinases, and regulation of NMDAR function by protein phosphorylation is well documented (Swope et al., 1992., Roche et al., 1994). In 1985, the laboratory of James Gurd reported that a major PSD-associated glycoprotein of molecular weight 180 000 (PSD-GP 180) was a prominent substrate for tyrosine kinases associated with the postsynaptic apparatus (Gurd, 1985., Gurd and Bissoon, 1985., Kearney and Gurd, 1986). Tyrosine phosphorylated PSD-GP180 was widely distributed in different brain regions, and its phosphorylation on tyrosine residues was developmentally regulated (Gurd and Bissoon, 1990., Souliere et al., 1994). Following this finding, Kennedy’s group (Moon et al., 1994) purified and sequenced individual bands in the PSD fraction that migrate at an apparent Mr of 180 kDa. One of the first proteins sequenced was quickly identified as the NR-2B subunit by searching the Genbank database (Moon et al., 1994). The
identification of PSD-GP180 as the NR-2B subunit of the NMDAR provided an important link between these earlier studies of the tyrosine phosphorylation of PSD-GP180 and more recent observations indicating that tyrosine phosphorylation plays an important role in the regulation of NMDAR channel function.

Several studies have described the possible linkage between tyrosine phosphorylation and the activity of the NMDAR channel. Earlier study has revealed that endogenous protein tyrosine kinase is closely correlated with the gating and regulation of NMDA channels (MacDonald et al., 1996). Inhibition of protein tyrosine kinase activity with genistein or lavendustin A reduced both NMDA currents and NMDAR-mediated intracellular Ca^{2+} signaling in spinal dorsal horn neurons (Wang and Salter, 1994). Furthermore, intracellular application of pp60^c-Src or tyrosine phosphatase inhibitor, sodium orthovanadate, resulted in the potentiation of NMDA currents in both spinal dorsal horn and hippocampal neurons (Wang and Salter, 1994). Salter’s group also provided evidence that protein tyrosine phosphatase play a role in the run-down of NMDAR-mediated currents during whole-cell patch recording. The data revealed that the application of exogenous protein tyrosine phosphatase to spinal dorsal horn neurons resulted in a decrease in NMDA single channel activity (Wang et al., 1996). The simplest explanation, but not the only, for regulation of channel function by tyrosine kinase is that it is caused by phosphorylation in one of the cytoplasmic domains of an NMDA channel subunit protein. There appears to be evidence for direct phosphorylation of tyrosine residues within NR-2A, and NR-2B subunits (Moon et al, 1994; Lau and Huganir, 1995, Rosenblum et al., 1996, Rostas et al, 1996). In studying recombinant NMDARs, Kohr and Seeburg (1996) found that whole-cell currents mediated by NR-
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1/NR-2A receptors expressed in HEK293 cells were enhanced by Src kinase. Furthermore, they observed that Src had no effect on mutant NR-1/NR-2A receptors in which the C-terminal intracellular tail of NR-2A had been deleted. A simple explanation for these findings could be that the target of functionally relevant tyrosine phosphorylation is NR-2A and that the site of phosphorylation is a tyrosine, or tyrosines, within the tail of the protein. Analysis of the consequences of tyrosine phosphorylation for the channel properties of heteromeric NMDARs of different subunit composition revealed, however, that the function of NR-1/NR-2B diheteromeric channels appears to be unaffected by tyrosine phosphorylation (Kohr and Seeburg, 1996). NR-2B extracted from rat brain is phosphorylated on tyrosine and NR-2B can be phosphorylated in vitro by the Src kinase family Fyn (Suzuki and Okamura-Noji., 1995). A simple explanation, which has yet to be studied, is that in recombinant NR1/NR-2B receptors the NR-2B subunit is already fully tyrosine phosphorylated by basal kinase activity. If this is the case, then there might be no non-phosphorylated substrate available for exogenously added Src, and thus its effects may have been occluded. Furthermore, a recent study (Zheng et al., 1998) indicated that the extracellular Zn$^{2+}$ contamination in the recording solution causes the recombinant NR1/NR-2A receptors to be tonically inhibited. The potentiation of NR1/NR-2A receptors by Src in HEK293 cells is due to the removal of Zn$^{2+}$-induced inhibition of NR1/NR-2A receptors in HEK293 cells. The NR1/NR-2B recombinant receptors, however, are not enhanced by Src in HEK293 cells, in part, because these recombinant receptors are less sensitive to Zn$^{2+}$-induced tonic inhibition. The findings may provide the evidence for the discrepancy between tyrosine phosphorylation and recombinant NR-1/NR-2B channel activity.
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What are the tyrosine kinases which are responsible for the phosphorylation of the NMDAR? Src and Fyn are both associated with the PSD and both phosphorylate NR-2B and NR 2A under in vitro conditions (Cudmore and Gurd, 1991., Suzuki and Okumura-Noji, 1995). Both Src and Fyn potentiated NMDA currents in transfected cells (Kohr and Seeburg, 1996). Subsequent work using a specific Src family activating peptide (EPQ(pY)EEPIA) and a Src function inhibiting antibody (anti-src1), identified Src as the endogenous tyrosine kinase associated with native NMDARs (Yu et al., 1997). Src was capable of regulating the function of both excised single NMDAR channels and synaptically activated NMDAR channel (Yu et al, 1997). The action of Src on NMDAR channels was dependent upon a region (amino acids 40-58) within the noncatalytic unique domain of the kinase. Src also co-immunoprecipitated with NMDA receptors revealed the physical interactions (Yu et al, 1997).

Tyrosine kinase is required for LTP induction and may act via regulation of NMDARs. The early evidence for the involvement of protein tyrosine kinases in LTP comes from studies with membrane-permeable tyrosine kinase inhibitors. The first evidence for the requirement of tyrosine kinase for LTP induction arose from the study of broad-spectrum tyrosine kinase inhibitors, genistein or lavendustin A (O'Dell et al., 1991). In this study, bath application of genistein or lavendustin A prevented the induction of LTP. It is interesting that these compounds have no effect on established LTP. Such observations suggest that although tyrosine kinase activity is necessary for the induction of LTP, persistent activation of tyrosine kinase is apparently not required for LTP expression.
Even though the studies with the tyrosine kinase inhibitor revealed the necessity for tyrosine activity during the induction of LTP, the data cannot provide insight into which tyrosine kinases in particular are involved. Since all tyrosine kinases share a highly conserved catalytic domain, which is the target of these inhibitors, this explains their broad-spectrum effects. One approach to studying the involvement of particular tyrosine kinases in LTP is the use of a genetic approach to generate knock out mice carrying null mutations in protein tyrosine kinases. In particular, LTP has been studied in mice containing a selective knockout of either p59\textsuperscript{fyn}, p60\textsuperscript{src}, p56\textsuperscript{lyn}, or p123\textsuperscript{yes}. Analysis of LTP in these mutant mice revealed that only Fyn mutant mice are deficient in LTP (Grant et al., 1992). Specifically, the threshold for induction of LTP is increased, and in any cases LTP is only partially reduced, but not absent.

These results, however, should be interpreted carefully. As Fyn kinase is involved in cellular differentiation and proliferation, deficient animals may have subtle developmental anomalies, which affect the establishment of a proper network of neurons. In fact, histological examination of the hippocampus in Fyn mutant mice revealed that anatomical defects in cell layers in hippocampus were present (Grant et al., 1992). Furthermore, recent evidence suggests that Fyn kinase is involved in axonal myelination (Umemori et al., 1994). Thus, the deficiency in LTP in the Fyn mutant mice might not involve mechanisms underlying normal LTP induction. Moreover, mice lacking Fyn had a 2-fold upregulation of Src expression. Fyn mutant mice revealed LTP deficit only after ten weeks when Src levels fall (Kojima et al., 1997). Since the tyrosine kinase inhibitors used were not selective, and since in the fyn gene knockouts upregulation occurred in
expression of Src kinase (Kojima et al., 1997), then the case for the involvement of specific tyrosine kinase in LTP induction is elusive.

1.3 Role of mGluRs in LTP induction

The metabotropic glutamate receptor (mGluR) was initially thought to be coupled exclusively to IP₃/Ca²⁺ signal transduction by its characterization in cell and tissue preparations or in the Xenopus oocyte (Pin and Duvosin, 1995, for review). The first cDNA clone of a mGluR subtype was isolated by functional expression cloning that combined electrophysiology and a Xenopus oocyte expression system (Houamed et al., 1991., Masu et al., 1991). This cloning effort was subsequently extended using cross-hybridization and the polymerase chain reaction (PCR), which disclosed that mGluRs form a receptor family consisting of at least eight different subtypes, termed mGluR1 to mGluR8. Recently, a Ca²⁺-sensing receptor was isolated from the parathyroid gland and was found to share a significant sequence similarity with the mGluR family, including its large extracellular domain (Brown et a., 1993). Thus, the superfamily of the G-protein-coupled receptors may thus be subdivided into three major subgroups: one comprising most of the G protein coupled receptors, such as adrenergic, muscarinic receptors. Second group is peptidergic receptors. The third group is mGluRs and Ca²⁺-sensing receptors. There is evidence that mGluRs are involved in triggering a variety of protein kinase activities (Conn and Pin., 1997, for review). Because mGluRs are upstream of cellular protein kinases, their manipulation should produce similar effects on the NMDAR and LTP as that produced by the manipulation of their downstream protein
kinases. The following section will briefly review work on the role of mGluRs in signaling transduction in general and in the induction of LTP at CA1 synapses.

1.3.A mGluR-mediated signal transduction

Signal transduction mechanisms and agonist selectivity of the eight subtypes of mGluRs have been studied after DNA transfection of individual receptor cDNA clones in animal cells (Figure 1.4). Among the eight subtypes, mGluR1 and mGluR5 are related more closely in their amino acid sequences (more than 60%) (Okamoto et al., 1994). Consistent with this sequence similarity, the closely related subtypes show the same signal transduction pathway and respond strongly to quisqualate (Abe et al., 1992). The other six subtypes are linked to the inhibitory cascade of cyclic AMP formation. These six subtypes can be subdivided further: mGluR2, mGluR3, and mGluR8 react effectively with 1-amino-cyclopentyl-1,3-dicarboxylate (1S, 3R-ACPD) (Tanabe et al., 1993), whereas mGluR4, mGluR6, and mGluR7 respond effectively to L(+)-2-amino-4-phosphonobutyric acid (L-AP4) (Okamoto et al., 1994). Therefore, the mGluR subtypes are subdivided into three groups according to their sequence similarities, signal transduction mechanisms, and agonist selectivity. Group I mGluRs (mGluR1 and mGluR5), which are linked to IP₃/PKC activity, are abundantly expressed in the hippocampus (Hollman and Heinemann, 1994). The rise in [Ca²⁺]ᵢ induces the translocation of Ca²⁺-dependent isoenzymes (PKCα/β/γ) from the cytosol to the membrane. This membrane-associated, but only partially activated kinase, becomes completely activated when DAG binds to the protein (Huang, 1993). DAG can be generated by receptor-mediated hydrolysis of phosphatidyl-inositol-diphosphate and/or
The eight subtypes of the mGluR family can be divided into three groups as indicated. Group I (mGluR1,5) is coupled to IP3/PKC. Group II (mGluR 2,3, and 6) is negatively linked cAMP, reacts to ACPD. Group III (mGluR4,6, and 7) is linked to cAMP, and reacts to L-AP4.
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after depletion of this small pool, by cleavage of phosphatidylycholine by phosphoinositol and phosphatidyl choline specific phospholipase C. Thus, a variety of receptors might be involved in generation of DAG after induction of LTP among them, group I mGluRs which are the center of interest in this thesis.

1.3.B Blocking mGluRs blocks LTP induction, "a molecular switch"

model

A first indication that mGluRs might be involved in LTP was uncovered by Reymann's group (Reymann and Matthies, 1989), who was able to block LTP expression at the CA1 region by application of L(+)-2-amino-4-phosphonobutyric acid (L-AP4) after the tetanus. This result was confirmed later by Collingridge's group (Bahir et al., 1993) using another relatively unspecific inhibitor of mGluRs, L(+)-2-amino-3-phosphonopropionic acid (L-AP3). Studies with a more specific antagonist of mGluRs, namely α-methyl-4-carboxyphenylglycine (MCPG) led to conflicting results. On the one hand, it was reported that MCPG, if present during tetanus, prevented LTP induction (Bashir et al., 1993). In that study, synaptic potentiation induced by tetanus in the presence of MCPG decayed within 30 min. On the other hand, another study found that MCPG had no effect at all on LTP induction (Chinestra et al., 1994). Possible discrepancies include experimental conditions (Manzoni et al., 1993), and possible preferences for specific mGluR subtypes (Chinestra et al., 1993). However, a more interesting possibility was that mGluR activates a "molecular switch" which can be turned "on" and "off". In molecular switch hypothesis, MCPG again reversibly blocked the induction of LTP, but not STP, in CA1. After washout of MCPG, LTP was induced, and responses were stimulus-matched
to control levels (i.e. the baseline was reset). MCPG then had no effect on further LTP induction. These experiments suggest that mGluRs, once activated by tetanus remain “on” or conditioned, so that further activation is no longer necessary. This is unlike NMDARs, which need to be activated each time. One important feature of the molecular switch model for mGluR involvement in LTP that allows this hypothesis to be tested is that the switch can be turned off by several minutes of low-frequency stimulation (Bortolotto et al., 1994). Thus, one possible explanation for the failure of MCPG to block the induction of LTP is that this molecular switch can be inadvertently activated, perhaps during slice preparation, thereby rendering LTP insensitive to MCPG (Bear and Malenka, 1994). However, consequent work by Thomas and O'Dell (1995) revealed normal LTP in the presence of MCPG, although the experiment was designed to closely match the conditions under which MCPG has been reported to block LTP (500 μM MCPG applied beginning 15 min. pre-tetanus). Moreover, MCPG still failed to block LTP in slice where, prior to attempting to induce LTP, low-frequency stimulation was delivered to turn off a molecular switch that might have been activated in the basal state of the hippocampal slices (Thomas and O'Dell, 1995). Therefore, a mGluR controlled switch-like cellular signaling events does not appear to explain the inability of MCPG to block LTP. In my view, MCPG-sensitive mGluRs may primarily have a regulatory role in the induction of LTP. This regulation may be quite complex, including both a facilitation of LTP induced by near-threshold patterns of synaptic stimulation, and perhaps the suppression of LTP induced by strong-intensity, high-frequency synaptic stimulation. In fact, the finding by Thomas and O'Dell revealed MCPG did block LTP induced by 5 Hz stimulation even though MCPG did not affect LTP produced by high frequency stimulation. Thus, a better
understanding of MCPG-sensitive mGluR-mediated cellular signaling events in different experimental conditions should yield some insights into the functional roles of mGluRs in the induction of LTP.

1.3.C Activation of mGluRs causes LTP

In a second line of research exploring the possible role of mGluRs in LTP, the effect of receptor agonists alone or in combination with different tetanus paradigms was studied. First, the activation of mGluRs by ACPD caused a slowly developing potentiation of synaptic transmission, which lasted up to 3 hours (Bortolotto and Collingridge 1993). Interestingly, this agonist-induced synaptic potentiation occluded tetanus-induced LTP. This so-called slow onset potentiation (SOP) is inducible only in hippocampal slices incubated in an interface chamber, but not under submerged conditions. Ca$^{2+}$-dependent PKC was already translocated into a membrane-associated state in submerged conditions but not in an interface slice preparation (Angenstein and Staak, 1997). Furthermore, basal endogenous protein phosphorylation patterns differ substantially between both types of slice incubation. After addition of phosphatase inhibitors to the incubation medium of submerged slices, phosphorylation profiles of proteins resemble that of interface slices thus pointing to a changed balance of kinase/phosphatase activity due to these different incubation conditions (Ramakers et al., 1997). These results support the position taken by the authors claiming a drastic influence of experimental conditions on the output of LTP experiments in slices. In my own work, the quality of prepared slices and the conditions of slice pre-incubation are the most important for the outcome of LTP. As the slices are kept in a holding chamber for a much longer time than that in recording chamber, the
interface chamber is much better than that submerged chamber for slice keeping due to enhanced viability. This was confirmed by the phase contrast appearance of neurons in hippocampal slices. Of course, it is only speculation that this shifted balance is the reason for the conflicting data concerning induction of SOP, but different basal activity of PKC is expected to influence the threshold for the induction of LTP (Stanton, 1995). Possibly for this reason other laboratories failed to induce SOP by mGluR stimulation (Behnisch and Reymann, 1993., Chinestra et al., 1994).

Second, coupling a STP-inducing “weak” tetanus with activation of mGluRs by ACPD induces LTP (Aniksztejn et al 1992). Very recently, these in vitro data were confirmed in in vivo experiments showing development of LTP after “weak” tetanus followed by activation of mGluRs by ACPD. Interestingly, this procedure is correlated with a transient increase in the activity of cytosolic PKC comparable to that observed after strong tetanus (as measured by Otani et al, 1993), but see (Angenstein et al 1996).

1.4 AMPAR potentiation and modifications in LTP

AMPARs share many of the functional properties of nicotinic Ach receptors in that they mediate fast excitatory synaptic responses. The excitatory synaptic currents (EPSCs), that evoked by released glutamate, are largely mediated by AMPARs. The following section discusses the molecular mechanisms of AMPAR modification that contribute to the expression of LTP at CA1 synapses.

1.4.A AMPA/kainate receptors
Four cloned AMPA receptor subunits have been described GluR1-4, or GluRA-D (Keinanen et al., 1990), while there are currently two groups of kainate receptors (GluR5-7 and KA1-KA2, see review Nakanishi 1992, Seeburg, 1993, Wisden and Seeburg, 1993, Westbrook, 1994). Each AMPA subunit has two splice variants in the sequence between TMIII and TMIV, termed “flip” and “flop”, resulting in different electrophysiological properties and ontological expression (Monyer et al., 1991). While GluR1-3 are expressed throughout the hippocampus, GluR4 is limited to the dentate gyrus and CA1 (Seeburg, 1993).

The kainate-preferring subunits presently consist of GluR5-7 and KA1 and KA2. While GluR6 and KA2 are expressed throughout the hippocampus, KA1 is restricted to the dentate gyrus and CA3, and GluR7 appears to be restricted to the dentate gyrus. Apparently, GluR5 has not been demonstrated in the hippocampus (Seeburg, 1993). In vitro, GluR7, and KA1 and KA2 are not functional as homomeric receptors, unlike GluR5 and GluR6. Co-expression revealed a variety of responses.

1.4.B AMPA/kainate receptors are phosphorylated by PKA

AMPA/kainate receptors are also regulated by changes in phosphorylation (MacDonald, 1997., Roche et al., 1994). Basal phosphorylation of glutamate subunits GluR1-3 seems critical in normal functioning. Prevention of “rundown” of kainate-induced currents has been demonstrated by the addition of the catalytic subunit of cAMP-dependent kinase A (PKA), while addition of cAMP, or the phosphatase inhibitor okadaic acid, potentiated kainate responses (Wang et al, 1991). In addition, the PKA activator forskolin increased both glutamate- and kainate-induced currents, in whole-cell and outside-out patches as
well as miniature EPSCs, while a peptide inhibitor of PKA not only blocked this potentiation, but inhibited these responses with respect to controls (Greengard et al, 1991). The regulation of AMPA/kainate receptor by PKA in cultured hippocampal neurons has been reported to require anchoring of PKA to A-kinase-anchoring proteins (AKAPs) (Rosenmund et al, 1994). Introduction of the catalytic subunit of PKA both increased the phosphorylation of GluR6 and potentiated kainate-induced currents. This effect was mimicked by addition of forskolin, and inhibited by the presence of the PKA inhibitor PKA(5-24). Site directed mutagenesis of ser684 was sufficient to abolish phosphorylation and enhancement of GluR6 (Wang et al, 1993).

1.4.C AMPA/kainate receptors are phosphorylated by PKC and CaMK-II

In a study of both recombinant GluR1, expressed in SF9 cells, and native GluR1 in synaptosomes and postsynaptic densities, GluR1 was rapidly phosphorylated by Ca\(^{2+}\)/calmodulin dependent kinase II (CaMK-II) and slowly phosphorylated by PKC, while PKA has no effect (McGlade-McCulloh et al., 1993). Similar results were seen in synaptosomal preparations, while in PSD preparations only endogenous CaMK-II exhibited an increased phosphorylation of immunoprecipitated GluRs. Using cultured hippocampal neurons, “rundown” of the kainate-induced current is prevented by the addition of Mg\(^{2+}\)/ATP in the recording pipette, while addition of microcystin LR, a phosphatase inhibitor, enhanced such currents by 1.5–2.0-fold, indicating that the GluR1 undertakes a basal phosphorylation to maintain the channel functions. Specific kinases involved in basal phosphorylation, however, were not investigated. Addition of
autophosphorylated CaMK-II in this preparation enhanced kainate-induced currents 3- to 4-fold. A later study by the same group used primary hippocampal cultures and immunoprecipitation to isolate NMDARs (Tan et al., 1994). The phosphorylation of AMPARs, GluR1-GluR3, occurred after addition of glutamate and glycine (Glu/Gly) to the cultures in the absence of Mg²⁺. The increase in phosphorylation of GluRs could be blocked by NMDAR antagonist, AP5. Thus, phosphorylation appears to be a result of NMDAR activation. Rapid increases in phosphorylation were also observed in response to ionomycin, and blocked by KN-62. Slow phosphorylation was caused by phorbol 12-myristate 13-acetate (PMA, a PKC activator), but not forskolin. Treatment with KN-62 reduced the basal phosphorylation of GluRs and CaMK-II and inhibited the response to glu/gly, with no effect on PMA response, further demonstrating that enhanced phosphorylation is probably mediated by CaMK-II. The authors conclude that activation of NMDARs could stimulate the phosphorylation of CaMK-II and AMPARs with minimal involvement of PKC and PKA, although AMPARs can be targets of PKA and PKC phosphorylation in vitro (Tan et al., 1994). Thus, phosphorylation of postsynaptic AMPA/kainate receptors by CaMK-II enhances their response, a possible mechanism for the potentiation of synaptic transmission seen in LTP.

1.4. D AMPAR modifications in LTP expression, “the CaMK-II hypothesis”

CaMK-II is involved in the induction of LTP. As suggested above, postsynaptic intracellular Ca²⁺ is likely to be a central mediator of LTP induction in the hippocampus, and consequently there has been widespread interest in understanding the mechanisms by
which changes in intracellular Ca\(^{2+}\) can lead to diverse, long-lasting biochemical and cellular changes. There are many proposed biochemical mechanisms for the induction of LTP (AMPAR potentiation). Prominent among them is the possibility that there is a persistent activation of one or more protein kinases. As Ca\(^{2+}\) enters the cytosol, it encounters a number of proteins that regulate its biochemical effects. Central among them is calmodulin (CaM), a small Ca\(^{2+}\) -binding protein, which can act as an intracellular Ca\(^{2+}\) sensor (Bachs et al., 1992). Ca\(^{2+}\)-CaM binds to a number of enzymes and modulates their activity. These include CaM-dependent protein kinase (CaMK). Of five CaMK isoforms that have been identified, CaMK-II and IV have been characterized the most (Deisseroth et al., 1998). Both have broad substrate specificity and, therefore, may regulate a number of Ca\(^{2+}\)-dependent biochemical events. CaMK-II has been extensively studied as a potential mediator of Ca\(^{2+}\) -dependent synaptic changes (Fukunaga et al., 1993). A number of studies on the localization, activation, and perturbation of CaMK-II present a compelling case that CaMK-II is directly involved in the regulation of LTP (Lisman et al., 1997). CaMK-II is enriched in the hippocampus and is concentrated both pre- and postsynaptically. CaMK II isolated from brain contains \(\alpha\) and \(\beta\) subunits in a ratio of about 4:1. Unlike protein kinase A (PKA) and PKC, in which there are separate catalytic and regulatory subunits, each subunit of CaMK-II has catalytic activity. In the resting state, an autoinhibitory domain of the kinase keeps the catalytic site inaccessible. This inhibition can be relieved by the binding of CaM to the autoinhibitory domain. The kinase thus activated can not only phosphorylate other substrates at serine residues, but also undergoes autophosphorylation at a number of sites (Brickey et al., 1994).
The autophosphorylation of CaMK-II causes the enzyme to switch to a Ca\(^{2+}\)-independent form, that is, the kinase maintains catalytic activity even after intracellular Ca\(^{2+}\) concentrations have returned to basal levels. Therefore, it can respond to small Ca\(^{2+}\) transients. This is the property of CaMK-II that initially led to the proposal that it could function as a "memory molecule" and might contribute to long term modulation of synaptic function (Soderling, 1995). Site-directed mutagenesis experiments indicate that of the multiple autophosphorylation sites, phosphorylation at Thr\(^{286}\) is both necessary and sufficient for the generation of Ca\(^{2+}\)-independent activity (Miller and Kennedy, 1986). Physiological stimuli lead to the activation of CaMK-II and to the generation of the Ca\(^{2+}\)-independent form of the enzyme. Particularly noteworthy are changes in CaMK-II activity that accompany the induction of LTP. Stimuli that give rise to LTP in hippocampal slices also lead to significant increase in CaMK-II enzymatic activity. Immunostaining of cultured hippocampal neurons with a phospho-specific antibody that specifically recognizes the Thr\(^{286}\)-phosphorylated form of CaMK-II indicates that activation of NMDAR, which is required for LTP induction, leads to autophosphorylation of CaMK-II at Thr\(^{286}\) (Soderling, 1996). Perturbation experiments also support a role for CaMK-II in the induction of LTP. For example, microinjection of a peptide that selectively inhibits CaMK-II activity by binding to its catalytic site prevents the induction of LTP in the CA1 region of the hippocampus (Malinow et al, 1989; Wang and Kelly, 1995), and a targeted gene disruption of the CaMK-II \(\alpha\)-subunit produces a deficiency in the induction of LTP (Silva et al., 1992).

**The enhancement of AMPARs by CaMK-II in LTP induction.** Because CaMK-II is thought to mediate the induction of LTP in the hippocampus, there is
considerable interest in identifying the substrates of this enzyme. Most putative targets of CaMK-II have been identified by *in vitro* kinase assays. In several instances physiological stimuli that lead to increase intracellular Ca\(^{2+}\) levels, result in the phosphorylation of these substrates at sites that are also phosphorylated by CaMK-II *in vivo*. Substrates defined in this way include GluR1 (Tan et al., 1994). GluR1 is involved in synaptic transmission, and its phosphorylation by CaMK-II may modulate synaptic efficacy. Indeed, phosphorylation of GluR1 by CaMK-II increases channel function. Such a mechanism may be involved in the change in postsynaptic response following the induction of LTP. More recently, the direct phosphorylation of AMPARs was shown in response to LTP (Barria, et al., 1997). This experiment revealed that AMPAR phosphorylation, following the induction of LTP, correlated with the activation and autophosphorylation of CaMK-II, and was blocked by the CaMK-II inhibitor, KN-62 (Barria, et al., 1997).

1.5 Pre- or post-synaptic locus for LTP expression

Even if we accept that LTP involves a postsynaptic modification, as described above, this does not rule out presynaptic changes. The issue of pre- or post-synaptic locus of LTP expression remains unresolved and contentious in recent years. It might be expected that the increase in both presynaptic and postsynaptic functions could increase a synaptic efficacy. For example, 1) increasing release probability; 2) increasing the amount of glutamate in each vesicle; 3) increasing the number of functional receptors in the postsynaptic membrane; or 4) increasing the size of the response caused by each functional receptor. A few observations suggest both presynaptic and postsynaptic loci
for LTP expression, but no agreement has yet been reached. The problem with almost all of the experiments in this debate of LTP expression is that they are indirect and interpretation is always based on assumptions that can not be directly confirmed.

**Quantal analysis.** LTP is associated with an increase in the coefficient of variation (CV = \( m^2/\sigma^2 \)) of the EPSCs recorded from individual cells (Bliss and Collingridge, 1993), where \( \sigma \) is the standard deviation, \( m \) is mean value of EPSCs, which is determined by \( n \) (the number of functional synapses), \( p \) (release probability) and \( q \) (the size of the postsynaptic response to a quantum of transmitter). That is, the trial to trial variability in the amplitude of EPSCs, normalized by its average amplitude, is larger after LTP induction than before. This is expected from a presynaptic site of expression. Two previous papers (Stevens and Wang, 1994; Bolshkov and Siegelbaum, 1995) supported the presynaptic hypothesis using techniques in which only one or a few synapses are activated. In this case, two components of synaptic strength could be separated. One is reliability (the fraction of stimuli that cause a EPSC). The second is potency (the averaged peak size of the EPSC). Furthermore, the synaptic responses could be estimated not only by the mean amplitude of the EPSCs, but also by identifying failures (responses with zero amplitude) and successes of transmission. With LTP, these authors report a change in the rate of successes, but no change in the mean amplitude of successes (the "potency"). They argue that such an observation is only compatible with an increased probability of transmitter release, indicating pre-synaptic mechanism. But, this is not a universal finding. Minimal stimulation experiments have, moreover, revealed an increase in quantal size, suggesting a postsynaptic contribution to LTP (Kullmann, 1994., Stricker et al., 1996). The weakness of this approach is precisely that it is an indirect one,
typically invoked when single quanta cannot be resolved. Generally this situation occurs when the quantal unit of interest is too close in magnitude to that of the background noise, is variable and / or cannot be distinguished from events due to activity in other inputs. In turn, these complications introduce large intrinsic standard errors that make it difficult to interpret changes in CV. In fact, this extensive simplifying model ignores both intrinsic variations of the individual parameters, regardless of whether one or more inputs to the postsynaptic cell are considered, and additional weighting factors associated with the activation of polysynaptic responses. With these considerations, other physiologically realistic hypotheses of the quanta analysis relevant to the expression of LTP were developed (Faber and Korn, 1991). For example CV could be corrected for the variance of the background noise, using the relation $\sigma^2 = \sigma^2_{\text{measurement}} - \sigma^2_{\text{noise}}$.

There is, however, an alternative interpretation for the increase in quantal content that occurs with LTP. An increase in the number of quanta of transmitter release could reflect a postsynaptic uncovering of previously silent AMPA receptors (Edwards, 1991, Malenka and Nicoll, 1997). If correct, this would have extensive implications for the significance of quantal parameters in the brain.

**Silent synapse.** The studies on the locus underlying LTP expression at CA1 synapses of the hippocampus have attracted attention to the possibility that changes in LTP can be due to activity-dependent changes in the number of functional receptors; that is a rapid conversion of non-functional synapses to functional ones. Therefore, the decrease in the rate of failures during LTP could be explained if there were an up-regulation of AMPARs at synapses that were silent before LTP (Edwards et al., 1991; Liao, et al., 1995). In this view, the expression of LTP at CA1 synapses is the result of
postsynaptic modifications. Support for this hypothesis first came from an estimate of NMDAR-mediated and AMPAR-mediated components of the unitary EPSCs. Kullmann (1994) studied the EPSCs, evoked with low frequency presynaptic stimulation, initially holding the neuron at a negative potential, and subsequently at a positive potential in the presence of AMPAR blockers. This allowed the trial to trial amplitude fluctuations of the AMPAR and NMDAR-mediated components of the EPSCs to be recorded consecutively, and appeared to be without altering presynaptic transmitter release. The CV of the AMPA component was consistently larger than that of the NMDA component. Since the CV is independent of the mean quantal amplitude, this implies that either the number of functional synapses (q) or the probability of neurotransmitter release (p) is larger for NMDARs than for AMPARs. AMPARs and NMDARs appear to be colocalized, at least at some synapses (Bekkers and Stevens, 1989), so it is difficult to see how p could be different for two receptor types. Instead, Kullmann (1994) proposed that there are some synapses where NMDARs are functional, but AMPARs are either nonfunctional or absent. In terms of quantal analysis, n is smaller for the AMPAR-mediated component. However, here the meaning of q is clearly different from that of classical quantal analysis, and rather reflects solely the number of postsynaptic sites containing active clusters of receptors. After LTP induction, the CV of the AMPA component increased, but there was little change in either the amplitude or the CV for the NMDA component, when compared with the corresponding values measured in another pathway that acted as a control. The CV of the AMPA component thus became similar to, but never less than, the CV of the NMDA component. This was interpreted as resulting from activation of AMPARs at synapses where only NMDARs were functional before the induction of LTP.
In other words, LTP induction causes an increase in $q$ for AMPARs, with little or no change in $q$ for NMDARs.

Analysis of long-term facilitation (LTF) at the crayfish neuromuscular junction has also shown that the activity could turn silent synapses to functional ones (Wojtowicz et al., 1991). LTF was induced by prolonged, high frequency (20 Hz) stimulation of an excitatory axon that innervates an individual muscle fiber by making multiple discrete synapses. Quantal analysis of neuromuscular synaptic EPSPs indicated that LTF could simply result from an increase in $n$, in which transmission was described as a simple probabilistic model such as binomial or Poisson, or as more complicated non-uniform model. An increase in the number of active zones in some neuromuscular synapses during LTF was consistent with the observations from the electrophysiological analysis (Wojtowicz et al., 1994).

The CV is not exclusively determined by $n$, $q$ and $p$, but also by quantal variability. Quantal variability describes trial to trial variability in the peak amplitude of the quantal response at an individual release site. The transmission was described as nonuniform model between sites, which reflect different synaptic properties and degrees of electrotonic attenuation. Although Kullmann (1994) suggested that quantal variability could not account for the discrepancy between the CV of the AMPA and NMDAR-mediated components, an independent test of the hypothesis that did not rely on indirect estimates of quantal parameters was required. This was provided by Liao et al (1995) and Isaac et al (1995), who used minimal stimulation of presynaptic fibers to examine postsynaptic events arising from the release of very small numbers of quantal. Both groups showed that a very low intensity stimulus that failed to elicit AMPAR-dependent
EPSCs at a negative holding potential could often evoke unitary NMDAR-mediated EPSCs at a positive holding potential. This implies that the stimulus was sufficient to cause glutamate release. This small amount of glutamate activated NMDARs, but that no AMPARs. The finding supports the hypothesis that AMPARs are absent or nonfunctional at a subset of synapses where NMDARs are present.

LTP can be induced by pairing the small intensity presynaptic stimulation with postsynaptic depolarization to $-10$ mV, to potentiate $Ca^{2+}$ entry through NMDAR channels (Liao et al 1995., Isaac et al., 1995). This caused AMPAR-mediated EPSCs to occur when the neuron was returned to more negative potentials, suggesting that clusters of AMPARs were uncovered at previously silent condition. The data showed that the proportion of trials resulting in failures of transmission decreased during the expression of LTP when the AMPAR-mediated EPSCs were measured at negative potentials. However, the failures of both AMPAR- and NMDAR-mediated EPSCs did not change when postsynaptic neuron was hold at positive potentials. This was again in agreement with a selective uncovering of the AMPARs, with no change in NMDARs.

These data evoke a novel interpretation for the increase in quantal content of AMPAR-mediated EPSCs during LTP. The increase reflects a change, not in the release probability ($p$), as was originally proposed with quantal analysis, but in the number of sites ($q$) at postsynaptic neuron. In contrast to the neuromuscular junction, $q$ is not simply the number of sites at which transmitter is released, since for AMPARs it is determined postsynaptically.

However, there are some limitations with the silent synapse hypothesis in LTP. First, the evidence showed that the NMDAR-mediated component of EPSCs, in fact,
increased with LTP (O'Connor et al, 1995; Clark & Collingridge 1995). If the potentiation of NMDAR-mediated EPSCs is also correlated with a change in quantal content, it will be necessary to determine whether this could result from activation of latent clusters of NMDARs in parallel with AMPARs, or from an increase in glutamate release.

Second, AMPARs have a lower affinity for glutamate than do NMDARs. The possibility is that at some cases, minimal stimulation-induced a small amount of glutamate activates only NMDARs, but not AMPARs. The conversion of these apparently non-functional receptors to functional ones during LTP could be due to some mechanism that increased the concentration of glutamate in the synaptic cleft; for example, an increase in vesicle filling or the simultaneous release of multiple vesicles. In fact, Malgaroli et al (1995) have reported an increase in synaptic vesicle cycling in hippocampal cultures exposed to brief episodes of high extracellular glutamate with low Mg\(^{2+}\), a stimulus shares some features with the processes of the induction of LTP. Vesicle recycling was studied by measuring the rate at which presynaptic terminals were labeled upon bath application of an antibody to the intraluminal domain of synaptotagmin, a synaptic vesicle membrane protein. Although this technique has the advantage of assaying presynaptic function directly, it remains to be determined whether similar changes in presynaptic function occur during conventional LTP studied in hippocampal slices.

If expression of LTP has a presynaptic component then it was some kind of communication from the postsynaptic back to the presynaptic neuron. One candidate for such a retrograde messenger is the gas nitric oxide (NO). The inhibitors of the enzyme nitric oxide synthase (NOS) have been reported to block LTP (Kullmann and Siegelbaum, 1995). The interpretation is that Ca\(^{2+}\) entry through postsynaptic NMDAR
activates CaMK-II, which in turn phosphorylates and activates NOS. The NO that is synthesized can then diffuse back across the synaptic cleft to the presynaptic terminal and in some way enhance release of the neurotransmitter glutamate. However, like so many other features of the presynaptic –versus-postsynaptic issue, the NO story is mired in controversy (Williams et al., 1993; Cummings et al., 1994). Subtle variations in experimental protocol will have to be resolved before a truly coherent picture of LTP at hippocampal synapses emerges.

1.6 The cellular events in NMDAR-independent LTP

at mossy fiber-CA3 synapses

The intensively studied and probably the best known form of NMDAR-independent LTP is expressed at the mossy fiber-CA3 synapses in the hippocampus. As mentioned (Fig 1.2), mossy fibers are the axons of dentate granule cells that synapse onto the proximal apical dendrites of CA3 pyramidal neurons. Mossy-fiber LTP was first observed by Alger (Alger and Teyler, 1976). Initial studies indicated that mossy fiber LTP shared several similarities with LTP recorded at the CA1 region. Tetanic stimulation of the mossy fiber caused a persistent increase in the efficacy of synaptic transmission, or LTP. Soon afterwards it was determined that NMDAR activation is not required for the induction of LTP at this synapse (Harris and Cotman, 1986; Zalutsky and Nicoll, 1990).

1.6.A The NMDAR is not required for the induction of mossy fiber
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Studies of the binding distribution of NMDARs indicated that there was remarkably less NMDAR binding sites in area stratum lucidum of CA3, the termination region of mossy fiber synapses, while NMDARs were observed in high concentration in most other regions of the hippocampus. The evidence gave the first implication that mossy fiber LTP might differ from that of CA1 or dentate gyrus synapses. The first direct evidence for the induction of NMDAR-independent LTP at the mossy fiber-CA3 synapses resulted from the observation that mossy fiber LTP was not blocked by NMDAR antagonist, AP5 (Harris and Cotman, 1986). These experiments were later confirmed using voltage-clamp recording (Zalutsky and Nicoll, 1990), in which LTP of the mossy fiber EPSCs was normally expressed even though the membrane potential was held at -70 mV.

Is postsynaptic Ca\(^{2+}\) required for mossy fiber LTP induction? The resistance of mossy fiber LTP to NMDAR antagonists led to an examination of alternate hypotheses for mossy fiber LTP induction. Alternatively, it could indicate that the rise in postsynaptic Ca\(^{2+}\) is required, but is not through NMDARs and instead occurs as result of voltage-gated Ca\(^{2+}\) channel activation; Ca\(^{2+}\) entry through a Ca\(^{2+}\) permeable AMPA/kainate channel; or is indirectly from intracellular release following mGluR activation. The observations on this issue are still controversial (Nicoll and Malenka, 1995). Some evidence suggested that mossy fiber LTP was independent of postsynaptic Ca\(^{2+}\) and postsynaptic membrane potential (Zalutsky and Nicoll, 1990). In these experiments, the effect on mossy fiber LTP of inhibition of glutamate receptor-mediated synaptic transmission was studied postsynaptically with by receptor antagonists, or presynaptically by removing extracellular Ca\(^{2+}\). Postsynaptic inhibition of mossy fiber
synapses during a tetanus had no effect on the magnitude of LTP observed following the washout of the glutamate receptor antagonists. However, removal of extracellular Ca$^{2+}$ to abolish any Ca$^{2+}$ entry into the presynaptic terminal prevented the induction of LTP. The findings suggest that mossy fiber LTP is fundamentally different from NMDAR-dependent LTP. The induction of mossy fiber LTP needs a rise in presynaptic Ca$^{2+}$ and is independent of postsynaptic Ca$^{2+}$ or postsynaptic membrane potential.

However, there is conflicting evidence to support a role for postsynaptic Ca$^{2+}$ and voltage-dependent processes in the induction of mossy fiber LTP. In these experiments, Williams and Johnston found (1989) that postsynaptic injection of Ca$^{2+}$ chelators into CA3 neurons prevented the induction of mossy fiber LTP, thus suggesting that a rise in postsynaptic Ca$^{2+}$ was indeed necessary for mossy fiber LTP. These experiments did not directly address the question of the source of Ca$^{2+}$ required for LTP—if LTP depended on Ca$^{2+}$ release from intracellular stores, a similar result would be expected. A strong test of the Ca$^{2+}$ channel hypothesis was performed by examining the voltage sensitivity of mossy fiber LTP. When cells were voltage-clamped at hyperpolarized potentials during tetanic stimulation (−80 mV), mossy fiber LTP was prevented. These data, together with the chelator experiments, strongly suggest that mossy fiber LTP depends on a voltage-dependent postsynaptic entry of Ca$^{2+}$. This is most likely through voltage-gated Ca$^{2+}$ channels, although the possibility of a novel voltage-dependent ligand-gated channel or second messenger system cannot be excluded. These findings led to the proposal that voltage-gated Ca$^{2+}$ channels might share a similar function for mossy fiber LTP as NMDARs for LTP in area CA1.
1.6.B The cellular biochemical events in mossy fiber LTP, “the PKA hypothesis”

The issue of the molecular mechanisms for mossy fiber LTP expression has received less attention than that for LTP in the CA1 region. The signaling pathway that connects the transient rise in Ca$^{2+}$ in the presynaptic terminal to the long lasting enhancement of evoked glutamate release is still not determined. Recent observations indicate that a brief elevation of cAMP in mossy fiber terminals causes a large and persistent increase in evoked glutamate release. The evidence that the action of forskolin and mossy fiber LTP occluded each other and that blockers of PKA prevented the induction of mossy fiber LTP, indicating that PKA activation is an essential event in mossy fiber LTP induction (Weisskopf et al., 1994; Huang et al., 1994). In agreement with this finding, the high-affinity forskolin-binding sites were found in the mossy fiber terminals (Worley et al., 1986). Furthermore, the mRNA for adenylyl cyclase I (AC1), a subtype of cyclase that is activated by Ca$^{2+}$/calmodulin, is expressed in high concentrations in dentate granule cells, which project mossy fiber onto CA3 region. Indeed, estimates of Ca$^{2+}$ concentrations in mossy fiber terminals during tetanus and which essentially activate AC1 are similar. These results indicate that the increase in cAMP and activation of PKA are necessary in this form of synaptic plasticity, and that increases in presynaptic Ca$^{2+}$ must be able to activate adenylyl cyclase.

How does activation of PKA enhance glutamate release? One possibility is by modulation of presynaptic Ca$^{2+}$ channels. Pharmacological evidence suggests that glutamate release from mossy fiber is mediated by both N- and P-type Ca$^{2+}$ channels.
When either N- or P-type channels are completely blocked during the tetanus, the normal magnitude of LTP could be evoked, indicating that the Ca\textsuperscript{2+} required for inducing LTP can enter through both of these channel types. The other possibility is that enhancement occurs at some step following Ca\textsuperscript{2+} entry. This latter possibility is particularly interesting in that some unknown elements in mossy fiber boutons may participate in regulating the synaptic vesicle release machinery.

1.7 Hypothesis and objectives

The cellular signaling events in the induction of these two distinct forms of LTP are summarized in Figure 1.5. In NMDAR-dependent LTP at the CA1 synapse, the Ca\textsuperscript{2+} influx through NMDAR in the dendritic spine, triggers the rapid Ca\textsuperscript{2+}-dependent autophosphorylation of Thr286 of CaMK-II and its activation. This activated CaMK-II then catalyzes slow Ca\textsuperscript{2+}-independent autophosphorylation and phosphorylation of GluR1 of AMPARs at a site that enhances AMPAR responsiveness.

The processes contributing to LTP induction require the activation of postsynaptic NMDARs at CA1 synapses. I hypothesize here that the cellular molecules, which act by regulating NMDAR functions, should participate in the events of LTP induction. Both the tyrosine kinase, Src, and the serine/threonine kinase, PKC, are known to up-regulate the functions of NMDARs. The role of Src kinase in LTP is not known. In addition, the basis for PKC activation in LTP is elusive. One source of PKC activation could result from the activation of the mGluR5. My specific objectives, in this part, were to analyze the role of Src kinase and mGluR5-coupled PKC in NMDAR-dependent LTP.
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In NMDAR-independent LTP at mossy fiber-CA3 synapses, however, the entry of Ca\(^{2+}\) into the presynaptic terminal, through Ca\(^{2+}\) channels, is responsible for the increased release of glutamate from synaptic vesicles. It is proposed that the tetanus-induced increase of Ca\(^{2+}\) bound to calmodulin, in turn stimulates adenylyl cyclase, thereby generating cAMP and activating PKA. PKA causes a long-lasting increase in Ca\(^{2+}\) channel activity or alternatively a long-lasting increase in the release process.

Chelatable Zn\(^{2+}\) is most abundantly localized in the vesicles of mossy fiber synaptic boutons and is released with glutamate upon the stimulation of presynaptic neurons. However, the relevance of endogenous Zn\(^{2+}\) to the induction of mossy fiber LTP is an enigma. In addition, the concentration of free extracellular Zn\(^{2+}\) at synapses in the CA3 region of the hippocampus was estimated to be as high as 300 µM. Zn\(^{2+}\)-deficient rats showed an deficits of high-frequency-induced mossy fiber-CA3 synaptic response (Hess, 1979). Zn\(^{2+}\) (50 µM) blocks NMDAR channels and enhances AMPAR-mediated currents in neuronal cultures (Christine and Choi, 1990, Mayer and Vyklucky, 1989, Mayer et al, 1989, Peters et al., 1987, Rassendren et al, 1990, Westbrook and Mayer, 1987). I hypothesize that mossy fiber bouton Zn\(^{2+}\) might be involved in modulating the efficacy of the glutamate-mediated synaptic transmission. My aim in this project was to investigate the role of Zn\(^{2+}\) in NMDAR-independent LTP. I postulate that the induction of NMDAR-dependent and NMDAR-independent LTP has distinct signaling cascades and separate mechanisms. Which, if any, is critical in learning and memory will also be discussed.
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Figure 1.5 Two Models for the Cellular Biochemical Events in LTP Induction

A. Postsynaptic receptor modifications in NMDAR-dependent LTP. Tetanus induces Ca\textsuperscript{2+} entry through NMDA channels. Ca\textsuperscript{2+} binds with CaM activates CaMK-II, which in turn phosphorylates and then up-regulates AMPARs.

B. Presynaptic potentiation in NMDAR-independent LTP. Tetanus causes Ca\textsuperscript{2+} entry via VSCC into the presynaptic terminals. Ca\textsuperscript{2+} binds with CaM via CaMK-II which activates PKA, thereby increasing the glutamate release.
CHAPTER TWO
EXPERIMENTAL PROCEDURES
Chapter two: Experimental Procedures

2.1 Hippocampal slice preparation

Hippocampal slices (400 μm) were prepared from 30- to 36-day-old Sprague Dawley rats for chapter three, and from 21- to 49-day old mice for chapter four. The animal was anesthetized deeply with halothane and decapitated; the brain was removed, and the hippocampi were dissected free. The hippocampi were mounted in a chamber containing ice-cold ACSF. Transverse slices (about 400 μm) were cut with a Tissue Slicer (Stoelting Co, Wood Dale, USA). The slices were allowed to recover in a holding chamber at the interface of saturated 95%O₂/5%CO₂ ACSF for at least 1 hr at room temperature. A single slice was then transferred to a submersion type recording chamber, where it was continuously superfused with artificial cerebrospinal fluid (ACSF) (2 ml/min) that had been saturated with 95% O₂-5% CO₂ at 30±1°C. The composition of ACSF (in mM) was 124 NaCl, 3 KCl, 1.25 NaH₂PO₄, 1.3, 2, or 3 MgCl₂, 2 CaCl₂, 26 NaHCO₃, 10 dextrose, 10 μM BMI. 1.3 mM MgCl₂ was used for the measurement of NMDA receptor-mediated currents (NMDA EPSCs) as indicated in individual figure descriptions. 3 mM MgCl₂ was used to record mossy fiber LTP to prevent epileptic discharges. 2 mM MgCl₂ was used in all other experiments.

2.2 The whole-cell recording configuration in slice

The hippocampal slice technique has greatly facilitated the investigation of the properties of neurons and the analysis of synaptic transmission between neurons. This is because in slices neurons remain healthy for many hours and some of the connections between neurons are preserved. The combination of the hippocamapal slice technique with the
power of the patch-clamp technique offers many advantages. Figure 2.1 shows the configurations of the recording in hippocampal slices.

2.2. A The patch pipettes and data acquisition

The whole-cell patch pipettes were pulled from borosilicate glass tubing (1.5 mm OD X 1.12 mm ID/Fiber, FHC Inc, Brunswick, USA), and heat polished immediately before use. For current clamp experiments, the patch pipette (3-5 MΩ) solution contained (in mM): 132.5 K-gluconate, 17.5 KMeSO₄, 10 HEPES, 0.2 EGTA, 2 Mg-ATP, 0.3 GTP, 5 QX-314 (pH 7.25, 290 mOsm). For voltage-clamp experiments, the patch pipette solution contained 132.5 mM Cs-gluconate, 17.5 mM CsCl, 10 mM Hepes, 0.2 mM EGTA, 2mM Mg-ATP, 0.3 mM guanosine triphosphate, and 5 mM QX-314 (pH 7.25, 290 mOsm). The pipette solution was freshly prepared directly before use. The pipette potential was balanced to zero current with ACSF solution in the bath before formation of the seal. The liquid junction potential was not corrected. Patch recordings were done using the "blind" patch method (Blanton et al., 1989). Raw data were amplified with Axopatch-1D amplifiers (Axon Instruments Inc, Foster City, CA). Currents were filtered at 2kHz with a low-pass filter. Data were digitized at a frequency of 8 kHz and stored on-line using the pclamp6 system.

2.2. B The measurement of series resistance and input resistance

Voltage-clamping. I assumed here a reasonably small cell (longest dimension ≤ 100 μM) with a resistance at rest $R_I$ on the order of 1 MΩ, and input capacitance $C$, on the order of 10-100 pF. The neuron was studied using a patch-clamp pipette that has a
Figure 2.1 Patch clamp configurations in the hippocampal slice

A. Whole-cell recording (WCR) was obtained by patching a pyramidal cell soma in the CA1 region. Field potential recording was obtained by placing an electrode on the dendritic layer in CA1. The stimulator was put on the track of the Schaffer collateral input. B. To record mossy fiber-CA3 responses, whole-cell recording was obtained by patching a pyramidal cell soma in the CA3 region. The stimulator was placed on the granule cell layer in the dentate gyrus.
resistance Rs during recording (Rs is different from the pipette resistance measured before contacting the cell). If the pipette potential (Vp), follows an imposed square impulse of amplitude ΔV, the current is I. If one assumes that Rs << RI. One obtains:

\[ Rs = \frac{\Delta V}{I_{in}} \]
\[ R_I = \frac{\Delta V}{I_{ss}} \]

And

\[ \tau = RsC \]

Where I_{in} is the "instantaneous current" obtained just after the jump, \( \tau \) the time constant of the current relaxation, and I_{ss} the steady-state current. I_{in}, I_{ss}, and \( \tau \) can be measured from the current recording. In my experiments, the input resistance (R_i) and series resistance (Rs) were monitored using voltage steps (-5 mV, 200 ms) at 5 minutes intervals throughout the period of the experiment. The measurements of R_i and Rs are shown in Figure 2.2. The range of R_i was 239 to 406 MΩ and Rs was from 16 to 38 MΩ at 5 minutes after breakthrough.

**Current clamping.** The pulse (0.2 nA, 200 ms) was injected. Rs ranged from 15-19 MΩ. R_i was 126 to 186 MΩ. The resting membrane potential was from -58 to 76 mV. Note that the estimate of Rs in current clamp model is not accurate because the instantaneous component is not accurately measured.

2.3 The measurement of synaptic responses

2.3.A EPSP recording
Figure 2.2 The series resistance and input resistance

A Voltage clamp recording mode. The pulse is -5 mV for 200 ms. Cell capacitance is cancelled. Series resistance ($R_s$) = pipette voltage ($V_p$) / instantaneous currents ($I_{in}$).

The input resistance ($R_{in}$) = $V_p$/steadystate current ($I_s$).
Chapter two: Experimental Procedures

The single cell excitatory postsynaptic potentials (EPSPs) were recorded with the current clamp approach. The EPSP slope, which is mediated by AMPAR responses, was calculated as the slope of the rising phase 10-65% of the peak response. Averaged EPSP slope values were determined for each 5 min period of recording. The baseline value of EPSP slope was that from the first 5-min period and was defined as 100%.

2.3.B EPSC recording

The single cell excitatory postsynaptic currents (EPSCs) were recorded with voltage clamp recordings. The amplitude of the AMPAR-mediated excitatory postsynaptic currents (AMPA EPSCs) was taken as the initial slope of the inward current and the 10-90% rising times were 0.6 – 2 ms. This rapid inward current was blocked by bath applied 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 5µM) and was unaffected by D-AP5 (50 µM). NMDAR-mediated excitatory postsynaptic currents (NMDAR EPSCs) were pharmacologically-isolated by bath applying 5 µM CNQX; these EPSCs were abolished by 50 µM D-AP5 (Figure 2.3). In principle, due to the different kinetics of the two synaptic components, the AMPA and NMDA receptor-mediated components of the EPSCs can be recorded within a single-evoked response by measuring at different latencies following the stimulus. This is indicated by the current-voltage (I-V) relationship (Figure 4.5); in which the initial slope measurement showed a slight outward rectification whereas measurement of the amplitude at a longer latency (50 ms after the stimulus) displayed a region of negative slope conductance, due to the voltage-dependent block of NMDA channels by Mg$^{2+}$. Unfortunately, however, the separation is only partial and cannot be measured accurately by this means. Therefore, the relative contributions of
The figure shows data from a neuron to illustrate the effects of 50 uM AP5. The traces are the single recording before (a) and 10 min after (b) the start of the addition of 50 uM D-AP5. Note that AP5 affected the peak EPSC measurement but had practically no effect on the initial slope.

Note also that sensitivity to AP5 increases with latency and that 100 ms is the optimum time point for obtaining an accurate measurement of the NMDAR EPSCs. Holding potential is -60 mV.
Chapter two: Experimental Procedures

AMPA and NMDA receptors to the EPSC were determined pharmacologically, using the specific NMDAR antagonist, D-AP5. At -60 mV, the percentage of the response that was sensitive to AP5 increased with time following the stimulus (Figure 2.3). At a latency of 100 ms, the proportion of the response mediated by NMDARs, estimated by sensitive to AP5, was 86%.

2.3.D fEPSPs recording

Extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded via a micropipette filled with ACSF (1 - 3 MΩ) placed at outside of the cells. The initial slope of fEPSP, which was mediated by AMPAR responses, was measured. Averaged field EPSP slope was calculated every minute, and normalized to the first 10-min responses (100%).

2.4 Stimulations

2.4.A The test stimuli

Synaptic responses were evoked by a bipolar tungsten electrode. In CA1 region, the location of electrode was at the Schaffer collateral pathway about 50 μm from the cell bodies. In the dentate gyrus, the location of the electrode was at the medial perforant pathway. For recording mossy fiber-CA3 synaptic responses, the location of the electrode was at the dentate gyrus granule cell layer on the hilar side. For the recording commisural-CA3 synaptic responses, the location of electrode was at the associational-commisural pathway on the CA1 side.
Test stimuli were delivered at a frequency of 0.1 Hz for recording CA1, dentate gyrus and commissural-CA3 responses, and 0.2 Hz for recording mossy fiber-CA3 synaptic responses. The stimulus intensity was set to 25% of the maximum response.

2.4.B The tetanic stimulation

The tetanic stimulation consisted of two of 100 Hz stimuli lasting 500 ms at the intertrain interval of 10s in chapter three. In controls, this produced LTP, which was at a stable level by 30 min. after tetanus and which persisted for greater than 1.5 hours. For clarity we show records of only the first 30 min after tetanus. In chapter four, the tetanus consisted of four of 100 Hz stimuli lasting 500 ms at an intertrain interval of 10s. In chapter five, the tetanus consisted of one of 100 Hz stimulation lasting for 500 ms. For mossy fiber LTP, 50 μM D-AP5 was applied during tetanus to prevent the contamination of NMDAR-dependent LTP from commisural inputs.

2.5 Src kinase Assay

Hippocampal slices were prepared, stimulated and extracellular fields recorded as described above. After baseline synaptic responses had been stable for at least 10 min then either tetanic stimulation was delivered or the test stimulation was continued for up to 5 min (control). The CA1 region was microdissected and immediately frozen on dry ice. Four CA1 regions (from control, or from 1 or 5 min post-tetanus) were pooled together. In each experiment an entire set of control, 1 min or 5 min pooled slices was taken from one animal. For the subsequent preparation and measurement the experimenter was unaware of the stimulation condition of the pooled slices. The Src
kinase assay was performed by Jonathan Davidow. The tissue was homogenized in ice-cold lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1% NP40, 1 mM Na orthovanadate, and protease inhibitors - pepstatin A (20 μg/mL), leupeptin (20 μg/mL) and aprotinin (20 μg/mL) and 1 mM phenylmethylsulfonyl fluoride. Insoluble material was removed by centrifugation at 14,000 g for 10 min at 4°C. Protein content of soluble material was determined by a Biorad Dc protein assay. Src activity was measured as Src autophosphorylation using an immune complex kinase assay. Soluble proteins (100 μg) were pre-cleared with sepharose beads and then incubated overnight with 2 μl anti-Src antibody, mab 327 (J. Bolen, DNAX, Palo Alto CA). Immune complexes were isolated by adding 40 μL of protein G-sepharose beads followed by incubation for 3 hrs at 4°C. Immunoprecipitates were washed five times with lysis buffer. Beads were then resuspended in kinase buffer which contained (in mM) 20 Tris-HCl (pH 7.6), 20 MgCl2, 2 MnCl2, 1 EDTA, 1 EGTA, 0.1 dithiothreitol, 10 μCi γ-32P ATP and enolase (3.2 mg/mL). Positive controls containing 0.1, 1 or 10 units of pp60c-Src (UBI) in kinase buffer were run in parallel. All of the samples were incubated for 5 min at 37°C and the reaction was stopped by adding 4x Laemmli sample buffer. Samples were subjected to SDS polyacrylamide gel electrophoresis (10% gel). Proteins were transferred to a nitrocellulose membrane and exposed overnight on a Phosphor Screen (Molecular Dynamics, Sunnyvale CA) for quantitation and analysis with ImageQuant software. Membranes were then immunoblotted with anti-Src antibody (1:500 dilution); the secondary antibody was coupled to HRP and was visualized by enhanced chemiluminescence.
2.6 Statistics

Averaged initial slope of EPSP (field recording or current-clamp recording), was calculated every minute, and normalized to the 10-min responses (100%) immediately before tetanus or drug application. For the measurement of EPSCs obtained by voltage-clamp recording the amplitude of EPSCs was averaged every minute, and normalized to the 10-min responses (100%) immediately before tetanus or drug application. Averaged normalized value in each group was expressed as mean ± SEM. To compare the significance between two groups, student t test was used. P < 0.01 indicates the significance between groups. n represents that numbers of recorded slices.

2.7 Materials

2.7.A The mutant mice
Src (-/-) and control background mice (129/sv X CD1 cross) were purchased from The Jackson Laboratory, USA. mGluR5 mutant mice were generated by a postdoctoral fellow, ZhenPing Jia, in Roder’s laboratory (see appendix A). Here, I would like to thank Dr ZhenPing Jia for providing mGluR5 mutants. ZhenPing screened N2 offspring of a 129/sv X CD1 cross. Littermates containing wildtype alleles (+/+) as controls for the homozygous (-/-) null mutants. These mice produced no detectable mGluR5 protein (see appendix A).

2.7.B Zn$^{2+}$-deficient rats. Sprague-Dawley rats were maintained on a freely accessible Zn$^{2+}$-deficient diet (Dyets Inc, Bethlehem, Pennsylvania, USA) beginning at
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the age of 32 days, and lasted for 70 to 78 days. The rats with Zn\textsuperscript{2+} deprivation revealed a variety of immune deficiency symptoms, and anorexia. Control rats maintained under the same environment, and were fed a Zn\textsuperscript{2+}-containing diet. For dietary Zn\textsuperscript{2+} supplements, the rats after 70 days of Zn\textsuperscript{2+} deprivation were given Zn\textsuperscript{2+}-supplemented food for 3-days prior to the experiments.

2.7.C Peptides and antibodies

The intracellular solution was supplemented as required with peptides or antibodies that were stored as 100X single-use, stock solutions prepared just before use. All peptides used in the study in chapter three were from Dr. J. Bell, Ottawa Regional Cancer Center, Ottawa. The peptides and antibodies were numbered and I was unaware of which was applied in all experiments except those shown in chapter three. The amino acid sequence of scrambled Src(40-58) was AGSHAPFPSPARAGVAPDA and was created by randomly ordering the sequence of Src(40-58). Anti-Src1 was obtained from S. Courtneidge, SUGEN, Redwood City, CA. PKCI was obtained from RBI.

2.7.D Chemicals

Dithizone solutions were made by suspending 100 mg of drug in 1 ml of ethanol. The drug was dissolved and protonated by the addition of 2 ml of NH\textsubscript{4}OH. The volume of the stock solution was then adjusted to 10 mM by the addition of normal saline. The same solutions, but containing no dithizone, were made as the control vehicle solutions. The final concentration of NH\textsubscript{4}OH was 0.02%.
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Dithizone, diethyldithiocarbamic acid, TTX, picrotoxin, and bicuculline were from Sigma chemical Co, St Louis. D-AP5, CNQX, NBQX, 1S,3R-ACPD, R,S-MCPG and QX-314 were from Research Chemicals Int.
CHAPTER THREE

SRC ACTIVATION MEDIATES THE INDUCTION OF LTP IN CA1 NEURON

This work was published as:


The work in all figures (Figure 3.1., 3.2., 3.3., 3.5., 3.6., 3.7., 3.8., 3.9., 3.10) were performed by me. The Src kinase assay (figure 3.4) was carried out by Jonathan Davidow in Dr Michael Salter’s lab.
3.1 INTRODUCTION

LTP is induced by a cascade of biochemical steps which, for the NMDAR-dependent form of LTP, occur in the postsynaptic neuron at the CA1 synapse (Malinow et al., 1989; Malenka et al., 1989). The first clear demonstration that protein tyrosine kinase was essential for the induction of LTP in CA1 neurons was provided by O’Dell (1991). The data revealed that the specific tyrosine kinase inhibitors, genistein or lavendustin A, selectively blocked the induction of LTP when applied in the bath, or injected into the postsynaptic neuron. By contrast, the inhibitors had no effects on established LTP, or on basal synaptic transmission, or on the neurotransmitter actions attributable to the actions of PKA or PKC. These data indicated that tyrosine kinase activity might be required for LTP induction.

The role of tyrosine kinase in LTP induction was reinforced by finding that enhanced tyrosine phosphorylation of the NR-2B subunit after the induction of LTP was observed (Rostas et al., 1996). Tyrosine phosphorylation of NR-2B was measured in two ways: binding of anti-phosphotyrosine antibodies (PY20) to a glycoprotein of 180 kDa purified on Con A-Sepharose, and binding of anti-NR-2B antibodies to tyrosine-phosphorylated proteins. Anti-NR-2B binding to tyrosine phosphorylated proteins after LTP induction was increased by 1.5-fold. This increase in the number of tyrosine phosphorylated NR-2B occurred without a change in the total number of NR-2B. The results suggested that tyrosine phosphorylation of the NMDAR contributes to LTP. However, this protein tyrosine kinase family has over nine members. The tyrosine kinase inhibitors lack the pharmacological specificity to identify which of those tyrosine kinases expressed in the central nervous system (CNS) is essential (Grant et al., 1992).
To facilitate identification of individual tyrosine kinase involved in LTP, mutant mice lacking the Src, Fyn, Yes or Abl were generated by Soriano and analyzed by Grant (1992). The results showed that mutations in Src, Yes, or Abl did not interfere with the induction of LTP. However, LTP induction was impaired in Fyn mutants. These results suggest that Fyn signaling may contribute to LTP. These results, however, should be interpreted cautiously. As Fyn kinase is involved in cellular differentiation and proliferation, animals deficient in Fyn kinase also have developmental anomalies which affect the establishment of properly functioning neurons. In fact, histological examination of the hippocampus in Fyn mutant mice revealed that anatomical defects were present (Grant et al., 1992). Furthermore, recent evidence suggests that Fyn kinase is involved in axonal myelination (Umemori et al., 1994). Thus the deficiency in LTP in the fyn mutant mice might not involve mechanisms underlying normal LTP induction. In addition, the analysis of the phenotype in Fyn deficient mice was complicated by the presence of Src compensations (Kojima et al., 1997). In Fyn mutants, the age-dependent impairment of LTP was associated with the changes of age-dependent Src activity. The Fyn deficient mice showed a clear deficit of LTP only between 14 weeks and 6 months of age. In younger Fyn mutant mice (less than 10 weeks) LTP was similar to that seen in the wildtype mice. Thus, the LTP phenotype in Fyn mutants is age dependent and appears only when the mice are over 10 weeks of age. Interestingly, at all ages Src is expressed at higher levels in Fyn mutant mice that in wildtype mice. At 14 weeks of age when the LTP deficit begins in Fyn mutants, the level of compensatory Src expression was reduced. This correlative data points out that Src may be involved in LTP in the context of Fyn mutants.
Because the tyrosine kinase inhibitors used were not selective, and because in the Fyn mutants alterations occurred in the levels of other Src family members, the clarity of roles to particular tyrosine kinase members in LTP induction is still problematical.

Tyrosine phosphorylation regulates the function of NMDARs (Wang et al., 1994; Kohr and Seeburg, 1996) via Src, a non-receptor protein tyrosine kinase (Yu et al., 1997). Since NMDARs are necessary for the induction of LTP at many synapses (Collingridge and Singer, 1990; Nicoll and Malenka, 1995), we made use of Src specific blockers and activators to determine whether Src participates in LTP.
3.2 RESULTS

3.2.A Src is necessary for the induction of LTP

We made whole-cell patch clamp recordings from pyramidal neurons in the CA1 region of rat hippocampal slices; field potentials were recorded by an extracellular electrode. Excitatory synaptic responses were evoked by stimulating the Schaffer-collateral inputs to CA1 neurons. LTP at these synapses is known to depend upon NMDA receptors. In order to determine whether Src is necessary for LTP induction we made use of a unique domain peptide fragment, Src(40-58), known to block Src function (Yu et al., 1997). Src(40-58) was applied directly into the neurons by diffusional exchange from the patch electrode (Figure 3.1). During application of Src(40-58), tetanic stimulation caused short-but not long-lasting potentiation of the intracellularly recorded excitatory post-synaptic potentials (EPSPs): the slope of the EPSPs was 99±5.7% (mean±SEM) of baseline by 30 min after tetanic stimulation (n=6 cells). However, the tetanic stimulation did produce a long-lasting increase in field EPSP slope to 182±24% baseline. Thus, Src(40-58) prevented induction of LTP in the cells in which it was administered intracellularly, but not in neighboring cells. A peptide with the same amino acid composition but in random order, scrambled Src(40-58), served as a control and did not prevent induction of LTP. Intracellular application of the antibody, anti-src1, which specifically blocks Src action (Roche et al., 1995), caused the EPSP slope to decline to 120±9.5% of baseline by 30 min after tetanus (n=7 cells; Figure 3.2) whereas the fEPSP slope was maintained at a sustained level 189±21% of baseline. In contrast, a non-specific IgG fraction did not affect LTP induction. With administration of Src(40-58), or of anti-src1, the tetanus
Figure 3.1 Blocking Src by Src(40-58) prevents induction of LTP

**Top.** A plot of EPSP slope from representative cells with intracellular administration of Src(40-58) or scrambled Src (40-58) (sSrc(40-58)). The traces in the insets are averages of 3 sweeps taken at the time points indicated by the letters below the x-axis. **Middle.** Averaged EPSP slope or experiments with Src(40-58) (n=6), or with sSrc(40-58) (n=5). Data were normalized to baseline value. Error bars are standard error in all figures. **Bottom.** Averaged, normalized field EPSP (EPSP) slope with Src (40-58), or with sSrc(40-58).
Figure 3.2 Blocking Src by anti-Src1 prevents induction of LTP

Top. A plot of EPSP slope from representative cells with intracellular administration of anti-Src1 or non-specific IgG. The traces in the insets are averages of 3 sweeps taken at the time point indicated by the letters below the x-axis. Middle. Averaged EPSP slope or experiments with anti Src1 (n=6) or with IgG (n=5). Data were normalized to baseline value. Error bars are standard error in all figures. Bottom. Averaged, normalized field EPSP (EPSP) slope with anti Src1, or with IgG.
produced post-tetanic potentiation, the peak of which was not different from that of the respective controls. Thus, Src was necessary for the induction of LTP.

**Src(40-58) has no effect on the induction of LTP in Src mutant mice.** As an additional control Src(40-58) was tested in mice lacking src and had no effect on LTP induction (EPSP slope 30 min after tetanus was 195±17% baseline, n=5 cells, p>0.1, compared to LTP recorded by application of sSrc(40-58) in Figure 3.1). Src(40-58) prevented LTP in wildtype mice from the same genetic background (EPSP slope 110±8% baseline, n=4 cells) and thus, the effect of this peptide required Src. It is necessary to mention that the requirement of Src for the induction of LTP observed here appeared to be inconsistent with the finding that normal LTP obtained in Src null mutant mice reported by Grant (Grant et al., 1992). Because multiple Src family members bind to the same receptor, the possibility is that various members may functionally substitute for each other if one of the kinases becomes inactivated (Kypta et al., 1990., Courtneidge et al., 1993). In fact, Fyn kinase, a member of src family, revealed a dramatic increase in Src null mutant mice (Stein et al., 1994). In addition, the analysis of the phenotype in Fyn deficient mice was complicated by the presence of Src compensations (Kojima et al., 1997). In Fyn mutants, the age-dependent impairment of LTP was associated with the changes of age-dependent Src activity. The Fyn deficient mice showed a clear deficit of LTP only between 14 weeks and 6 months of age. In younger Fyn mutant mice (less than 10 weeks) LTP was similar to that seen in the wildtype mice. Thus, the LTP phenotype in Fyn mutants is age dependent and appears only when the mice over 14 weeks of age are used. Interestingly, at all ages Src is expressed at higher levels (about three-fold higher than that in of control) in Fyn mutant mice that in wildtype mice. At 14 weeks of age
when the LTP deficit begins in Fyn mutants, the level of compensatory Src expression was reduced. This correlative data point out that Src may be involved in LTP in the context of Fyn mutants.

**Src(40-58) has no effect on basal synaptic transmission** In the adult CNS there is a basal level of Src activity (Grant et al., 1992) and in CA1 neurons this might produce a tonic enhancement of NMDA channel function. Then the blockade of LTP might have been through abolishing ongoing enhancement of NMDA channels, in which case blocking Src would be expected to reduce basal synaptic NMDA responses. However, in voltage-clamp experiments administering Src(40-58) had no effect on pharmacologically-isolated NMDAR-mediated excitatory post-synaptic currents (NMDAR EPSCs) (Figure 3.3A). Moreover, Src(40-58) had no effect on AMPAR-mediated (AMPAR) EPSCs (Figure 3.3B). Thus, neither synaptic NMDARs nor synaptic AMPARs was enhanced tonically by basal Src function.

**3.2.B Src activity is increased by LTP inducing stimulation**

We hypothesized that Src might be activated during LTP induction. To test this we measured Src catalytic activity by means of an immune-complex kinase assay. The $^{32}$P incorporation produced by Src immunopurified from slices that had received tetanic stimulation was greater than that from control slices that had received only test stimulation (Figure 3.4A,B). Because there was no difference in the level of Src protein (Figure 3.4A), tetanus caused an increase in Src activity.
Figure 3.3  Src(40-58) had no effect on basal synaptic transmission

A. Src(40-58) had no effect on basal NMDAR EPSCs. Top. from a cell with administration of Src(40-58) and of CNQX. EPSCs evoked at potentials from -80 to +60 mV, in 20 mV increments. The I-V relationship was determined during first 5 min period (left) or 30 min after the start of recording (right). Bottom. I-V relationship for amplitudes of NMDAR EPSCs (n=3) during the first 5 min and the period of 30 to 35 min. B. No effect of Src(40-58) on basal AMPA EPSCs. Top. EPSCs from a cell with administration of Src (40-58) at potentials from -80 mV to +60 mV, from the first 5 min or at 30 min after the start of recording. Bottom. averaged I-V relationship for peak amplitude of AMPA EPSCs (n=3) from first 5 min or for 30 to 35 min.
3.2.C Src is sufficient for the induction of LTP

To determine whether increasing Src activity affects synaptic responses we administered exogenous recombinant Src (pp60\textsuperscript{c-Src}) which was found to increase EPSP slope, to 185±24% baseline (n=7 cells). In contrast, heat-inactivated pp60\textsuperscript{c-Src} had no effect (Figure 3.5). To examine the effect of activating endogenous Src we used the high-affinity peptide, EPQ(pY)EEIPIA, an activator of tyrosine kinases in the Src family (Roche et al., 1995). EPQ(pY)EEIPIA was applied alone, or with Src(40-58) to determine whether the effects required Src itself. Application of EPQ(pY)EEIPIA produced an increase in EPSP slope to a sustained level 226±22% of baseline (n=8 cells; Figure 3.6A). On the other hand the nonphosphorylated form of the peptide, EPQYEEIPIA which does not activate tyrosine kinases, did not affect EPSPs (n=4 cells). Moreover, during administration of Src(40-58) perfusion with EPQ(pY)EEIPIA had no effect on EPSP slope (n=6 cells, Figure 3.6B). However, during administration of scrambled Src(40-58), perfusing EPQ(pY)EEIPIA did produce an increase in EPSP slope to 200±19% baseline (n=5 cells). Thus endogenous Src was necessary for the enhancement of EPSPs by EPQ(pY)EEIPIA. In voltage-clamp recordings, EPQ(pY)EEIPIA potentiates AMPAR EPSCs through an increase in AMPAR EPSC conductance with no change in driving force (Figure 3.7). Overall, activating Src was sufficient to enhance EPSPs.

If endogenous Src participates in LTP produced by tetanic stimulation then LTP and the enhancement by the activating peptide may occlude each other. This was investigated by applying EPQ(pY)EEIPIA, and when the EPSPs had been maximally
Figure 3.4 Src activity is increased by tetanus

A. Immune complex kinase assays were done with Src immunoprecipitated after control stimulation or 1 min or 5 min after tetanus. Proteins were separated by SDS-PAGE and transferred to nitrocellulose, and 32P was detected by exposure to a Phosphor Screen. Results of a representative immune complex kinase experiment are shown; phosphorylation of Src or of enolase is indicated by the arrows. The corresponding anti-src immunoblot is shown below. B. 32P labeling was quantified for Src (solid bars) and enolase (open bars). Data from 1 min- and 5 min times were normalized as percentage of control (n=4 experiments).
Figure 3.5 Exogenous Src caused synaptic potentiation

*Top.* A plot of EPSP slope during intracellular application of pp60c-src or heat-inactivated (boiled) pp60c-src. *Bottom.* Averaged EPSP slope with application of pp60c-src (n=5).
Figure 3.6 Activation of endogenous Src caused synaptic potentiation

A. Top: A plot of EPSP slope during application of EPQ(pY)EEIPIA (1 mM), or EPQ(Y)EEIPIA (1 mM). Bottom: Averaged EPSP slope during application of EPQ(pY)EEIPIA (n=8) or EPQ(y)EEIPIA (n=4). B. Top. A record of EPSP slope during application of Src(40-58) or sSrc(40-58). During the period indicated by the upper horizontal line EPQ(pY)EEIPIA was administered by perfusing the patch electrode.
Figure 3.7 Activation of Src increased the AMPA conductance

I-V relationship for EPSCs evoked during application of EPQ(pY)EEIPIA. EPSCs were evoked at membrane potentials from -80 mV to +60 mV. Top. The records are superimposed EPSC traces collected during the first 5 min or 30 min after the start recording. Bottom. Averaged peak amplitudes of AMPAR EPSCs are plotted in the graph (n=4) with first 5 min or 30 min.
enhanced, tetanic stimulation was delivered (Figure 3.8A). This stimulation caused post-tetanic potentiation but produced no long-lasting increase in EPSP slope. In contrast, when EPQYEIEIPIA was administered, delivering tetanic stimulation at the same time after beginning the recording caused a long-lasting increase in EPSP slope (212±5.3% baseline, n=5 cells). In other experiments, tetanus produced a lasting potentiation of EPSP slope (212±28% baseline, n=5 cells) but there was no further increase when EPQ(pY)EEIPIA was applied intracellularly (Figure 3.8B). On the other hand, in cells not conditioned by tetanic stimulation perfusing EPQ(pY)EEIPIA, at the same time after beginning recording, caused a progressive enhancement of EPSP slope which reached a stable level 225±23% of baseline (n=4 cells). Thus, Src-induced enhancement of EPSPs and tetanus-induced LTP were mutually occluded.

3.2.D NMDAR is required for Src action on LTP

Because LTP in CA1 neurons depends upon raising intracellular [Ca^{2+}], we next determined whether the Src-induced enhancement of AMPAR EPSCs might be linked to a rise in [Ca^{2+}]. In previous experiments AMPA receptors appeared not to be regulated by Src (Yu et al., 1997) but in those experiments, in contrast to the present ones, a high level of intracellular Ca^{2+} buffering was used. To determine whether Src-induced enhancement of AMPAR EPSCs requires raised intracellular [Ca^{2+}] we increased the buffering capacity of the intracellular solution. With the high Ca^{2+}-buffering solution (10 mM EGTA) EPQ(pY)EEIPIA had no effect on AMPAR EPSCs (Figure 3.9A). EPQ(pY)EEIPIA remains active in the high Ca^{2+}-buffering solution because there was an
Figure 3.8 Src-induced potentiation and LTP occlude each other

A. Effect of tetanic stimulation (arrow) during intracellular application of EPQ(pY)EEIPIA or EPQYEEIPIA. Recordings of EPSP slope during application of EPQ(pY)EEIPIA (n=5) or EPQYEEIPIA (n=5) from representative cells are plotted at the top. Averaged EPSP slope in the bottom. B. Effect of tetanus on action of EPQ(pY)EEIPIA. Representative recordings of EPSP slope when tetanus was delivered 10 min after the start of recording or without tetanus. EPQ(pY)EEIPIA was actively perfused. Averaged EPSP slope is plotted in the bottom graph (n=5 with tetanus, n=4 without tetanus).
increase in NMDAR EPSCs (Figure 3.9A,B). The increase in NMDAR EPSCs was associated with no change in driving force or in I-V relationship (Figure 3.9B) and was produced with low Ca\(^{2+}\)-buffering solution (Liu et al., 1993). Also, the enhancement of NMDA currents by EPQ(pY)EEIPIA was prevented by Src(40-58) and was not produced by EPQYEEIPIA. Thus, the Src-induced increase in AMPAR EPSCs was Ca\(^{2+}\)-dependent but the potentiation of NMDAR EPSCs was Ca\(^{2+}\)-independent.

Because Src is associated with and upregulates the function of NMDA receptors (Yu et al., 1997), we questioned whether NMDA receptors are required for Src-induced enhancement of AMPAR EPSCs. We blocked NMDA receptors by bath-applying the antagonist, MK-801, during experiments with low Ca\(^{2+}\)-buffering intracellular solution. When MK-801 was applied starting just prior to whole-cell recording, administering EPQ(pY)EEIPIA produced no change in EPSP slope (Figure 3.10A). In other experiments, after synaptic responses had been potentiated by EPQ(pY)EEIPIA, MK-801 had no effect on AMPAR EPSPs (Figure 3.10B). Thus, NMDA receptor activation was necessary to induce, but not to sustain, the Src-induced potentiation of AMPAR-mediated synaptic currents.
Figure 3.9 Src-induced enhancement of AMPAR responses depends on [Ca^{2+}]_{i}

A. Potentiation of AMPAR EPSCs is Ca^{2+} dependent but potentiation of NMDAR EPSCs is Ca^{2+} independent. Top. Plot of amplitude of AMPAR EPSCs and NMDAR component during a recording with high Ca^{2+} buffering intracellular solution and application of EPQ(pY)EEPIA. Bottom. Effects of EPQ(pY)EEPIA on averaged AMPAR EPSCs or NMDAR component during recordings with high Ca^{2+} buffer solution (n=5). Data were normalized to EPSCs in the first min of recording. B. I-V relationship for pharmacologically-isolated NMDAR EPSCs during application of EPQ(pY)EEPIA. Top. Superimposed NMDAR EPSCs evoked at membrane potentials from -80 to +60 mV. Traces are from the first 5 min and after 30 min recording. Bottom. Averaged amplitudes of NMDAR EPSCs (n=3) from the first 5 min or after 30 min.
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Figure 3.10 Src-induced enhancement of AMPAR responses depend on NMDARs

C. MK-801 (10 uM) was bath applied just before the start of recording with intracellular solution containing EPQ(pY)EEPIA. The upper graph shows a representative plot of EPSP slope from one cell and the lower graph is averaged, normalized data (n=4). B. EPQ(pY)EEPIA was administered intracellularly and MK-801 (10 uM) was bath applied during the period indicated by the short horizontal line, after the potentiation of EPSP slope was administered intracellularly and MK-801 was bath applied during the period indicated by the short horizontal line, after the potentiation of EPSP slope was established. One example is shown in the upper graph and averaged, normalized EPSP slope is plotted below (n=5).
DISCUSSION

SRC KINASE FULFILLS NECESSARY AND SUFFICIENT CONDITIONS AS THE MEDIATORS OF LTP INDUCTION
3.3.A Src activation mediates LTP induction

I have shown that the blockade of Src prevented the induction of LTP and activating Src, or administering recombinant Src, induced lasting potentiation which occluded LTP induction. Like tetanus-induced LTP, the potentiation produced by directly activating Src depended upon a rise in intracellular \([\text{Ca}^{2+}]\) and upon NMDA receptors. Thus, Src fulfills necessary and sufficient conditions to be considered a mediator of LTP induction at Schaffer collateral-CA1 synapses. Although there is a basal level of Src function this did not appear to contribute to LTP, but rather the activation of Src, as a consequence of tetanic stimulation, was required to induce LTP. Thus, activation of Src provides a biochemical mechanism for gating the induction of LTP.

3.3.B Src acts via regulation of NMDARs in LTP induction

The requirement for Src activation in LTP induction has been attributed to its enhancement of NMDAR activity, because an NMDAR antagonist, MK-801, blocks its effect. Models of biochemical events underlying induction of LTP in hippocampal CA1 focus on the signalling cascade(s) initiated by \(\text{Ca}^{2+}\) influx through NMDA receptors (Malenka et al., 1988; Malenka et al., 1992; Wyllie et al., 1994). The most parsimonious explanation for our findings during induction of LTP is that Src is rapidly activated which leads to enhanced NMDA receptor function. Enhancing NMDA receptor function results in increased \(\text{Ca}^{2+}\) entry which may trigger the downstream signalling cascade. Hence, the present results indicate that for LTP induction there is a hitherto unexpected step upstream of NMDA receptors. Src is widely expressed in the nervous system and thus, Src may have a common role in plasticity of excitatory synaptic transmission in many
3.3.C Possible mechanisms whereby Src activates NMDARs during LTP induction

Src phosphorylates NMDARs The simplest explanation for modifications of NMDAR channel function by Src is that the NMDAR subunits themselves are directly phosphorylated leading to changes in channel activity. There is good evidence for direct phosphorylation of tyrosine residues within NR-1, NR-2A, and NR-2B subunits (Lin et al., 1993; Moon et al., 1994; Lau and Huganir, 1995; Rosenblum et al., 1996; Rostas et al., 1996). The functional effect of this phosphorylation has been found on the receptors composed of NR-1 and NR-2A subunits (Kohr et al., 1996). Furthermore, the deletion of the C-terminal domain of NR-2A eliminates the potentiation of NR-1/NR-2A-receptor currents by Src and results in deficits of LTP induction. It has been proposed that a unique tyrosine phosphorylation site on the C-terminal of NR-2A may confer sensitivity to Src and be critical for the induction of LTP (Sprengel et al., 1998).

Src may target one or more NMDAR associated PSD proteins NR-2B subunits are phosphorylated by one of the Src family of tyrosine kinases (Moon et al., 1994, Miyakawa et al., 1997), but no functional effect of this phosphorylation has been identified. This discrepancy between biochemical and physiological data raises the possibility that tyrosine phosphorylation of NR-2B could affect receptor structure rather than channel activity. For example Src phosphorylation of the PSD complex could lead to synaptic re-organization, which in turn could regulate NMDAR channel activity.
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NMDARs have been reported to be physically associated with a variety of proteins present within PSD complex (Kornau et al., 1995; Muller et al., 1996; Ehlers et al., 1996). Some insight into the mechanisms that modulate NMDAR function by PSD proteins has been revealed from studies of PSD-95 (Kennedy, 1997). An example of such modification is the reduction of NMDAR activity by direct binding of calmodulin to NR-1 subunits, which inactivates NMDARs (Ehlers et al., 1996).

**Src may regulate the homeostasis of \([Ca^{2+}]_i\)**  
\[Ca^{2+}\] is believed to be a central mediator of LTP induction (Lynch, 1984., Bliss and Collingridge, 1993). The induction of LTP in CA1 region requires the activation of NMDARs and the subsequent influx of \(Ca^{2+}\) into the postsynaptic cell. A computer simulation model of the NMDAR has suggested that \(Ca^{2+}\) influx may be highly dependent, not only on the permeability of the NMDAR channels, but also the properties of \(Ca^{2+}\) buffers and the location of the spine on the dendrite at different sites (Reynolds and Miller, 1990). Direct imaging of \([Ca^{2+}]_i\) with \(Ca^{2+}\)-sensitive dyes has provided important insights into the regulation of \([Ca^{2+}]_i\) in neurons and its biological consequences in LTP induction (Regehr et al., 1989). As \(Ca^{2+}\) enters the cytosol, it encounters a number of proteins that regulate its biochemical effects. Central among them is calmodulin. \(Ca^{2+}/calmodulin\) binds to a number of enzymes and can act locally at the synapse by inducing the modifications of pre-existing synaptic proteins, which lead to the induction of LTP. It has been reported that tyrosine kinase regulates the frequency and the amplitude of \(Ca^{2+}\) oscillations in cultured hippocampal neurons (Sakai et al., 1997). In addition, \(Ca^{2+}\) release from a distinct intracellular pool associated with tyrosine kinase activation was observed (Jonas et al., 1997). The studies
raise the possibility that Src may regulate the efficacy of \([Ca^{2+}]_i\) around the NMDAR channel, which in turn accelerates the activation of calmodulin at synaptic spines.

### 3.3.D Model for a Src-mediated signalling pathway in LTP induction

*Pyk-2 may be required for activation of Src in LTP.* The most plausible explanation that accounts for the roles of Src in LTP is that during the induction of LTP Src is rapidly activated which leads to enhanced NMDA receptor function. How might Src be activated so as to contribute to a network of kinase activity in LTP? Given the model for Src regulation, there are a variety of ways to initiate activation of Src in the cell.

Src could be activated directly after tetanic stimulation: for example, the \(Ca^{2+}\) influx mediated by the NMDA receptor might directly trigger the tyrosine kinase Src. However, Src is \(Ca^{2+}\)-independent enzyme (Brown and Cooper., 1996). Therefore, Src might be activated indirectly by protein kinase, such as proline-rich tyrosine kinase 2 (Pyk-2). Several lines of evidence support this idea. The protein tyrosine kinase PyK-2, which is highly expressed in the CA1 region of the hippocampus (Lev et al., 1995), is rapidly phosphorylated on tyrosine residues in response to various stimuli that elevate intracellular \(Ca^{2+}\), for example, by applying glutamate or high \(K^+\) to induce neuronal depolarization in the rat hippocampus. More recently, there is also evidence showing that tyrosine phosphorylated PyK-2 directly binds to the SH2 domain of Src leading to Src activation (Dikic et al., 1996). This raises the possibility that Src links PyK-2 with the efficacy of the NMDAR channel gating in the initial events of LTP induction.
The further direction on this part is to clarify this possibly cellular signaling pathway. It will be necessary to determine whether PyK-2 is required for LTP induction. The endogenous PyK-2 should produce synaptic potentiation which has the same features as Src-potentiation. Blockade of Src function should prevent PyK-2 action on LTP.

**Src may interact with CaMK-II in LTP induction.** Once activated, how does Src exert its effects on the AMPA receptor potentiation? It is known that Src is not a Ca\(^{2+}\) -dependent enzyme, and yet the enhancement of AMPA responses produced by activating Src is dependent upon Ca\(^{2+}\). Therefore, Src seems to upregulate AMPARs indirectly through one or more steps requiring Ca\(^{2+}\), for example, CaMK-II. Models of biochemical events underlying the induction of LTP in hippocampal CA1 focus on the signalling cascade(s) initiated by the Ca\(^{2+}\) influx through NMDARs (Barria et al., 1997). It was postulated that the rise in intracellular Ca\(^{2+}\) leads to the activation of CaMK-II which phosphorylates and thereby upregulates the function of AMPARs. This is supported by several lines of evidence. Both broad-spectrum and more selective inhibitors, that target the two major Ca\(^{2+}\) -activated CaMK-II isoforms, are able to inhibit the induction of LTP at the CA1 synapses. Furthermore, injection of Ca\(^{2+}\) /calmodulin or CaMK-II into CA1 pyramidal cells leads to the induction of LTP. In addition, Ca\(^{2+}\) /calmodulin- or CaMK-II-induced and tetanus-induced potentiations occluded one another (Wang and Kelly, 1995). Finally, gene knockout mice lacking the gene for the \(\alpha\) subunit of CaMK-II showed greatly impaired LTP (Silva et al., 1992). Based on these observations combined with our results, a new model for the cellular biochemical cascades in LTP is summarized in Figure 3.11.
Figure 3.11 A new model for Src signaling cascades in LTP Induction

Tetanic stimulation induces a "weak" Ca\(^{2+}\) rise in postsynaptic neurons. A rise in [Ca\(^{2+}\)] stimulates PyK-2, which in turn binds to SH2 domain of Src and activates Src. The activation of Src up-regulates NMDA channel activities, and causes a "strong" Ca\(^{2+}\) entry. This "strong" Ca\(^{2+}\) with calmodulin (CaM) stimulates CaMK-II, which phosphorylates and then up-regulates the function of AMPAR.
CHAPTER FOUR

THE mGluR5-COUPLED PKC PATHWAY IS NECESSARY FOR THE INDUCTION OF THE NMDAR-MEDIATED COMPONENT OF LTP

This work was published as:


The work in all figures of this chapter was carried out by me. mGluR5 mutant mice were provided by ZhenPing Jia in Dr Roder's Lab.
4.1 INTRODUCTION

Of the eight mGluRs that have been molecularly characterized (Nakanishi, 1994), those linked to PKC activity (Abe et al., 1992) have been implicated most strongly in LTP (Pin & Duvoisin, 1995., Conn and Pin, 1997). mGluR5 is expressed most strongly on post-synaptic neurons in the CA1 region of the hippocampus (Romano et al., 1995; Minakami et al., 1993), whereas mGlu1α is absent (Shigemoto et al., 1993). mGluR5 is found mainly in the post-synaptic dendrites and spines of CA1 cells upon EM analysis (Romano et al., 1995). Immunogold staining for mGluR5 shows localization in a peri-synaptic annulus on dendritic spines of CA1 pyramidal cells, whereas AMPAR and NMDAR proteins were concentrated in the centre of the post-synaptic density (Lujan, et al., 1996). Presumably, mGluR5 can only respond to high levels of glutamate, such as may be released during high frequency tetanic stimulation, or co-incident firing at lower frequencies (i.e. theta rhythms). One study using gene targeting techniques has demonstrated that mGluR1 is not required for LTP in CA1 (Conquet et al., 1994), although a second study did report impaired LTP in this region (Aiba et al., 1994). In fact, LTP could be produced normally in all pathways except in the mossy fibre/CA3 synapses, where activation of the NMDA receptor is not required for LTP induction. These studies indicate that mGluR1 may not be involved in NMDAR-dependent LTP, which is not surprising since mGluR1 is not expressed in CA1 pyramidal neurons (Lujan et al., 1996). The involvement of mGluR5 is unknown since there are no currently available agonists, or antagonists, specific for mGluR5 (Hayashi et al., 1994; Pin & Duvoisin, 1995; Kingston et al., 1995). In the absence of selective agonists, or
antagonists, we made use a null mutant mice lacking mGluR5 to critically test the role of mGluR5-linked PKC signaling in the induction of LTP.
4.2 RESULTS

SELECTIVE IMPAIRMENT OF THE LTP_{NMDA} IN mGluR5 MUTANTS
4.2.A mGluR5 is the main high-affinity ACPD receptor in CA1 synapse

The mGluR agonist, (1S,3R)-1-amino-cyclopentane-1,3-dicarboxylic acid (ACPD), has been shown to produce several effects including a reversible depression of synaptic transmission in the hippocampus (Pin and Duvoisin, 1995). In the CA1 area, multiple subtypes of mGluRs have been implicated in the depression in fEPSP, namely Group I and III (Gereau and Conn, 1995). Since ACPD is not active at Group III mGluRs, its depressant effects are likely caused by Group I (mGluR1/5). This depression was not altered in the mGluR1 mutant mice (Aiba et al., 1994). To examine whether or not ACPD-induced depression is mediated through mGluR5, the field excitatory postsynaptic potential (fEPSP) in the CA1 region of the hippocampus was compared, between the mGluR5 mutants and wildtype controls, in the presence of various concentrations of ACPD (Figure 4.1A). In wildtype slices 10, 25, 50 or 100 µM ACPD, 10 min. after agonist application, decreased fEPSP by 14.3 ± 1.21, 28.8 ± 2.6, 55.5 ± 4.6 and 98.1 ± 9.2 %, respectively. However, the effects of ACPD on fEPSP were very much attenuated in the mutant slices. For example, 25 µM ACPD did not decrease fEPSP at all in mGluR5 mutants, and 50µM of ACPD produced only a 12 ± 1.0 % depression in fEPSP. However, application of 300 µM ACPD decreased fEPSP by 64.4 ± 5.4 %, even in the mutant slices. Such an apparent shift in the sensitivity to ACPD indicates that the effects of ACPD on fEPSP, at low concentrations (below 50 µM), may be exclusively mediated by the activation of mGluR5. Other mGluRs, such as mGluR1, may be activated at higher ACPD concentrations.

The lack of effect of low concentrations of ACPD (25 or 50 µM) on CA1 fEPSP in the mGluR5 mutant mice was also supported by our whole-cell recordings from individual
Figure 4.1 Decreased response to ACPD in mGluR5 mutants

A. Depression of fEPSP slope induced by 1S, 3R-ACPD. Data are expressed as mean percent of the control values in mutant mice (open circle, 6 slices, 6 animals) and wildtype mice (filled circle, 6 slices, 6 animals). Traces taken from representative experiments show the effects of ACPD on evoked fEPSP. Each trace is an average of six sweeps recorded immediately prior to drug application (0), or after 10 min. in the agonist at a concentrations of 10 μM (1), 25 μM (2), 50 μM (3), 100 μM (4), and 300 μM (5). B. Whole-cell current clamp recording showing the time-course of EPSP depression by bath application of 1S, 3R-ACPD in a single wildtype (•) or mutant (o) CA1 cell, representative of 4 cells. Representative time courses of EPSP depression in whole-cell current clamp recordings in the medial perforant pathway of the dentate granule neurons in the presence or absence of ACPD and 1 mM (RS) MCPG, in wildtype (•) and mutant (o) mice. Insets in (B) and (C) are representative EPSP before (0), during (1) and after (2) ACPD application. D. Mean (± SEM) % fEPSP following L-AP4 application to CA1 from control (•) and mutant (o) mice (n=4). E. Mean fEPSP following carbachol addition to control (•) and mutant (o) mice (six slices, 3 animals).
neurons, where the application of 50 μM of ACPD had no effect on EPSP in the mutants, whereas the same concentration of ACPD produced 64.2 ± 5.1% (n=4) depression of EPSP in the wildtype controls (n=4) (Figure 4.1B). Neuronal depolarization in response to ACPD in wildtypes was 8.3 ± 0.76 mV (n=4), compared to no changes in mutants. In the dentate gyrus, in contrast to the CA1 region, the response to ACPD (25, 50 μM) application in slices was indistinguishable between mGluR5 mutant and wildtype controls (Figure 4.1C). In addition, all depression was completely blocked by the addition of 1 mM of MCPG in the dentate region.

To examine if the lack of actions of ACPD on fEPSP in the CA1 region of mGluR5 mutant mice was selective, the effects of both L-AP4, a selective agonist for group III mGluRs, and carbachol, an agonist for the muscarinic acetylcholine receptor, were compared between mutants and wildtype controls. The depression in fEPSP after application of various concentrations of L-AP4, or carbachol, was the same in these two groups, suggesting that functional effects mediated by group III mGluRs, and muscarinic acetylcholine receptors, was not altered in the mutant mice (Figure 4.1D, E).

4.2.B NMDAR EPSCs were reduced in mGluR5 mutants

The efficacy of excitatory synaptic transmission in the Schaffer collateral-commissural pathway in the CA1 region of the hippocampus in the mGluR5 mutants and control mice was examined. The amplitude, time course and current-voltage relations of the AMPA component (CNQX sensitive) of synaptic currents, obtained with whole-cell recordings, revealed normal responses in the mutant mice (Figure 4.2A, B). However, the NMDAR channel current, expressed as the ratio to the non-NMDA component, was significantly
Figure 4.2 Reduced NMDA EPSCs in mGluR5 mutants

A. The EPSC traces were recorded from CA1 neurons in whole-cell voltage-clamp mode and were averages of 6 successive sweeps before (0) and 20 min. after (1) the addition of 10 μM CNQX. The holding membrane potentials are indicated between the traces. B. The averaged amplitudes of AMPA- and NMDA-mediated responses in +/+ (●) and +/- (○) mice, normalized to the 5 ms peak of AMPA EPSC at -80 mV which was 342.6 ± 38 pA (n=10) in control and 318.8 ± 34 pA (n=11) in mutant mice. The AMPA component at - 80 mV was taken as 100% and all other current amplitudes were scaled and expressed as a percentage of the AMPA current. C. The data in (B) shown as the NMDA component of EPSCs which differed between wildtype (crossed bars) and mutants (open). *significant difference (p<0.01, t-test). D. The magnitude of the paired-pulse facilitation of fEPSPs in the CA1 area of the two groups (6 slices from 3 animals for each genotype). (P1 first response, P2 second response applied at the indicated intervals on the x-axis).
(p<0.01) reduced in the mutant slices at the holding potentials between -20 to +60 mV (Figure 4.2C). The NMDARs retained their usual voltage dependence (Figure 4.2B). The extent of paired-pulse facilitation, a measure of presynaptic function, showed no significant difference over an interpulse interval range of 20-500ms (Figure 4.2D).

4.2.C NMDAR-dependent LTP is partially impaired in mGluR5 mutants

To examine the possible consequences of the reduced NMDA currents we examined the ability of three hippocampal pathways to undergo LTP in mice lacking mGluR5 (Figure 4.3). In this series of experiments fEPSPs were measured at 5-95% of the peak and LTP was induced by 4 trains of tetani. In the CA1 region, the magnitude of LTP was significantly reduced (by 35%) in the mutant slices compared to the wildtype controls. Averaged LTP in the last 30 min. after tetanus was 50.2 above the (100%) baseline ± 5.7% from 19 mutant slices (19 animals) and 77.6 ± 10.2 % above the baseline from 19 wildtype slices (19 animals) (F(1,32) = 6.45, p=0.016) (Figure 4.3A). When we measured fEPSP in CA1 at 10-60% of the peak, LTP in wildtypes was 168 ± 14 (19 slices from 19 animals) (Table 1), which was not significantly different (p = 0.28) than LTP in mutants 148 ± 11 (19 slices from 19 animals). The slope measurement at 5-95% of the peak, in fig. 20, included both an AMPAR- and a NMDAR-mediated component, since addition of AP5 removed approx. 15% of the potential; whereas CNQX removed 85% of the potential. Therefore, about half of the missing LTP in CA1 may be accounted for at the level of induction. The other missing portion may lie at the level of expression, since repeat experiments in 50 μM AP5, 20 min. after LTP induction removed some of the difference between wildtype and mGluR5 mutant mice (Table 1). These data indicate that the NMDA
Figure 4.3 Partially impairment of NMDAR-dependent LTP in mGluR5 mutants

The mean ± SEM of the 5-95% slope of the fEPSP, normalized with respect to 10 min. immediately preceding the tetanus (↑) for hippocampal slices obtained from control (*) or mutant (o) mice in (A) area CA1, (B) dentate gyrus medial perforant pathway, and (C) the CA3 mossy fibre pathway. LTP in CA1 was induced by 4 trains of 100 Hz tetanic stimulation. LTP in the dentate gyrus was induced by 4 trains of tetanus in the presence of 100 µM picrotoxin. LTP in CA3 was induced by 1 tetanic train in the presence of 50 µM AP5. Representative traces (average of 6 sweeps) of fEPSP obtained immediately before (0) and 60 min. following (1) the tetanus are shown for a control (a) and mutant (b) mouse respectively.
Table 1 The lack of genetic background effects on the LTP phenotype

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<th>129 X C57</th>
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<td>(-/-)</td>
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<td>LTP_{AMPA} by ACPD</td>
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<td>238.4 ± 86.1 n=8</td>
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<td>LTP_{NMDA} by tetanus</td>
<td>241.2 ± 96.4</td>
<td>111.9 ± 13.2</td>
<td>239.9 ± 98.9 n=4</td>
</tr>
<tr>
<td>Saturation of LTP_{NMDA}</td>
<td>323.6 ± 47.6 n=8</td>
<td>107.9 ± 11.1 n=8</td>
<td>318.6 ± 126.6 n=3</td>
</tr>
</tbody>
</table>

Note: 1. The values of LTP_{AMPA} from both mutants and wildtype controls are no significant difference between or within the strains (p > 0.2).

2. The values of LTP_{NMDA} in mutants are significant difference from that in Wildtypes between or within strains (p < 0.005).

3. The values of LTP_{NMDA} from mutants are no significant difference between strains (p > 0.3).

4. The values of LTP_{NMDA} from wildtypes are no significant difference between strains (p > 0.3).
receptor-mediated component contributed significantly to the field EPSP, and the reduced LTP recorded with fEPSP in mGluR5 mutants was likely due to elimination of this component.

A significant reduction in the degree of LTP was also seen in the medial perforant pathway of dentate gyrus, where the averaged LTP from 9 slices (5 animals for each genotype) was $40.4 \pm 5.7 \%$ above baseline for the mutants, compared to $66.9 \pm 9.2\%$ for the wildtype controls ($F(1,12)=6.2; p=0.028$) when fEPSP was measured at 5-95% of the peak (Figure 4.3B). However, when the mossy fibre LTP in the CA3 region was compared between the mutants and the wildtype controls, we could not detect any significant difference in either magnitude or time course (Figure 4.3C). The averaged mossy fibre LTP from 9 slices (5 animals for both groups) was $60.3 \pm 14.7 \%$ for mutants and $50.6 \pm 5.8 \%$ for wildtype controls ($F(1,12)=0.31$, NS). Therefore LTP was produced in all three pathways of hippocampus, but was selectively attenuated only in CA1 and the dentate gyrus.

**4.2.D Impaired LTP\textsubscript{NMDA} but normal LTP\textsubscript{AMPA} in mGluR5 mutants in response to tetanus**

LTP was also analyzed using voltage clamp recordings in the Schaeffer collateral-CA1 synapse in hippocampal slices. The NMDAR-mediated component of the evoked excitatory post-synaptic currents (NMDA EPSCs) was estimated at 100 ms using voltage clamp recordings. The AMPAR-mediated component was measured at 9 ms (AMPA EPSCs), and was separated from the NMDAR-mediated component using the NMDAR antagonist D-2-amino-5-phosphonovaleric acid (D-AP5). In 14 wildtype neurons from control littermates
(n=8 mice), the LTP of the NMDA receptor-mediated component (LTP\textsubscript{NMDA}) paralleled that of the AMPA receptor-mediated component (LTP\textsubscript{AMPA}), at a holding potential of -50 mV, and was stable for at least 1 hr. The peak of LTP\textsubscript{NMDA} was 241 ± 34%, compared to LTP\textsubscript{AMPA} at 249 ± 35% 30 min. following tetanic stimulation (Figure 4.4A). In 12 mutant neurons (n= 8 mice) however, the transient increase in NMDA\textsubscript{EPSCs}, after the tetanic stimulation, had almost returned to baseline values (112 ± 4.1 %) within 10 min, while LTP\textsubscript{AMPA} at 30 min (238 ± 30 %) (Figure 4.4B) was not different from that in wildtype (p > 0.1). The NMDAR-mediated currents were contaminated < 15% by AMPA currents. Similar numbers of failures to induce any LTP (i.e. both LTP\textsubscript{NMDA} and LTP\textsubscript{AMPA} were absent) were observed in control and mutant neurons (approx. 20 %). The amplitude of NMDA\textsubscript{EPSCs} was also measured in 5 μM CNQX. Lower doses of CNQX (2 μM) were also effective. The remaining EPSCs in 2 μM CNQX were pure NMDA EPSCs as the addition of 50 μM AP5 completely abolished the currents under these experimental conditions. LTP\textsubscript{NMDA} was also eliminated at all potentials between -70 to +20 mV without observable voltage-dependency in the mutants (8 neurons, n= 4 mice) (Figure 4.5A,B). This corresponds with previous results showing that NMDA EPSCs prior to and following LTP induction were not altered in the rise and decay times, or the reversal potential, at all voltage levels in control neurons (Bashir et al., 1991). Differences in L-type voltage-gated Ca\textsuperscript{2+} channels played little role in these experiments since 10 μM nimodipine did not affect wildtype LTP\textsubscript{NMDA} or LTP\textsubscript{AMPA}, nor mutant LTP\textsubscript{AMPA} levels (8 neurons, n= 4 animals; data not shown). Only the amplitude of post-tetanic potentiation was attenuated by nimodipine.
Figure 4.4 Impaired $\text{LTP}_{\text{NMDA}}$ with intact $\text{LTP}_{\text{AMPA}}$ in mGluR5 mutants

$LTP_{\text{AMPA}}$ and $LTP_{\text{NMDA}}$ in CA1 synapse of $+/+$ (A) and $-/-$ (B) mice is plotted as a function of time. Horizontal bars in each panel indicate that 50 μM AP5 was applied prior to, and following tetanus. Superimposed traces were taken at the indicated times on the axis. 1-0 indicates subtraction of trace 1 from trace 0. 2-3 indicates subtraction of trace 2 from trace 3. The holding potential was -50 mV.
Figure 4.5  LTP\textsubscript{NMDA} was impaired at all voltages in mGluR5 mutants

A. Superimposed traces of NMDA EPSCs at three holding potentials before (0) and 30 min (1) LTP induction. NMDA EPSCs were measured at 25 ms in CNQX. B. I-V relationship of the NMDA EPSCs before (open circles) and after (filled circles) LTP induction. At the end of the experiment, the CNQX-resistant EPSCs was completely blocked by 50 \textmu M AP5.
As the basal NMDAR-mediated synaptic responses (expressed as the ratio of NMDA EPSCs to AMPA EPSCs) in mutant mice lacking mGluR5 were slightly reduced at the holding potentials from -40 to +60 mV shown above, it was possible that NMDA channel function in mutant neurons was initially set at a depressed level prior to the tetanic stimulation and as a consequence of this set value, the threshold may be increased for LTP induction. If so, one would expect to see LTP_{NMDA} at saturating levels of tetanic stimulation. However, as shown in Figure 4.6, LTP_{NMDA} was not seen at all in neurons from mGluR5 mutant mice (16 neurons, n = 8 animal/genotype), even when the number of trains (100 Hz, 500 ms, 10s intervals) was increased to 4 (p < 0.05). Like LTP induction in wildtype slices, the induction of LTP_{AMPA} in mutants was blocked when D-AP5 (50 μM) was present during the tetanic stimulation (6 neurons, n=3 animals/genotype). These data indicate that the basal NMDA channel was functional in mutant mice, but was uncoupled from mGluR5 activation. In addition, to avoid saturation of responses we stimulated at 25% of the maximum NMDA EPSCs and always observed single component LTP_{AMPA} in response to tetanic stimulation in the mutants. Pre-synaptic function as measured by paired pulse facilitation was normal in the mutants.

4.2.E Impaired LTP_{NMDA} in response to ACPD in mGluR5 mutants

It has been shown that selective activation of mGluRs with ACPD can evoke a long lasting enhancement of synaptic transmission which was very similar to that following tetanic stimulation in the hippocampus (Bashir et al., 1993; Bortolotto & Collingridge, 1993; O'Connor, 1994). We find here that ACPD application to wildtype slices induced an initial
Figure 4.6 Saturating tetanus failed to induce LTP\textsubscript{NMDA} in mGluR5 mutants.

Trains of tetani (100 Hz, 500 ms) (arrows) were given to wildtype or mutant neurons in the presence of CNQX at 10-min intervals until saturation of LTP\textsubscript{NMDA} was obtained (n=8). The number of trains is indicated by the arrows. Stimulation intensity to evoke baseline NMDA EPSCs were set at 25-30% of the max. amplitude. The superimposed traces were obtained as indicated by the letters on the axis.
depression of both AMPA EPSCs and NMDA EPSCs, followed by a slowly rising potentiation of both components, which reached a peak amplitude about 15 min. following ACPD application and persisted for at least 30 min, without significant decay (6 neurons from 4 animals for each dose). At a concentration of 60 μM, the mean amplitudes of LTP_{NMDA} and LTP_{AMPA} at 30 min following ACPD application were measured at 160.4 ± 19.3% and 161.8 ± 21.2% respectively, in wildtype neurons (24 neurons from 12 mice) at a holding potential of -50 mV (Figure 4.7A). In mutants, we found only a slight initial depression of EPSCs and a single LTP_{AMPA}, at 156.7 ± 15.6% with no rebound of NMDA receptor-mediated EPSCs (105±3.3%) following 60 μM ACPD (Figure 4.7B; 25 neurons, 13 mice; p <.01). The responses of the initial depression and following potentiation to ACPD were never observed at a concentration of less than 40 μM in the mutants neurons (4 neurons from 3 mice/genotype).

4.2.F The involvement of PKC in the induction of LTP_{NMDA}

The signal downstream of mGluR5 could involve PKC, since PKC-mediated NMDAR phosphorylation is thought to be involved in LTP_{NMDA} induced by tetanic stimulation, or ACPD (Hu et al., 1987; Malinow et al., 1989; Aniksztejn et al., 1992). We investigated the synaptic response to PKC activation. A PKC stimulator,4β-phorbol-12,13-dibutyrate (PDBu, 20 μM) was bath applied before (Figure 4.8A; 16 neurons, 8 mice/genotype), or after tetanic stimulation (Figure 4.8B; 16 neurons, 8 mice/genotype). Prior potentiation by PDBu occluded subsequent potentiation of NMDA EPSCs by tetanus. Likewise, prior tetanus occluded subsequent PDBu induced potentiation. Therefore, tetanus and PDBu-induced LTP_{NMDA} may share similar pathways, but other explanations are also possible.
Figure 4.7 Impaired LTP$_{NMDA}$ in mGluR5 mutants responding to ACPD

A. Bath perfusion of 60 uM ACPD for 5 min evoked a large initial depression followed by a parallel long lasting potentiation of the dual components of EPSCs in +/+ mice (n=12).  
B. In the -/- mice, ACPD caused a small initial inhibition of EPSCs followed by a single LTP$_{AMPA}$ without LTP$_{NMDA}$.  
30 min after LTP induction, 50 uM AP5 was applied for 5 min to isolate the NMDA component (n=13).  
Superimposed traces were taken at the indicated points on the X axis.
This PDBu treatment completely restored the missing LTP\textsubscript{NMDA} in mutants. Since the concentration of phorbol 12-myristate 13-acetate (PMA) or 4-beta-phorbol-12,13-dibutyrate (PDBu) in the range 10 \(\mu\text{M}\) (Muller et al., 1988., Gustafsson et al., 1988., Parfitt and Madison, 1993., Staak et al., 1995) to 40\(\mu\text{M}\) (Zhao et al., 1994) were shown to cause long-lasting synaptic potentiation, 20 \(\mu\text{M}\) PDBu was used for my occlusion experiments. Since the effect of PDBu can be pre- or post-synaptic, or both, and since PDBu-induced synaptic facilitation appeared to differ from LTP (Muller et al., 1988., Gustafsson et al., 1988), we repeated PDBu application combined with post-synaptic injection of PKC peptide inhibitor (19-36), a potent pseudosubstrate blocker of PKC activity (PKCI) (Malinow, 1989; Hvalby et al., 1994). Since at the concentration of 50 \(\mu\text{M}\), PKC(19-36) inhibits the activity of CaMK-II (Hvalby et al., 1994), 20 \(\mu\text{M}\) PKC(19-36) was used in my experiments. The application of PKC(19-36) completely prevented the effect of PDBu on LTP\textsubscript{NMDA} in 4/4 CA1 neurons, but had no significant effect on LTP\textsubscript{AMPA}. Therefore, PKC activity is at least required for LTP\textsubscript{NMDA} induced by PDBu. It is reasonable to propose that differential modulation of LTP\textsubscript{AMPA} and LTP\textsubscript{NMDA} occurs. PKC, or one of its isoforms, may be involved in LTP\textsubscript{NMDA} expression, while other enzymes (i.e. \(\alpha\text{CaMKII}\)) or other isoforms of PKC could be responsible for LTP\textsubscript{AMPA}. The mutant mice lacking mGluR5 may not activate the PKC pathway required for LTP\textsubscript{NMDA}.

4.2.6 The lack of genetic background effects on the LTP phenotype

We believe the phenotype ascribed to the loss of mGluR5 cannot be accounted for by the differential inheritance of background genes because (1) we find no difference in LTP in the parental 129 and CD1 strains (Jia et al., 1996); (2) the phenotype of mutants was
Figure 4.8 PKC stimulation rescues the impaired LTP_{NMDA} in mGluR5 mutants.

A. Bath perfusion of 20 uM PDBu for 6 min. followed by tetanus (arrow) in wildtype (filled circles) and mutants (open circles) in the presence of 2 uM CNQX. The CNQX-resistant EPSCs was blocked by 50 uM AP5. B. Initial tetanus in wildtype (filled circles) and mutant (open circles) was follows by bath applied PDBu (20 uM) for 6 min.
observed in both 129 x CD1 and 129 x C57Bl/6 offspring (Table 1); (3) the backcrossing of our 129 chimera to the 129 strain showed the same results of selectively impaired LTP\textsubscript{NMAD} in the absence of mGluR5 (Table 1). Therefore, the phenotype is consistent across different genetic backgrounds.
4.3 DISCUSSION

$m\text{GluR}5$ IS REQUIRED FOR $\text{LTP}_{\text{NMDA}}$ INDUCTION, BUT

NOT FOR $\text{LTP}_{\text{AMPA}}$ INDUCTION
4.3.A mGluR5 is the dominant receptor responding to ACPD in CA1 region

The data show that mGluR5 deficient mice develop normally at a gross neuroanatomical level (Jia et al., 1998), but exhibit changes in the synaptic responses of hippocampal neurons which may be associated with deficits in spatial learning and memory (Lu et al., 1997). The responses of hippocampal neurons to group III agonists (L-AP4), or carbachol, which activates muscarinic acetylcholine receptors, were normal. However, the responses to ACPD in CA1 was dramatically reduced in mGluR5 mutants. In contrast, the ACPD effects were not altered in mGluR1 deficient mice (Aiba et al., 1994). Therefore, mGluR5 is perhaps the best candidate to fulfil the role of the ACPD-mediated responses since it is localized both pre- and postsynaptically at CA1 synapses (Romano et al., 1995). In contrast to the CA1 area, the response to ACPD at various concentrations in the perforant pathway of the dentate gyrus was indistinguishable between the wildtype and mGluR5 mutant mice. Since the depression effect of ACPD was completely blocked by addition of MCPG in both groups, it is reasonable to speculate that such an effect is mediated by mGluR1, mGluR2 or additional unknown members of Group I mGluRs. Analysis of mGluR1 and mGluR1/5 double deficient mice will be valuable in distinguishing these possibilities. The inhibitory ACPD action on mGluR5 may not be relevant to LTP, since LTP was decreased in the dentate gyrus, where mGluR5 did not seem to respond to ACPD.

4.3.B mGluR5 plays a role in NMDAR-dependent LTP
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The results also suggest that the mGluR5 receptor plays an important regulatory role in LTP in NMDA-dependent pathways in the hippocampus. Although it is generally agreed that the induction of LTP in both the CA1 region, and dentate gyrus, is dependent upon activation of NMDA receptors; whether or not mGluRs are also required in this process has been controversial (Bliss & Collingridge, 1993; Nakashiba, 1994). The pharmacological profile of the effects of various agonists and antagonists of mGluRs suggests Group I mGluRs (mGluR1 and mGluR5) are potential candidates for LTP modulation. Given the fact that mGluR1α is mainly expressed in the interneurons in the CA1 region of the hippocampus, but not in pyramidal neurons (Baude et al., 1993), its role as a postsynaptic modulator of CA1-LTP is not likely. Indeed, when the mGluR1 gene was mutated, LTP could still be elicited with full strength in NMDA-dependent pathways of the hippocampus but was impaired in NMDA independent LTP in the mossy fibre synapses on CA3 (Conquet et al., 1994; but see Aiba et al., 1994). Conquet obtained expected results but his mutant is more problematic than that reported by Aiba. For example, The mutant mice generated by Aiba were the true null mutations. Whereas, mGluR1 mutants from Conquet’s lab resulted from a mGluR1 N-terminal fusion with β-galactosidase. The mGluR1/galactosidase fusion could act in a dominant/negative fashion on some unknown pathways in LTP. While these studies indicate that mGluR1 does not need to be coactivated to induce, or modulate, LTP in either the CA1 area or dentate gyrus, it does not rule out the involvement of other members of the Group I family. The only other known member of Group I mGluRs, mGluR5, fits the pharmacological profile and is richly expressed in pyramidal neurons in the CA1 area. It was therefore the best candidate molecule to serve as a modulator in the induction of LTP in CA1 and indeed, our data shows partially reduced LTP in the absence of mGluR5.
Similar results were obtained at the perforant pathway in the dentate gyrus. The fact that the mossy fibre LTP was normal in the mGluR5 mutants, but severely diminished in the mGluR1 mutants, suggests that mGluR5 is important in specifically modulating NMDAR-dependent LTP, whereas mGluR1 specifically modulates NMDAR-independent LTP. The mechanism by which mGluR5 modulates LTP could involve its regulation of NMDAR function at the induction and/or expression level of LTP. Hence in mGluR5 mutant mice we saw a reduction in NMDAR mediated current in the CA1 area of the hippocampus. fEPSPs measured at 5-95% of the slope contained an NMDAR mediated component (15%) which was removed in AP5 (Figure 4.9). Therefore, about one half of the missing LTP in CA1 could lie at the level of LTP induction. The remainder could lie at the level of LTP expression, since addition of AP5 after tetanus reduced LTP and removed part of the difference in LTP between wildtype and mGluR5 mutants.

4.3.C mGluR5 is required for the induction of LTP_{NMDA}

Our finding that LTP of the NMDA receptor-mediated component of synaptic transmission is selectively eliminated in mGluR5 mutant mice suggests that activation of mGluR5 is normally required for the enhancement of the NMDAR component, but not the AMPAR component. This has several important implications in synaptic plasticity.

Although it is generally agreed that induction of LTP in the CA1 region of the hippocampus requires the activation of post-synaptic NMDARs, whether or not the expression phase of LTP is also expressed in the post-synaptic neuron, remains a matter of debate (Bliss and Collingridge 1993; Nicoll & Malenka, 1995; Kullmann & Siegelbaum, 1995). One approach that has been used to address the nature of LTP was to separately
Figure 4.9 The AP5-sensitive component in LTP maintaining phase in mGluR5 mutants

A. A plot of averaged normalized the peak of fEPSP from mGluR5 (+/+; n=12) and (-/-; n=14). B. A plot of averaged normalized initial slope (10-60%) of fEPSP from the same recording in A. C. A plot of averaged normalized peak of fEPSP from mGluR5 (+/+; n=8) and (-/-; n=10). 50 uM AP5 was applied after LTP induction. D. A plot averaged normalized initial slope of fEPSP from the same recording in C.
analyze the two components of the glutaminergic signal mediated by NMDARs and AMPARs. Differential enhancement of these two components would support a post-synaptic locus for LTP, whereas equal potentiation of both signals would argue for a pre-synaptic view of LTP (Kullmann and Siegelman 1995). Some studies have previously shown equal enhancement of NMDA EPSC and AMPA EPSC following tetanic stimulation (O'Connor et al, 1995; Clark & Collingridge 1995), whereas others showed only a small NMDA EPSC (Kullmann et al 1996; Xie et al 1992; Aniksztejn & Ben-Ari, 1995; Asztely et al 1992; Muller et al 1992; Tsien & Malinow, 1990) or no LTP NMDA (Perkel & Nicoll, 1993, Selig et al, 1995, Kauer et al., 1988). The reason for these discrepancies are not known, but clearly depend on the experimental conditions used to induce LTP. For example, strong stimulation favored the generation of LTP NMDA, whereas weak stimulation only generated LTP AMPA (Aniksztejn & Ben-Ari, 1995). The use of pairing procedures rather than tetanus probably also elicited LTP differentially. In the experiments described in the present paper we used a relatively strong stimulation protocol. Therefore, equal enhancement in both components after tetanus was obtained in wild type mice of several different genetic backgrounds (129, CD1, 129XCD1, 129XC57). A similar result was obtained with agonist, ACPD, application in wild type mice. However, in the absence of mGluR5, LTP NMDA was not observed in any neurons with either tetanic stimulation or ACPD application, whereas LTP AMPA remained intact. Therefore, activity of mGluR5 is required only for enhancement of NMDAR mediated signals during LTP. This differential utilization of mGluR5 may explain some of the discrepancies in obtaining LTP NMDA by other investigators, as described above. Since mGluR5 is localized perisynaptically (Lujan et al., 1996), activation of the receptors would be expected only when glutamate in the
synaptic cleft reaches a sufficiently high level, either by strong stimulation or by a reduction in glutamate uptake (Scanziani et al., 1997). Therefore, strong LTP inducing trains would lead to LTP\textsubscript{NMDA}, as well as LTP\textsubscript{AMPA}, as a result of the activation of mGluR5, whereas weaker stimuli, or pairing protocols, may not be sufficient to activate mGluR5. Therefore, only LTP\textsubscript{AMPA} was generated since expression of LTP\textsubscript{AMPA} does not depend on mGluR5. Our data would also explain why in a brief anoxic-aglycemic episode, LTP\textsubscript{NMDA} could be obtained much more readily, as excessive neurotransmitter release may have favoured the activation of mGluR5.

The selective loss of LTP\textsubscript{NMDA} in mGluR5 mutants would strongly support the hypothesis that the underlying mechanism for the expression of LTP\textsubscript{NMDA} and LTP\textsubscript{AMPA} are distinct. Therefore, post-synaptic neurons would be the predominant locus for LTP. We cannot rule out the possibility that, in mutants, the properties of NMDARs were altered in such a way that they now have a changed sensitivity to glutamate, and therefore, were not able to respond to increased neurotransmitter release during LTP. This seems unlikely since the NMDAR mediated current increased as the intensity of stimulation went up, and LTP\textsubscript{AMPA}, which requires activation of NMDAR, is normal in mutants.

Finally, our data elucidates the identity of a mGluR with respect to its role in modulating synaptic transmission and plasticity. This issue has also been controversial. While some studies using MCPG, a broadly selective antagonist, showed that co-activation of an unidentified mGluR may be necessary to induce LTP (Bashir et al., 1993, Bortolotto et al., 1994), others were not able to observe any effects of MCPG on LTP (Manzoni et al., 1993, Chinestra et al., 1993). Moreover, as there are no available subtype specific agonists or antagonists, the question of which one of the eight known
mGluRs is important cannot be answered pharmacologically. Although LTP\textsubscript{NMDA} was absent in mGluR5 \(-/-\) mice stimulated by ACPD application, LTP\textsubscript{AMPA} could still be elicited, suggesting that it is mediated by a separate mGluR. The nature of this subtype is currently unknown but analysis of various double or triple knockouts (ie. mGLuR5\textsubscript{X}mGluR1, mGluR5\textsubscript{X}mGluR4/2/7) would help to resolve this issue. It is also possible that the effect of ACPD in generating LTP\textsubscript{AMPA} is mediated by an unknown mGluR yet to be discovered (see review by Pin and Duvoisin, 1995, Conn and Pin, 1997).

4.3.D mGluR5 coupled PKC signaling pathways in LTP\textsubscript{NMDA} induction

The signal transduction pathway underlying potentiation of the NMDAR, as a result of the activation of mGluR5 is not completely defined in the present paper, but the activation of PKC may be required. PDBu induced LTP\textsubscript{NMDA} normally in mGluR5 \(-/-\), which suggests that the G-protein coupled signalling involved in the activation of PKC may not be altered in the mutants. In addition, augmentation of NMDA EPSCs by PDBu could be blocked by injecting a carefully selected dose of PKCl into the post-synaptic neuron (unpublished data). The blocked PKC was correlated with the lack of LTP\textsubscript{NMDA} and, therefore, it is reasonable to hypothesize that mGluR5 may be coupled to PKC activity, which in turn, is required for enhancement of the NMDAR component. Several other lines of evidence support a role for PKC in LTP. Post-synaptic injection of the selective 19-31 PKC inhibitory peptide blocked the induction of LTP in the hippocampus (Malinow, 1989; Malenka et al., 1989; O'Connor, 1995) and a PKC\(\gamma\) knockout also impaired LTP induction (Abeliovitch et al., 1993). Conversely, the injection of PKC into CA1 pyramidal neurons induced a kind of LTP (Hu et
PKC may enhance LTP by upregulating the function of the NMDAR (Ben-Ari et al., 1987). However, as the regulation of NMDARs can be very complex, potentially involving additional signaling pathways such as protein tyrosine kinases (Wang et al., 1994; Yu et al., 1997). Activation of an independent pathway could well compensate for the loss of the signal caused by lack of mGluR5. Whether or not the IP3 signal generated by the activation of mGluR5 is also important for LTP_{NMDA} is unclear. Dissecting the details of these components and how they are linked to the potentiation of the NMDAR will be the focus of future studies.

4.3.E mGluR5-mediated LTP_{NMDA} may underlie spatial learning and memory

If LTP is involved in learning and memory, then the mGluR5 mutants should show some impairment in learning and memory tasks, since hippocampal NMDAR-dependent LTP was significantly reduced in the mGluR5 mutant mice. Our results show that indeed mGluR5 mutant mice were significantly impaired in two different spatial learning tasks (see appendix B), which are known to depend on an intact hippocampus (Philips & LeDoux, 1992; Morris, 1990). In the water maze (Morris, 1990), subjects must actively escape from an aversive situation, while in contextual fear conditioning the subjects show passive fear response, (freezing) in the context in which they previously experienced shock (Philips & LeDoux, 1992). Each of the above paradigms include nonspatial control tests in which nonspatial learning was evaluated. mGluR5 mutants performance in all nonspatial control tests was comparable to control animals. Since we did not find any changes in motor and exploratory activities of mGluR5 mutants, the impairment in their spatial learning seems to be selective.
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The learning acquisition impairment in the water maze was confirmed in the probe trial. While controls showed persistent search for the platform, spent significantly longer time in the target quadrant and more often crossed the platform side, the mutants did not stay significantly longer in the target quadrant, and never persistently searched the area of the platform site. The longer latencies of mutants were not caused by their floating in water, inactivity near the wall, or thigmotaxic swimming along the wall. Also, their motivation, visual acuity, swimming abilities and learning of the association between a single cue and the platform position was the same as in control animals when the platform was marked by a flag. Mutants also showed impairment in the fear conditioning test, which requires the association of background contextual information with the US, but showed normal learning of association between a tone (CS) and a shock (US). Animals from both groups explored a shock chamber during training at the similar rate and responded identically to US. Our results are compatible with the injection of the Class I mGluR5 antagonist, MCPG into rats, which reduced spatial learning; whereas a Class I agonist applied after learning facilitated memory recall (Riedel et al., 1996).

A variety of other studies have tried to test correlation between LTP and learning and memory (Martinez & Derrick, 1996). Pharmacological, physiological and surgical perturbations have been performed, but the results are inconclusive. However, all genetic disruptions (9/9) that impaired spatial learning and memory also impaired LTP in the Schaeffer collateral pathway to CA1. This includes the genes for fyn (Grant et al., 1992); αCaMKII (Silva et al., 1992); CREB αδ (Bourtchuladze et al., 1994); PKCγ (Abeliovitch et al., 1993); NMDAR2A (Sakimura et al., 1995); CaMKII-D286 (Bach et al., 1995 and Mayford et al., 1996); calbindin D-28 (Molinari et al., 1996); and NMDAR1 (Tsien et al.,
Gene deletion systems that excised the NMDAR1 gene only in the CA1 region, several weeks after birth, also yielded mice that showed impaired LTP and spatial learning and memory (Tsien et al., 1996). Conversely, gene disruptions that did not impair spatial learning and memory did not alter the Schaeffer collateral pathway in the spatial LTP on CA1 cells (Huang et al., 1995; Nosten-Bertrand et al., 1996). This emphasizes the importance of the Schaeffer collateral pathway in learning and memory. Two studies tended to dissociate LTP and learning and memory in the perforant path and mossy fibre pathways of the hippocampus. The disruption of the thy-1 gene disrupted granule cell LTP in the perforant pathway of the hippocampus (Nosten-Bertrand et al., 1996), whereas learning and memory were normal. Disruption of PKA selectively decreased CA3 LTP in the mossy fibre pathway, but learning and memory was also normal (Huang et al., 1995). The above evidence and our own data here in which mGluR5 mutants showed normal mossy fibre LTP, but decreased spatial learning, provides a double dissociation between them. Therefore, the best remaining correlation between LTP and learning and memory resides in the CA1 region. In the absence of perforant paths, or mossy fibre LTP in the system, information relevant to spatial learning could reach CA1 directly from the entrohinal cortex (Huang, 1995).

These genetic connections between LTP and some forms of learning and memory are also supported by a locally applied NMDAR antagonist (AP5), which blocked induction of LTP in hippocampus, and appeared to block "spatial" learning but not a simple visual association task (Davis, 1992; Morris, 1990). However, more recent studies show that AP5 only impairs spatial learning in task-naive animals, while subjects pre-trained in a spatial task resist this inhibition (Bannerman, 1995). Since both NMDA antagonists AP5 and NPC
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17742, blocked LTP but failed to block spatial learning in pre-trained rats (Saucier, 1995), the relationship between LTP and spatial learning is not direct. Whether or not NMDAR is necessary for learning spatial strategies, or simply refining motor skills rather than spatial maps, requires more work.

4.3.F Summary

We made use of a novel mutant strain deficient in expression of mGluR5 but normal in the expression of other glutamate receptors. Analysis of the mutant mice showed that mGluR5 is not required for the development of the CNS. A first study analyzing field potentials (fEPSP) in these mice showed a small decrease in the induction of LTP in CA1 and the dentate gyrus but normal induction in the CA3 region of the hippocampus (Lu et al., 1997). The difference between genotypes was abrogated by adding AP5 after the induction of LTP (Lu et al., 1997). We show next using voltage clamp analysis that the expression phase of LTP<sub>NMDA</sub> was completely lost, whereas LTP<sub>AMPA</sub> was normal. The selective loss of LTP<sub>NMDA</sub> in mGluR5 <sup>−/−</sup> mice, suggests that distinct mechanisms must exist in CA1 neurons for LTP<sub>NMDA</sub> and LTP<sub>AMPA</sub> components of LTP. Therefore, these mutant mice provide a valuable new tool to dissect the various elements which are differentially used during synaptic plasticity in the mammalian CNS. In addition, the mice show a selective loss in two tasks of spatial learning and memory which supports the role of CA1 in this process as well (see appendix B).
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UMI
CHAPTER FIVE

ENDOGENOUS Zn\(^{2+}\) IS SPECIFICALLY REQUIRED FOR THE INDUCTION OF NMDAR-INDEPENDENT LTP

This work was submitted as:


The work in all figures (Figure 5.2., 5.3., 5.4., 5.5., 5.6., 5.7., 5.8., 5.9., 5.10., 5.11) in this chapter was performed by me. Zn\(^{2+}\) fluorescence staining in Figure 5.1 was carried out by Franco Taverna.
5.1 INTRODUCTION

In the central nervous system, chelatable Zn$^{2+}$ is most abundantly localized in the vesicles of hippocampal mossy fiber synaptic boutons (Danscher et al. 1985; Frederickson, 1989; Frederickson & Moncrieff, 1994; Frederickson et al. 1982; Haug, 1967), and is released with glutamate upon the stimulation of presynaptic neurons (Assaf & Chung, 1984; Aniksztejn et al. 1987; Charton et al. 1985). The concentration of free extracellular Zn$^{2+}$ at synapses in the CA3 subregion of the hippocampus is estimated to be as high as 300 μM; compared to 20 μM in other regions (Harrison & Gibbons, 1994; Xie & Smart, 1991). Rats, fed a Zn$^{2+}$ deficient diet, showed a normal basal mossy fiber-CA3 synaptic transmission, but had an impairment of high frequency-induced mossy fiber-CA3 synaptic response (Hesse, 1979) indicating that Zn$^{2+}$ participates in some aspect of synaptic activity. Zn$^{2+}$ (50 μM) blocks N-methyl-D-aspartate (NMDA) receptor channels and enhances α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor-mediated currents in neuronal cultures (Christine & Choi, 1990; Mayer & Vyklicky, 1989; Mayer et al. 1989; Peters et al. 1987; Rassendren et al. 1990; Westbrook & Mayer, 1987). These studies suggest that endogenous Zn$^{2+}$ may be involved in modulating the efficacy of the glutamate-mediated synaptic transmission, but its role in synaptic plasticity is unknown.

LTP in the hippocampus is believed to be a possible molecular substrate for learning and memory (Bliss & Collingridge, 1993; Bliss & Lynch, 1988; Gustafsson & Wigstrom, 1988; Malenka & Nicoll, 1993). In the CA1 region, LTP at Schaffer collateral synapses is induced postsynaptically through activation of NMDA receptors (Bliss & Lynch, 1988; Collingridge & Singer, 1990). In contrast, at mossy fiber-CA3 synapses, a
A distinct type of LTP occurs that is independent of NMDA receptor activation and is believed to be a stable potentiation of the presynaptic elements (Staubli et al. 1990; Weisskopf et al. 1994; Zalutsky & Nicoll, 1990). In the classic model for mossy-fiber LTP, tetanic stimulation causes Ca\(^{2+}\) accumulation in presynaptic terminals and subsequently activates a Ca\(^{2+}\)/calmodulin-sensitive adenylate cyclase (Tong et al. 1996). The participation of the endogenous Zn\(^{2+}\) in this process is not known. Therefore, I investigated LTP in the hippocampal slices from Zn\(^{2+}\)-deficient rats or in the normal rat hippocampal slices treated with the membrane-permeable Zn\(^{2+}\) chelators, diphenylthiocarbazone (dithizone) or diethylthiocarbamate (DEDTC), as well as the membrane-impermeable Zn\(^{2+}\) chelator, Ca\(^{2+}\)EDTA. Those agents have been shown to selectively bind Zn\(^{2+}\), and remove free Zn\(^{2+}\) from mossy fiber presynaptic boutons, with little effect on other ions such as Ca\(^{2+}\) and Mg\(^{2+}\) (Koh et al. 1996; Frederickson et al. 1988).
5.2 RESULTS

Presynaptic $\text{Zn}^{2+}$ is necessary for the induction of LTP at mossy fiber-CA3 synapses
5.2.A The induction of the mossy-fiber LTP was selectively impaired in Zn\(^{2+}\)-deficient rats

Staining of rat hippocampal slices with the Zn\(^{2+}\)-specific fluorescent dye N-(6-methoxy-8-quinolyl)-para-toluenesulfonamide (TSQ) exhibited that dense fluorescence was relatively appeared in the mossy fiber presynaptic terminals at the dentate granule cells in the hilus and CA3 regions (Figure 5.1A). A chronic dietary deficiency of Zn\(^{2+}\) resulted in depletion of Zn\(^{2+}\)-fluorescence from the mossy fibers (Figure 5.1B). The Zn\(^{2+}\)-deficient rats with dietary Zn\(^{2+}\) supplements for 48 hours after Zn\(^{2+}\) deprivation revealed that Zn\(^{2+}\) fluorescence in presynaptic fibers returned to normal (data not shown). Figure 5.1C showed that in the presence of dithizone (100 \(\mu\)M for 5 min), Zn\(^{2+}\) fluorescence was removed.

To examine the possible consequences of Zn\(^{2+}\) deprivation in the mossy fiber terminals, I studied the ability of both the mossy fiber- and the commissual fiber-CA3 synapses to undergo LTP in Zn\(^{2+}\)-deficient rats (Figure 5.2A). In this series of experiments fEPSP were measured at the initial slope (10-60%) of the peak, and LTP was induced by one train of tetanus. In control rats, the tetanic stimulation produced fEPSP potentiation. Although the degree of potentiation varied among control slices, the enhancement of fEPSP was clearly evident. The averaged normalized the fEPSP slope 30 min. after tetanic stimulation was 209±28% of baseline and the potentiation was stable for at least one hour (Figure 5.2A). In Zn\(^{2+}\)-deficient rats, however, only in 2 (2 rats) of the 12 slices (12 rats) did tetanic stimulation produce fEPSP potentiation. Although there was an initial small post-tetanic potentiation (PTP) in other 10 rats, which decayed to the baseline within 5 min, the averaged normalized value of fEPSP slope 30 min. after tetanic stimulation was 118±12% of baseline (Figure 5.2A, n=12). The Zn\(^{2+}\)-deficient rats fed
Figure 5.1 Staining of hippocampal slices with $\text{Zn}^{2+}$-fluorescent chelator TSQ

A. Fluorescent photomicrograph of normal rat hippocampus after staining with TSQ, showing dense fluorescence in the hilus and the stratum lucidum of the CA3 and dentate gyrus. Little $\text{Zn}^{2+}$ fluorescence is observed in the stratum pyramidal of CA1.

B. The slice was treated by dithizone (100 µM for 5 min). $\text{Zn}^{2+}$ fluorescence is largely reduced at both CA3 and dentate gyrus.

C. A hippocampal slice from $\text{Zn}^{2+}$-deficient rat. The dense of TSQ staining at CA3 and dentate gyrus is largely reduced.
Figure 5.2 Impaired LTP at mossy fiber-CA3 synapses in Zn$^{2+}$ deficient rats

A  The mossy fiber-CA3 LTP

B  The commisural-CA3 LTP

The mean ± SEM of the initial slope of the fEPSP at the mossy fiber-CA3 synapses (A), and commisural-CA3 pathways (B) of the hippocampal slices, obtained from control (*, n=10), Zn$^{2+}$-deficient rats (○, n=12), or the deficient rats with Zn$^{2+}$ supplementation (△, n=6).
with dietary Zn^{2+} supplements rescued the deficits of LTP, and returned LTP to normal levels. The mean amplitude of fEPSP slope 30 min. after tetanus was 187±19% of the baseline (n=9, p=0.1, compared to control, Figure 5.2A).

However, when LTP at the commisural fiber-CA3 synapse was compared between the Zn^{2+}-deficient rats and the controls, we could not detect any significant difference in either magnitude or time course (Figure 5.2B). The averaged commisural fiber LTP from 14 slices (8 rats) was 174±21% for Zn^{2+}-deficient rats and 181±19% (12 slice from 8 rats) for controls (p>0.1).

5.2.B The chelation of Zn^{2+} blocked LTP induction in Zn^{2+}-containing mossy fiber-CA3 synapses

To further determine the necessary of endogenous Zn^{2+} in the induction of mossy fiber LTP, LTP was analyzed using voltage clamp recordings in mossy fibre-CA3 synapses of control rat hippocampal slices in bath application of the membrane-permeable Zn^{2+} chelator, dithizone, or control solution. The mean amplitude of excitatory postsynaptic currents (EPSCs) 30 min. after tetanic stimulation was 194±22% of baseline in control and the potentiation was stable throughout the recording session. (Figure 5.3A). The application of dithizone (100 μM, 30 min.) completely blocked the induction of LTP in each of the six neurons tested (Figure 5.3B), although there was an initial small post-tetanic potentiation (PTP), which decayed to the baseline within 10 min. The mean amplitude of EPSCs 30 min. after tetanus was 108 ± 14% of baseline in dithizone-treated slices. Lower concentrations of dithizone (60 μM, 30 min.) also blocked LTP induction,
Figure 5.3 The chelation of Zn$^{2+}$ blocked tetanus-induced mossy fiber LTP

A. A plot of the averaged normalized initial slope of mossy fiber-CA3 EPSCs, and error bars are SEM (n=5). Tetanic stimulation was applied in the presence of control solution. B. A plot of the averaged normalized initial slope of mossy fiber-CA3 EPSCs, and error bars are SEM (n=6). The tetanic stimulation was applied in the presence of 100 μM dithizone.

The superimposed traces (EPSCs and the post-pulse by the voltage steps) before (a) and after (b) tetanus were the averages of 6 sweeps.
with at higher concentrations (200 μM) the blocking effect was more rapid (10 min., data not shown). To determine if dithizone could act on the maintenance of LTP, it was applied after LTP was induced. Application of 100 μM dithizone for 30 min. after the induction of LTP did not affect the established potentiation (199±20% of baseline, n=6, p>0.1, Figure 5.4A). The concentrations of 100 μM dithizone was sufficient to remove Zn$^{2+}$ from the mossy fiber zone (Danscher and Haug, 1971., Lenglet et al., 1984), and had little effect on Ca$^{2+}$ and Mg$^{2+}$ (Koh et al., 1996., Frederickson et al., 1988). This was confirmed in my own experiment in which 100 μM dithizone removed Zn$^{2+}$-TSQ fluorescence in the mossy fiber system of the hippocampus (Figure 5.1).

To support the idea that LTP blockade by dithizone was due to the chelation of Zn$^{2+}$, another Zn$^{2+}$ chelator, diethyldithiocarbamic acid (DEPTC) (Xu and Mitchell, 1993) was tested. Figure 5.4B shows that bath application of DEPTC (200 μM, 30 min.) also completely blocked the induction of LTP at mossy fiber-CA3 synapses. The averaged amplitude of EPSCs 30 min. after tetanus was 101±12% (n= 5) of baseline.

5.2.C Dithizone had no effect on LTP induction at two non-Zn$^{2+}$ containing synapses

To determine whether dithizone blocking of LTP was specific for mossy fiber-CA3 synapses, we studied LTP induction, in the presence of dithizone, at two non-Zn$^{2+}$ containing pathways. First, we recorded LTP at the associational-commisural-CA3 synapses, which do not contain a significant quantity of Zn$^{2+}$ at the EM level (Haug, 1967; Frederickson, 1989). LTP induction was not blocked by dithizone in this synapse.
Figure 5.4 Dithizone had no effect on the expression of LTP

A. A plot of the averaged normalized initial slope of mossy fiber-CA3 EPSCs (n=6). 100 uM dithizone was bath applied immediately after tetanus. B. A plot of the averaged normalized initial slope of mossy fiber-CA3 EPSCs (n=5). Tetanic stimulation was applied in the presence of DEPTC. The superimposed traces EPSCs and the post-pulse by the voltage steps) before (a) and after (b) tetanus were the averages of 6 sweeps.
Figure 5.5 Dithizone caused no change of LTP at non-\(\text{Zn}^{2+}\)-containing synapses

A. A plot of the averaged normalized associational commisural-CA3 EPSC slope, and the tetanus was applied in the presence of the control solution (n=6) or in the presence of dithizone (n=6). B. A plot of the averaged normalized Schaffer collateral-CA1 EPSC slope. The tetanus was applied in the presence of control solution (n=5) or in the presence of dithizone (n=6). The traces of single recording were before (a) and after (b) tetanus.
The averaged amplitude of EPSCs, 30 min. after tetanus, was 182 ± 21% of baseline (n=6) for control and 193 ± 20% in the presence of 100 μM dithizone for 30 min. (n=6, p>0.1, Figure 5.5A). Next, we recorded LTP at the Schaffer-collateral CA1 synapse. Again, application of 100 μM dithizone for 30 min. failed to change the induction of LTP at this synapse. The averaged LTP in dithizone treated slices (174±21%, n=6) was not different from that in control (168±16%, n=5, p>0.1, Figure 5.5B). These findings indicate that dithizone alters synaptic efficacy only at Zn$^{2+}$-containing synapses.

5.2.D Dithizone action was reversible

Mossy fiber-CA3 field EPSP was recorded from hippocampal slices. In the absence of dithizone, tetanic stimulation caused long lasting potentiation of the initial slope of field EPSP. The averaged normalized EPSP slope was 209±22% of the baseline (Figure 5.6A, n=8). To test the reversibility of dithizone action on mossy fiber LTP, hippocampal slice was treated with 100 μM dithizone for 30 min., and then continuously washed out with control solution. LTP inducing stimulation was then delivered at 60-min. interval after 30-min. dithizone. As shown in Figure 5.6B a 3-hour washing out was necessary to restore the capacity of the dithizone-treated slices for the induction of mossy-fiber LTP. The averaged normalized initial slope of mossy fiber-CA3 field EPSP was 203±22% of the baseline (n=6), 30 min. after the fourth tetanus. To determine whether exogenous Zn$^{2+}$ could reverse the dithizone action on mossy-fiber LTP, 100 μM Zn$^{2+}$ was applied for 30 min. immediately following dithizone. Figure 5.7 showed that the normal LTP (216 ± 24%, n=6) was induced by tetanus immediately after the addition of Zn$^{2+}$. These
Figure 5.6 Dithizone action was reversible

A. A plot of the averaged normalized mossy fiber-CA3 field EPSP slope (n=8). Tetanus was applied in the presence of control buffer. B. A plot of the averaged normalized mossy fiber-CA3 field EPSP slope (n=6). The first tetanus (arrow) was applied in the presence of dithizone. The following tetani were given in control buffer. The traces in graph are the averages of 2 recordings taken at the time point by the letters below the axis.
Figure 5.7 Dithizone action was counteracted by Zn$^{2+}$

A plot of the averaged normalized mossy fiber-CA3 field EPSP slope (n=6). The first tetanus was applied in the presence of dithizone. The second tetanus (arrow) was given after 30-min application of 100 uM Zn$^{2+}$. The traces in the graph are the averages of 2 recordings taken at the time point by the letters below the axis.
results strengthen the hypothesis that dithizone acts on the induction of LTP by a selective chelation of the synaptically localized Zn$^{2+}$.

5.2. E Intracellular or bath application of Ca$^{2+}$EDTA caused no change in mossy fiber LTP

The resting level of intracellular free ([Zn$^{2+}$]i) was estimated to be as low as 3 nM (Kalfakakou & Simons, 1990), while stimulation of neuronal cultures resulted in a sustained increase in [Zn$^{2+}$]i, reaching about 50 nM and lasting a few minutes (Sensi et al. 1997). To determine whether excessive Zn$^{2+}$ released into the postsynaptic neurons could be responsible for the mossy fiber LTP induction, we made use of a cell membrane-impermeable Zn$^{2+}$ chelator, Ca$^{2+}$EDTA, which is known to selectively block Zn$^{2+}$ function (Koh et al. 1996). 1 mM Ca$^{2+}$EDTA was applied directly into the neurons by diffusional exchange from the patch electrode (Figure 5.10A). During application of Ca$^{2+}$EDTA, tetanic stimulation caused normal levels of both post-tetanic potentiation (PTP), and long-lasting potentiation of the mossy fiber-CA3 EPSCs. The amplitude of EPSCs was $208 \pm 23\%$ (mean $\pm$ SEM, n=7 patches) of the baseline level by 30 min. after tetanic stimulation. Thus, Zn$^{2+}$ in postsynaptic neurons was not necessary for the induction of mossy fiber LTP.

To test whether Zn$^{2+}$ released into the synaptic cleft does not itself affect LTP, 1 mM Ca$^{2+}$EDTA was applied in the bath. As shown in Figure 5.10B, bath administration of Ca$^{2+}$EDTA produced no change in the behaviors of mossy fiber LTP. The averaged LTP was $196 \pm 23\%$ (mean $\pm$ SEM, n=6 patches), which was not different from LTP in the control group.
Figure 5.8 Ca^{2+}EDTA had no effect on mossy fiber-CA3 LTP

A. A plot of the averaged normalized initial slope of mossy fiber-CA3 EPSC with intracellular administration of 1 mM Ca^{2+}EDTA in CA3 cells (n=7). B. A plot of the averaged normalized initial slope of mossy fiber-CA3 EPSCs following bath application of 1 mM Ca^{2+}EDTA (n=6). Traces of single recording were before (0) and after (1) tetanus at the holding potential -70 mV.
5.2.F Bath application of Ca$^{2+}$EDTA potentiated NMDA conductance

The failure of Ca$^{2+}$EDTA above to effect on mossy fiber LTP could arise from the failure of Ca$^{2+}$EDTA to sufficiently bind endogenous Zn$^{2+}$. To rule out this possibility, NMDA receptor mediated synaptic currents (NMDA EPSCs) were monitored. It is reasonable to expect that NMDA EPSCs would be enhanced if endogenous Zn$^{2+}$ could be chelated, because Zn$^{2+}$ blocks NMDA channels in neuronal cultures at a concentration of 30 to 50 μM (Peters et al. 1987; Westbrook & Mayer, 1987), and because endogenous Zn$^{2+}$ in the CA3 region of hippocampus is estimated to be as high as 100 - 300 μM (Harrison & Gibbons, 1994; Xie & Smart 1991). In mossy fiber-CA3 synapses, the mean amplitude of NMDA EPSCs 10 min. after 1 mM Ca$^{2+}$EDTA was increased by 36 ± 6% (mean ± SEM, n=5) relative to the mean value 10 min. prior to Ca$^{2+}$EDTA application (data not shown). Ca$^{2+}$EDTA-induced increase of NMDA EPSCs was observed over a range of -80 to +60 mV with no change in the reversal potential (Figure 5.11A, n=4). In contrast, the efficacy of AMPA receptor-mediated EPSCs (AMPA EPSCs) were not changed, with application of Ca$^{2+}$EDTA (Figure 5.11B, n=4). The data suggest that bath application of Ca$^{2+}$EDTA can remove Zn$^{2+}$ in the synaptic cleft. The results are consistent with previous findings showing synaptically recorded AMPA EPSCs in the hippocampus were not significantly altered by Zn$^{2+}$, at concentrations that blocked NMDA EPSCs (Harrison & Gibbons, 1994).
Figure 5.9 Ca\textsuperscript{2+}EDTA potentiated NMDA conductance, but not AMPA

A. I-V curves for averaged amplitudes of NMDA EPSCs 5 min. before and immediately after bath application of CaEDTA (n=5). Traces from a cell without (solid line) or with CaEDTA (dotted line) and in the presence of 10 uM CNQX. Each trace is the average of 6 NMDA EPSCs evoked at potentials at -20 mV (a), +40 mV (b), and +80 mV (c).

B. I-V curves for averaged amplitudes of AMPA EPSCs 5 min. before and immediately after bath application of CaEDTA (n=4). Traces from a cell without (solid line) or with CaEDTA (dotted line). Each trace is the average of 6 NMDA EPSCs evoked at potentials at -80 mV (a), -40 mV (b), and +20 mV (c), and +40 mV (d).
5.3 DISCUSSION

ROLE OF Zn\(^{2+}\) IN THE INDUCTION OF LTP
5.3.A Endogenous Zn$^{2+}$ is specifically required for the induction of the mossy fiber LTP

My data provide compelling evidence, for the first time, that endogenous Zn$^{2+}$ is necessary for the induction of LTP at the Zn$^{2+}$-containing synapses of the hippocampus. Four observations support this idea. First, a one-to-one correlation between LTP deficits and Zn$^{2+}$ depletion in Zn$^{2+}$ deficient rats was observed at the mossy fiber synapses. Second, the block of mossy-fiber LTP by the membrane-permeable chelators was related to the removal of endogenous Zn$^{2+}$, since the blocking was time- and concentration-dependent. Third, the blocking effect was reversible, and was overcome by application of exogenous Zn$^{2+}$. Finally, the blocking was specific since dithizone blocked LTP only at Zn$^{2+}$-containing mossy fiber synapses and not at other synapses in the hippocampus which lack Zn$^{2+}$.

Our finding that Zn$^{2+}$ chelators block LTP in the mossy fiber pathway is consistent with earlier work on tissue specificity. Evoked Zn$^{2+}$ release from hippocampal slices was found specifically in the mossy fiber zone and was associated with depletion of mossy fiber Timm’s staining (Charton et al. 1985; Aniksztejn et al. 1987). Our studies with chelators took advantage of the fact these chelators bind with Zn$^{2+}$ ions, and block the development of a typical Timm pattern (Otsuka et al. 1975; Frederickson et al. 1987). The absence of endogenous Zn$^{2+}$ was verified using the Zn$^{2+}$-selective fluorescence marker, TSQ, for visualizing vesicular Zn$^{2+}$ (Frederickson et al., 1988). This direct fluorescence technique exhibited a specific pattern of Zn$^{2+}$ loss in dithizone-treated hippocampal slices, identical to that observed in the slices from Zn$^{2+}$-deficient rats.
Although Zn\(^{2+}\) chelators used here are not specific for Zn\(^{2+}\) and can also chelate endogenous Cu\(^{2+}\) and Fe\(^{2+}\), the levels of these metals were normal in Zn\(^{2+}\)-deficient rats, therefore, the effects of these endogenous metals on mossy fiber LTP can be excluded.

5.3.B Selective impairment of the mossy fiber LTP in Zn\(^{2+}\) deficient rats is due to the absence of endogenous Zn\(^{2+}\) in neurotransmitter vesicles

The impairment of the mossy fiber LTP in Zn\(^{2+}\)-deficient rats could result from structural injury within dentate granule cell axons, such as aberrant axonogenesis or abnormal formation of the glutamate vesicles, rather than a specific loss of the endogenous Zn\(^{2+}\) in mossy fiber boutons. Electron micrographs of dentate granule cells revealed normal ultrastructural integrity of the mossy fiber pathway in Zn\(^{2+}\)-deficient rats. A axonal tracing exhibited no abnormalities in the density and trajectory of the mossy fiber bundle in Zn\(^{2+}\)-deficient rats. In addition, Zn\(^{2+}\)-deficient rats demonstrated no abnormalities in basal synaptic transmission. Stimulus-response curves obtained from Zn\(^{2+}\)-deficient rats and controls were not significantly different, and the slope of a fEPSP elicited by a given presynaptic fiber volley did not differ between two groups (data not shown). Thus, the deficits in the mossy fiber LTP is due to the impairment of the events during LTP induction in Zn\(^{2+}\)-deficient rats.

5.3.C A possible model for Zn\(^{2+}\) action on the mossy fiber LTP

Much evidence has accumulated that the induction of mossy fiber LTP requires a rise of presynaptic Ca\(^{2+}\). Our data is consistent with a presynaptic expression mechanism (Nicoll
and showed that mossy-fiber LTP was accompanied by a large increase in the release frequency. Dithizone abolished the tetanus-induced increase in the release of quanta, indicating that the locus of dithizone action might be the presynaptic terminals (data not shown). There is, however, an alternative interpretation for the increase in quantal content with LTP: rather than indicating an increase in the number of quanta of transmitter release, it reflects a postsynaptic uncovering of clusters of silent AMPARs (Edwards, 1991). However, the presynaptic hypothesis was further supported by the observations that application of membrane-impermeable Zn$^{2+}$ chelator, Ca$^{2+}$EDTA, into the postsynaptic neurons did not affect on the induction of mossy fiber LTP. Since Ca$^{2+}$EDTA was also ineffective when applied in the bath, Zn$^{2+}$ must be exerting its effect within the presynaptic terminals rather than in the synaptic cleft where it is released by activity.

The cellular signalling events in the terminals that lead to the induction of mossy fiber LTP could include the activation of Ca$^{2+}$/calmodulin-sensitive adenylate cyclase, which leads to a long-lasting increase in transmitter release (Tong et al. 1996). Here, we performed experiments to provide direct evidence that endogenous Zn$^{2+}$ in mossy fiber boutons mediates mossy fiber LTP induction. How could Zn$^{2+}$ act? Zn$^{2+}$ was proposed to be a stabilizer of granule contents, such as insulin, in the secretory granules of pancreatic β-cells (Emdin et al. 1980; Zalewski et al. 1994), or nerve growth factor (NGF) in the secretory granules of salivary gland cells (Pattison & Dunn, 1976). From this point of view, the role of bouton Zn$^{2+}$ in mossy fibers may function as a packaging agent to stabilize the co-localized neurotransmitter, glutamate. However, since Zn$^{2+}$-deficient rats
revealed apparently normal glutamate vesicles in mossy fiber boutons. It would be appear that endogenous \( \text{Zn}^2+ \) is not necessary for the bouton vesicle formation at CA3 synapses.

Since the induction of LTP involves the activities of several protein kinases and enzymatic processes (Nicoll and Malenka, 1995), and since \( \text{Zn}^2+ \) participates in the functional activities of many enzymes (Berg & Shi, 1996), presynaptic bouton \( \text{Zn}^2+ \) might be involved in the protein kinase-mediated cellular signaling cascades underlying mossy fiber LTP induction.

5.3.D The removal of the endogenous \( \text{Zn}^2+ \) could in theory induce NMDAR-dependent LTP at mossy fiber-CA3 synapse

LTP has been extensively described at the CA3 and the CA1 regions of the hippocampus (Nicoll & Malenka, 1995; Williams & Johnston, 1988). It is well known that blockade of NMDA receptors prevents the induction of LTP in both the CA1 and the associational commisural-CA3 synapses, but is without effect on mossy-fiber LTP (Lynch et al. 1983; Malenka et al. 1988). A striking feature of the NMDA receptor is that, in contrast to most other ion channels, it is a ligand- and voltage-gated channel. Opening of the channel requires the binding of glutamate as well as a depolarization of the membrane to remove the block \( \text{Mg}^2+ \) from NMDA channels (Kleckner & Dingledine, 1988; Nowak et al. 1984). In addition, NMDA receptors are blocked by \( \text{Zn}^2+ \) in a voltage-independent manner (Christine & Choi, 1990; Peters, 1987; Rassendren et al. 1990; Westbrook & Mayer, 1987). As endogenous \( \text{Zn}^2+ \) is highly concentrated in mossy fiber–CA3 synapses (Danscher et al. 1985; Frederickson, 1989; Frederickson & Moncrieff, 1994; Frederickson, 1982; Haug, 1967), it is possible that the chelation of endogenous \( \text{Zn}^2+ \)}
would relieve Zn$^{2+}$ block from the NMDA channels, and lead to a form of NMDA receptor-dependent LTP at the Zn$^{2+}$-containing mossy fiber synapses. This hypothesis, however, cannot be directly tested because mossy-fiber LTP is classically measured in the presence of NMDA receptor antagonist to prevent the contamination with an NMDA receptor-dependent associational commisural pathway converging on CA3 neurons.

Zn$^{2+}$ is required by many cellular biological events (Vallee & Falchuk, 1993), and plays a critical role in the control of the gene transcription and metalloenzyme functions (Berg & Shi, 1996). The central effects of Zn$^{2+}$ deficiency are often manifested in behavioral disorders, such as learning deficits (Hurley & Shrader, 1972). On the other hand, a surfeit of Zn$^{2+}$ during development may contribute to other disorders (Dvergsten et al. 1983). My results demonstrated a new role of Zn$^{2+}$ in synaptic plasticity and may provide the common cellular basis for some of its pathological and physiological functions.
CHAPTER SIX

THE MAJOR FINDINGS, FURTHER DIRECTIONS AND NEW QUESTIONS ARISING FROM THIS THESIS
6.1 The initial biochemical events which lay the groundwork for the induction of LTP

6.1.A Introduction

In reflecting on what my observations have added to the studies of the initial biochemical events underlying two components of LTP in hippocampal CA1 synapses, it seemed worthwhile to bring together my findings concerning the role of Src kinase and mGluR5-coupled PKC in the induction of LTP and the evidence from others in the field concerning protein signaling pathways in LTP. Thus, I undertook to synthesize a conceptual framework which can be used to summarize the main results of this thesis. This conceptual framework was developed in the form of a model for cellular signaling events in the induction of LTP. In my view, the model provides a good explanation for the initial biochemical events in LTP consistent with the available evidence. The model could be seen as both a hypothesis and a summary current observations.

The core of the model is represented in Figure 6.1 which shows the most parsimonious view of the possible contributions of Src kinase and mGluR5-coupled PKC to two component of LTP. It is possible to incorporate the role of presynaptic Zn\(^{2+}\) in the induction of mossy fiber LTP into the model using the results and conclusions in Chapter 5. However, for the sake of clarity the Zn\(^{2+}\) findings will not be discussed here.

6.1 B A proposed model for the role of Src and mGluR5-coupled PKC in LTP
Figure 6.1 A new model for the biochemical events in LTP Induction

Tetanic stimulation causes a rise in postsynaptic neuron $[\text{Ca}^{2+}]_i$. This transient rise in $[\text{Ca}^{2+}]_i$ stimulates Pyk-2, which in turn binds to SH2 domain of Src and activates Src. The activation of Src up-regulates NMDA channel activities, and causes a "strong" Ca$^{2+}$ entry. This "strong" Ca$^{2+}$ with calmodulin (CaM) stimulates CaMK-II, which phosphorylates and then up-regulates the function of AMPAR. Differing from the expression of AMPAR-mediated component of LTP, the expression of NMDAR-mediated component of LTP could result from the activation of mGluR5-coupled PKC pathway. Activation of PKC causes a persistent enhancement of NMDAR function.
The model for the initial biochemical events in LTP is based on the following earlier observations:

- **Involvement of glutamate receptors in LTP.** Two main subtypes of glutamate receptors are involved in the LTP induction at CA1 synapses: the AMPARs and NMDARs. Their distinctive roles have been established using specific antagonists of excitatory amino-acid receptors (Watkins and Evans, 1981, McLennan and Liu, 1982). Ionophoretically applied DGG, an antagonist of both the AMPARs and NMDARs blocks both synaptic transmission and the induction of LTP by tetanic stimulation. Following wash-out of DGG, a similar tetanic stimulation induces LTP. Furthermore, NMDAR activation is required for LTP induction, as LTP is blocked by a variety of NMDAR antagonists acting at the various sites on the receptor. These include the competitive antagonist AP-5 (Collingridge et al., 1983, Harris et al., 1984). MK-801 (Coan et al., 1987), and an antagonist of the glycine modulatory site, 7-chlorokynurenate (Bashir et al., 1990).

- **Ca²⁺ signals induced by synaptic activation of NMDARs.** The input specificity of LTP is determined by Ca²⁺ entry through NMDARs into dendritic spines. The changes of [Ca²⁺]i in dendritic spines have been measured by combining whole-cell patch-clamp recording with confocal imaging of [Ca²⁺]i (Alford et al., 1993). Early experiments showed that tetanic stimulation results in a transient increase in [Ca²⁺]i, rather than a sustained change (Regehr and Tank, 1992, Perkel et al., 1993). Voltage-gated Ca²⁺ channels are also activated during the glutamate-induced depolarization. The component of Ca²⁺ entry through glutamate receptors
was isolated from that through voltage-gated Ca$^{2+}$ channels by using whole-cell voltage clamp with ATP-free intracellular solutions (Alford et al., 1993). Under these conditions, synaptic stimulation results in a Ca$^{2+}$ signal in the dendrites that is blocked by AP-5. The signal is insensitive to CNQX, indicating that it does not involve Ca$^{2+}$-permeable AMPARs. Its voltage- and Mg$^{2+}$-dependence further support its entry through NMDARs. In the presence of Mg$^{2+}$ the signal increased between –90 and –35 mV, the potential range over which the current-voltage (I-V) curve has a negative slope, owing to the voltage-dependent block of NMDARs by Mg$^{2+}$. In contrast, in Mg$^{2+}$-free solutions, where the I-V curve is linear, the Ca$^{2+}$ signal decreases with depolarization, as expected from the reduction in ionic driving force.

- **CaMK-II plays a key role in NMDAR-dependent-LTP.** CaMK-II is a multi-subunit enzyme (see Chapter one). Each of the 10-12 subunits in the holoenzyme has a catalytic domain and an autophosphorylation domain. CaMK-II, upon activation by Ca$^{2+}$ and CaM, can be autophosphorylated by an intersubunit, intraholoenzyme reaction. The autophosphorylated enzyme is autonomously active, that is, it is active independent of the continued presence of Ca$^{2+}$ and CaM. This mechanism sets up a self-perpetuating CaMK-II activation that may be sufficient for LTP, independent of any subsequent changes in gene expression (Lisman, 1985., 1994). A number of studies have indicated a necessary role for CaMK-II in LTP (see Chapter one). The evidence showed that LTP was blocked by CaMK-II inhibitors, such as KN-62 (Ito, et al., 1991); CaMK-II inhibitor peptide CaMK-II$^{273-302}$ (Malinow et al., 1989); inhibitors of the CaM such as trifluoperazine (Finn et al., 1989),
calmidazolium (Malenka et al., 1989), and CaM binding peptides, CBP (Malenka et al., 1989). Direct assays of CaMK-II activity have implicated a role for its autonomous activation in LTP expression (Fukunaga et al., 1993). An increase in both autonomous and second-messenger-dependent CaMK-II activity occurs 5 min. following LTP induction and lasts for at least 60 min. (Fukunaga et al., 1993). Pettit et al (1994) had shown that activation of CaMK-II is sufficient to increase synaptic efficacy. These authors applied active CaMK-II into hippocampal neurons and showed an augmentation of synaptic transmission and an occlusion of the subsequent induction of LTP. In an elegant series of experiments, Soderling’s group has demonstrated that CaMK-II can phosphorylate and activate AMPA receptors, a mechanism that clearly could contribute to the expression of LTP (see chapter one).

**Based on my own observations the model has been modified:**

- **Src kinase may be the first step in the initial events for LTP induction.** All previous models of LTP begin with the activation of the NMDARs as the first step (Bliss and Collingridge, 1993). My data suggest, for the first time, that Src activation may precede NMDAR activation. As shown in chapter three, the blockade of Src kinase blocked the induction of LTP and activation of endogenous Src, or application of recombinant pp60c-Src, evoked long-lasting potentiation which occlude tetanus-induced LTP. Thus, Src fulfills necessary and sufficient conditions to mediate LTP induction at CA1 synapses. Like tetanus-induced LTP, the enhancement of synaptic transmission caused by directly activating Src appear to require a rise in $[\text{Ca}^{2+}]_i$ and activation of NMDARs, because the NMDAR antagonist, MK-801,
blocks Src activation induced synaptic potentiation. Hence, the most parsimonious explanation for my findings during the induction of LTP is that Src is rapidly activated first, which then leads to enhanced NMDAR function. Note that Src does not require Ca\(^{2+}\) for its action on the NMDAR. The potentiation of NMDAR function results in increased Ca\(^{2+}\) entry (sufficient amount) which will trigger the downstream signaling cascade. Ca\(^{2+}\) entry through NMDAR channels is the main source of the rise in [Ca\(^{2+}\)]\(_i\). Thus, my results suggest, for the first time, that for LTP induction there is a unexpected step (Src activation) upstream of NMDARs. NMDARs are phosphorylated by tyrosine kinases during LTP (see chapter one). The tyrosine kinase family are required for the induction, but not for the expression of LTP, indicating that NMDAR phosphorylation could be one of the initial events that induce LTP. In addition, the time window between tetanic stimulation and the ensuing a rise in postsynaptic [Ca\(^{2+}\)]\(_i\) level sufficient for LTP induction is about 3 seconds (Malenka et al., 1992). This relatively long time window might involve new unknown events, such as Src activation, which then enhances NMDAR function.

The model represented in Figure 6.1 describes the boost in Ca\(^{2+}\) entry as a consequence of increased NMDAR channel activity. An alternative possibility for Src action may be that an unknown target provides a binding site for an SH2 domain containing protein, which triggers the cellular cascades underlying LTP induction. Some proteins which contain SH2 domains are also signalling proteins that could come together to form the complexes in PSD. Thus, the interaction of Src with the Src target protein could recruit a signalling complex to the NMDARs. The recruitment may activate the complex and evoke a cascade of downstream signalling.
This mechanism could involve consolidation of the later phases of LTP expression and might contribute to the engagement of other signalling cascades such as the Ras-GAP-MAP kinase pathway (Brambilla, et al., 1997).

- **Activation of mGluR5 mediates the expression of LTP\textsubscript{NMDA}.** My results that the NMDAR-mediated component of LTP (LTP\textsubscript{NMDA}) is selectively eliminated in mGluR5 mutant mice suggest that activation of mGluR5 is normally required for the enhancement of the NMDAR component of EPSCs, but not the AMPAR-mediated component of EPSCs. The selective loss of LTP\textsubscript{NMDA} in mGluR5 mutants would strongly support the hypothesis that the underlying mechanism for the expression of LTP\textsubscript{NMDA} and LTP\textsubscript{AMPA} are distinct. Furthermore, postsynaptic neurons would be the predominant locus for LTP.

The postsynaptic expression mechanism proposed above is based on the events of all-or-non-up-regulation of both AMPARs and NMDARs following LTP. Regarding the increased release of neurotransmitter, glutamate, from the presynaptic terminals, the equal enhancement of EPSC\textsubscript{NMDA} and EPSC\textsubscript{AMPA} following tetanic stimulation observed from control mice in chapter four, could argue for a presynaptic view of LTP expression. The possibility that the selective loss of LTP\textsubscript{NMDA} in mGluR5 mutants could result from the persistent response of NMDARs to the increased glutamate required for the mGluR5-coupled PKC pathway in postsynaptic neuron. Thus, my data from chapter four might suggest that both pre- and postsynaptic modifications have been carried out during LTP. Consistent with this hypothesis, more recently, Nicoll’s group reported (Liedo et al., 1998) that postsynaptic fusion
events, which could be involved in both retrograde signaling and in regulating postsynaptic receptor function, contribute to LTP.

- mGluR5-coupled PKC activation could be the major step in the expression of LTP\textsubscript{NMDA}. Generally, receptor mediated activation of PKC can be described as following: \([\text{Ca}^{2+}]_i\) is increased as result of transmitter-induced \(\text{Ca}^{2+}\) influx (e.g. activation of NMDARs) or enhanced \(\text{Ca}^{2+}\) release from internal stores mediated by IP3. The rise in \([\text{Ca}^{2+}]_i\) induces the translocation of PKC from cytosol to the membrane. This membrane-associated but only partially active kinase becomes completely activated when DAG binds to the protein (Huang and Haung, 1993). DAG can be generated by receptor-mediated hydrolysis of phosphatidylinositol-diphosphate and/or after depletion of this small pool, after cleavage of phosphatidylcholine by PLC. Thus a variety of G-protein linked receptors might be involved in generating DAG after the induction of LTP. Among them, mGluR5 is the center of the receptors (see Chapter one). Klann et al (1991) showed that a lasting increase in autonomous PKC activity is associated with LTP. However, activation of PKC seems to be not necessary for the expression of LTP\textsubscript{AMPA}, because sphingosine, which competes with second-messenger activation of PKC, blocks LTP induction but not the expression of LTP\textsubscript{AMPA} (Malinow et al., 1988). Furthermore, activation of PKC with phorbol esters does not result in long-lasting potentiation; AMPAR-mediated synaptic response returned to baseline following washout of the drug (Muller et al., 1988), indicating that a transient increase in PKC activity is not sufficient to induce long-lasting potentiation of AMPAR-mediated EPSCs. As there is no evidence for the effects of PKC activation on the expression of LTP\textsubscript{NMDA}. My observation that selective
Chapter six: Future Directions

elimination of \( \text{LTP}_{\text{NMDA}} \) in mGluR5 mutants implies that persistent activation of PKC through mGluR5 is required for \( \text{LTP}_{\text{NMDA}} \) expression. The fact is that the deficit of \( \text{LTP}_{\text{NMDA}} \) in mGluR5 mutants was rescued by the stimulation of PKC (see chapter 4). Taking these considerations together, the hypothesis on the cellular signaling cascade for the expression of \( \text{LTP}_{\text{NMDA}} \) could be the following: mGluR5-coupled PKC activation leads to the persistent potentiation of NMDAR function.

6.1.C Areas of this thesis require further investigation

We have focused our attention on the cellular mechanisms underlying two very distinct forms of LTP induction. The CA1 form of LTP is induced by a transient rise in postsynaptic \( \text{Ca}^{2+} \). Our studies, on this part, provide the new insights into the postsynaptic signaling events in the induction of LTP. First, we found a key component, Src, which may lie upstream of the NMDARs during the induction of LTP. This finding opens a new field to explore the initial signaling cascades that gate the NMDAR function to induce LTP. Second, the mGluR5 coupled PKC signaling pathway is specifically responsible for the induction of \( \text{LTP}_{\text{NMDA}} \), but not for \( \text{LTP}_{\text{AMPA}} \). The finding raises the possibility that the underlying mechanism for the induction of \( \text{LTP}_{\text{NMDA}} \) and \( \text{LTP}_{\text{AMPA}} \) are distinct. Further work will identify the distinctive biochemical components, in these two pathways.

- **How is Src kinase activated after tetanic stimulation?** Src could be activated directly after tetanic stimulation: for example, the \( \text{Ca}^{2+} \) influx mediated by the NMDA receptor might directly trigger the tyrosine kinase Src. However, Src is \( \text{Ca}^{2+} \)-independent enzyme. Therefore, Src might be activated indirectly by protein kinase, such as proline-rich tyrosine kinase 2 (Pyk-2). A several lines of evidence support this
idea. The protein tyrosine kinase Pyk-2, which is highly expressed in the CA1 region of the hippocampus (Levis et al., 1995), is rapidly phosphorylated on tyrosine residues in response to various stimuli that elevate intracellular Ca\(^{2+}\), for example, by applying glutamate or high K\(^{+}\) to induce neuronal depolarization in the rat hippocampus. More recently, there is also evidence showing that tyrosine phosphorylated Pyk-2 directly binds to the SH2 domain of Src leading to Src activation (Dikic et al., 1996). This raises the possibility that Src links Pyk-2 with the efficacy of the NMDAR channel gating in the initial events of LTP induction. It will be necessary to determine whether Pyk-2 is required for LTP induction. The endogenous Pyk-2 should produce synaptic potentiation which has the same features as Src-potentiation. Blockade of Src function should prevent Pyk-2 action on LTP. By the way, Pyk-2 was also found to regulate the delayed rectifier-type K\(^{+}\) channel, termed Kv1.2, which is highly expressed in hippocampus (Lev et al., 1995). It is also necessary to determine if Pyk-2-mediated regulation of K\(^{+}\) channel activity could involve in the events of Src signaling pathway for inducing LTP.

More recently, MacDonald’s group found that Src was required for the PKC-mediated potentiation of NMDAR channel activity in neuronal cultures (Lu et al., 1998). The data revealed that the administration of Src(40-58) prevented PKC induced enhancement of NMDAR channel activity. Moreover, mice lacking Src showed the deficits in PKC action on NMDAR channel activity. The results suggested that PKC might be upstream of Src for the up-regulation of NMDAR channels. However, it is not know how PKC activates Src. Further study is required to determine the signaling...
pathway for PKC, Pyk-2, and Src. How these protein signalling components interact with each other.

The presynaptic LTP. The induction of mossy fiber LTP is fundamentally different from the CA1 type of LTP. The postsynaptic neuron plays no role, either in the induction or the expression of this form of LTP. My work, shows for the first time that presynaptically localized Zn\(^{2+}\) participates in the LTP induction. Zn\(^{2+}\) functions as a co-factor or structural component of more than 200 enzymes and therefore plays a role in a broad array of biological processes. The finding that Zn\(^{2+}\) is specifically required for the induction of mossy fiber LTP, but not other forms of LTP, may arise from the fact that Zn\(^{2+}\) is highly concentrated in mossy fiber boutons. The specific cellular components that interact with Zn\(^{2+}\) in the events underlying mossy fiber LTP induction need to be explored.

In summary, the process of LTP induction, at the excitatory synapses in the hippocampus, is carried out by glutamate receptor channels and signaling molecules, which are clustered in postsynaptic neurons. Despite the similar end result of strengthening synaptic efficacy in all forms of LTP, each form of LTP has a distinct cellular signaling pathway.

6.2. New questions

Could we get direct meaningful relationship between LTP and spatial learning?

6.2.A Does the manipulation of LTP affect learning

If LTP is involved in learning & memory, the manipulating any one of the necessary signaling molecules studied above should alter the performance on learning tasks in
behavioring animals. On one hand, our results revealed that indeed mGluR5 null mutant mice were significantly impaired in two different spatial learning tasks (Lu et al., 1997). These data are compatible with the injection of the Class I mGluR5 antagonist, MCPG into rats, which reduced spatial learning; whereas a Class I agonist applied after learning facilitated memory recall (Riedel et al., 1996). However, it should be borne in mind that although our mGluR5 mutant mice were unable to express one component of LTP ($\text{LTP}_{\text{NMDA}}$), they were able to induce the other component ($\text{LTP}_{\text{AMPA}}$) at the normal level. From this point view, a major component of LTP, $\text{LTP}_{\text{AMPA}}$, seems not to be responsible for learning tasks. The finding suggests that there does not appear to be simple relationship between LTP induction and ability in spatial learning in the mutant mice.

Furthermore, Bach et al (1995) found an impairment of learning in a spatial task (the Barnes circular maze) in CaMK-II mutant mice. These animals were unimpaired in LTP when this was induced using standard laboratory parameters (100Hz) (Mayford et al., 1995). However, when they were tetanized at lower frequencies, closer to the theta range, they began to express depression rather potentiation. This finding suggests that ordinary tetanic LTP might differ in important ways from the LTP mechanisms that operate during learning, and that LTP triggered with lower frequencies of stimulation might be more appropriate way of testing the LTP/learning hypothesis. The question then arises: how many different stimulation paradigms must be tested before it can be concluded that a given mutant will never express LTP? In addition, mutant mice deficient in the PKCy showed profoundly impaired LTP in CA1, and yet are only mildly impaired on spatial tasks and contextual fear conditioning (Abeliovich et al., 1993a,b).
These findings raise a serious question about the interpretation of complicated results in the mutant mice: namely, if LTP is impaired under some conditions but not others, how can we ever know which conditions correspond to components of the learning tasks in behavioral animals.

6.2.B Non-synapse specific forms of LTP

At the heart of the LTP-learning debate lies Hebb’s postulate on synapse-specificity. It states that a synapse should increase in strength if the pre- and post-synaptic components are simultaneously active. The Hebbian basis of LTP induction is now well understood, and hippocampal LTP itself is considered synapse-specificity (Bliss and Collingridge, 1993). However, there exists, at present, no Hebbian experimental model of learning. Given that learning is clearly too complex a phenomenon to constrain the interpretation of LTP experiments, the logical next step would appear to be look more closely at the cellular correlates of non-synaptic activities during LTP.

Under some circumstances, synapse specificity has intuitive appeal because it provides the necessary structure for a huge memory capacity, well over and beyond that could be achieved through somatic potentiation. However, the modifications induced by LTP are rarely, if ever, limited to synapses. For example, in the original description of LTP, Bliss and Lomo (1973) reported a phenomenon since referred to as E/S potentiation. Following LTP induction, an increase in population spike amplitude (S) and a reduced threshold for cell firing can be observed even if the magnitude of the excitatory synaptic potential (E) is held constant. This indicates that the tetanus-induced modification is not limited to the synapse. Using an extracellular population spike as a dependent measure, an
increase in efficacy can reflect changes which occur exclusively in the soma, along the entire membrane, at synaptic terminals, or some combination of the three. Using an extracellular excitatory postsynaptic potential (EPSP) as the dependent measure, on the other hand, does not allow one to measure the changes that may lie on other cellular loci. Thus, changes at the soma, such as the nonsynaptic forms of potentiation which typically accompany an increase in synaptic efficacy are virtually ignored.

There is no a priori reason to require that a memory mechanism be limited only to modifications of synapses. At this point, it should be noted that the plastic changes associated with learning often are not limited to the synapses active during the learning event. I feel that these nonspecific responses should not be dismissed, but rather, should be appreciated as providing potential clues about the neural mechanisms of learning.
APPENDIX A

CNS DEVELOPMENT IS NORMAL IN mGluR5 MUTANTS

(Data from Zhengping Jia and Jeffrey T. Henderson)

In a previous study we showed that the mGluR5 null allele was inherited in a Mendelian fashion and mGluR5 -/- mice showed undetectable levels of mGluR5 protein, but normal levels of other glutamate receptors (Figure appendix 1). Immunocytochemistry using antibodies specific for both mGluR5 isoforms (Minikami et al., 1993) showed no mGluR5 in the hippocampus but normal mGluR1 α expression.

To examine mGluR5 mutant mice for potential developmental abnormalities, several sets of serial sections were obtained from mGluR5 -/- and +/- control littermates. Thionin-stained, ten-micron serial paraffin sections were taken through the entire extent of the cranium at 300 μm intervals, in both the sagittal and coronal planes (n=3), for both mGluR5 -/- and +/- littermates (45-60 dpn). Thirty micron serial cryostat sections were also obtained at intervals of 150 microns for mGluR5 +/- and -/- mice through the entire horizontal and sagittal plane (n=3 for each plane). As shown for regions known to express mGluR5 (hippocampus, cortex, striatum, olfactory bulb), mutant mice revealed no significant neurodevelopmental abnormalities for these, or other, structures within the CNS (Figure appendix 2). In addition, 30 μm serial cryostat sections (coronal plane) of mGluR5 -/- and +/- mice (300 micron intervals) were stained for cytochrome C oxidase activity, a gross indicator of the level of pre-synaptic input. These also showed no significant differences between the two groups (n=2 animals per group) (data not shown). Thus, mGluR5 -/- mice
were found to be without major neuroanatomical abnormalities, possessing all major 
neuroanatomic loci and fibre pathways in normal proportions.
Figure appendix 1  Production of mGluR5 null mutants (Jia et al., 1998)

(A). The 129 mGluR5 genomic clone (exon 1, open box), targeting vector and mutant mGluR5 locus expected following homologous recombination. P, PstI; B, BamH1; X, XhoI; H, Hind III; Xb, XbaI; N, NotI. (B). Southern blot analysis of genomic DNA isolated from a representative litter of 9 mice derived from a heterozygous (+/-) F1 inter-cross. DNA was digested with PstI and probed with an external 1.2 kb fragment (above) yielding a 11.5 kb restriction fragment from wildtype (+/+), and a 7.5 kb diagnostic band from mutant alleles (+/-). (C). Western blots of crude whole brain plasma membranes prepared from homozygote mGluR5 mutant (-/-), heterozygote (+/-) and wild type (+/+) mice, and probed with the antibodies indicated. The amount of mGluR5 detected using antibody to the C-terminus (Romano et al., 1995) relative to wildtype was 50 % in +/- and 0 % in -/- (n=3 per group). (D). Immunocytochemical staining of +/- (a,c) and -/- (b,d) mouse hippocampi, using antibodies selective for mGluR5 (a,b) and mGluR1a (c,d). The entire hippocampus (a, b) or the polymorph region of the dentate gyrus (c, d) is shown. Bar = 500 µ (a, b), 200 µ (c,d). Serial sections through the entire CNS (n=3) revealed normal neuroanatomical organization.
Figure appendix 2. Neuroanatomy in mGluR5 mice (Lu et al., 1997)

Pairwise comparisons of adult mGluR5 +/- (above) and -/- (below) litter mates. Thirty micron cryostat sections at comparable levels are shown. (A) parasagittal sections showing the hippocampal formation (rostral-right, caudal-left); (B) horizontal sections through the forebrain and thalamus (2.8 mm ventral to the dorsal aspect of brain); (C) horizontal sections through the hindbrain (4.5 mm ventral to the dorsal aspect of brain), (D) parasagittal sections of the forebrain (300 microns from midline); (E) parasagittal sections showing structures of the diencephalon, hippocampus, and splenium of the corpus callosum (300 microns from midline), (F) parasagittal sections of caudal aspect of the brain, showing regions of the superior and inferior colliculus and brain stem (450 microns from the midline). For photomicrograph pairs B-F, bar represents 1000 microns; for photomicrograph pair A, bar represents 500 microns. Labelled structures represent regions previously shown to express high levels of mGluR5. c1 - hippocampal subfield CA1, c3 - hippocampal subfield CA3, ct - neocortex, dg - dentate gyrus, hp - hippocampus, na - nucleus accumbens, ob - olfactory bulb, s - subiculum, sp - medial septal region (low in mGluR5 expression), str - striatum, tu - olfactory tubercle.
APPENDIX B

THE IMPAIRMENTS OF SPATIAL LEARNING

IN mGluR5 MUTANTS

(Data from Christopher Janus)

Descriptive behavioral observations

The mGluR5 mutants were indistinguishable in their weight and morphology. No changes in locomotor and other behaviours were apparent in home cages. When moved to new cages, mGluR5 mutants were soon engaged in normal exploratory behaviour, sawdust, digging, rearing and cage top climbing. Mutant males were also engaged in occasional bursts of fighting, at the same rate as males in control groups. However, mGluR5 mutants were unusually reactive to handling. They were difficult to catch in their home cage and often jumped off the experimenter's hand, or off the cage. This reactivity to handling showed slow habituation. To ensure a blind experiment all cages were coded, and handling and behaviour recording were carried out by different experimenters.

Behaviour in the open-field test

In order to examine motility and response to a novel environment, control and mutant mice were observed in an open-field test. Since no differences were found between fields crossed and time of walking, or between behaviours in the periphery and centre of the arena, we removed field crossing from the analysis and pooled peripheral and central fields data together. The analysis of behaviours (walking, pausing, wall leaning, rearing, grooming) performed by mice did not reveal any significant differences between groups (MANOVA:
Appendix: mGluR5 and Spatial Learning

F(5,16) = 0.25; p = 0.93 (Table appendix 1). In addition, the mGluR5 mutants did not differ from controls in their latency of central fields exploration (t (9)=0.33; p>0.05). The results indicated that mGluR5 mutant mice did not differ from control animals in their locomotor and exploratory behaviour in the new environment.

**Decreased learning acquisition in mGluR5 mutants in the water maze.** Control (n=27) and mGluR5 mutant (n=22) mice were trained for 6 days, in the hidden platform version of the water maze with 6 trials per day. Analysis of 3-trial blocks revealed a significant difference in learning between control and mutant mice (F(1,47) = 20.45; p < 0.001) and between days (F(3, 235) = 28.43; p < 0.001). Also, group by days and group by blocks interactions were significant (F(3, 235) = 3.88; p <0.01, and F(1,47) = 12.79; p <0.001, respectively). Both interactions indicated a significant difference in learning acquisition between groups. Throughout the experiment mutant mice always had longer latencies in finding the platform but beyond the third day of training their latencies levelled at a 35-40 s, while the control mice showed a steady decrease reaching an average of 10-15 s by the end of training (Figure appendix 3). Planned comparisons revealed that the most significant differences between groups occurred after the third day of training (6th 3-trial block, Figure appendix 3A). To elucidate further the nature of the above interactions, an analysis of simple effects with trend analysis and an effect size (Stevens, 1992) were performed for each group. For the control group we found a significant decrease in latencies over time (F(5,286) = 17.94, p <0.001) with 92% of the variance ( η2 = 0.916) accounted for the change in learning. Two trends were found significant, linear and quadratic (F(1,26) = 74.8, p <0.001, F(1,26) = 5.2, p <0.05, respectively). the linear trend accounted for 74% and the quadratic for 17% of the variance in learning acquisition. Accordingly, there was a
significant change in learning in the mutant group (F (5,231) = 5.63, p < 0.001) with 69% ($\eta^2 = 0.688$) of the variance explained by the change in learning. Again, only linear and quadratic trends were found to be significant (F (1,21) = 9.5, p< 0.01, F (1,21) = 15.9, p < 0.001) accounting for 31% and 43% of the variance. In conclusion, although both groups learned to find the hidden platform, the character of learning change was different in controls (mostly linear) and in mutants (mostly quadratic). At the end of the training period, all animals were given one probe trial, in which the platform was removed and the mice were allowed to search the pool for 60 seconds. The control mice spent more time in the target quadrant than in the other three quadrants (F (2, 78) = 59.21; p < 0.001), whereas mutant mice did not (Figure appendix 3B). The search time in the target quadrant by the mutant mice was significantly shorter than by controls (p < 0.001) and the mutant mice crossed the platform site less frequently than the controls (1.05 ± 0.26; 2.74 ± 0.38 for mutants and controls respectively; t(47) = 3.52, p < 0.001).

In the visible platform version of the water maze test, control (n=10) and mutant (n=10) mice were tested for two days because their learning rate in this test progressed rapidly. The analysis performed on 3-trial blocks showed no apparent differences between mutant and control mice in the latency to reach the platform. The significant results of day (p < 0.001), block (p < 0.001) and day by block interaction (p < 0.001) confirmed rapid learning across training blocks in both groups. A significant group by day interaction (p < 0.05) was caused by longer latencies of mutant mice (p < 0.05) during the first day of training (Figure appendix 3C).

**Decreased contextual fear conditioning in mGluR5 mutants.** This paradigm (Philips & LeDoux, 1992) allowed us to test (i) the subjects' unconditioned response (UR) to shock
Appendix: mGluR5 and Spatial Learning

(US) (ii), their ability to associate simple pairing between a shock and a discrete stimulus-like tone (CS), and (iii) the context in which they received the shock. Since the changes in duration and frequency of freezing were similar, we present only the total time of freezing responses for controls (n=15) and mGluR5 mutant mice (n=15) (Figure appendix 4). The control and mutant subjects showed a similar freezing response to shock during training, which suggest similar perception and response to aversive foot shock. The two groups were also not different in exploratory behaviours during the training session. In the context test carried out 24 h after training, the mutant mice showed a significant reduction in the freezing time especially at the beginning of the test (Figure appendix 4B)(MANOVA; F (1.28) = 8.7; p<0.01). Both control and mutant mice showed similar freezing to tone in the CS test carried out 2 h after the context test (Figure appendix 4C). The groups also did not differ in other exploratory behaviours during each phase (1st 3 min with no CS and 2nd 3 min with CS) of the CS test.
Table appendix 1 Normal locomotor behaviours performed by mGluR5 mutant mice in 5 min open-field test (Lu et al., 1997)

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Controls (n = 12)</th>
<th>mGluR5 Mutants (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Walking</td>
<td>202.2 ± 12.5</td>
<td>186.2 ± 12.8</td>
</tr>
<tr>
<td>Pause</td>
<td>59.4 ± 14.6</td>
<td>74.3 ± 16.0</td>
</tr>
<tr>
<td>Wall leaning</td>
<td>24.7 ± 3.8</td>
<td>25.9 ± 5.9</td>
</tr>
<tr>
<td>Rearing</td>
<td>3.8 ± 2.5</td>
<td>4.4 ± 1.9</td>
</tr>
<tr>
<td>Grooming</td>
<td>6.2 ± 1.0</td>
<td>5.9 ± 1.3</td>
</tr>
</tbody>
</table>

Data represent the average total time (sec) ± SEM. See text for detailed descriptions of observed behaviours.
Repeated measures did not reveal any significant differences between mutant and controls. No effect was observed in the avoidance of the visible platform in each 2-day block (2 days of testing is presented). ANOVA with repeated measures revealed no significant difference in the time spent in the four quadrants (2 days of testing). The average time spent in the right (NW) quadrant was significantly longer than in the left (SW) quadrant. Control animals searched significantly longer in the left (SW) quadrant than in the right (NW) quadrant. The average time spent searching in each quadrant was significantly different between groups. The average time spent in each quadrant was significantly different between groups. The average time spent searching in each quadrant was significantly different between groups. The average time spent searching in each quadrant was significantly different between groups. The average time spent searching in each quadrant was significantly different between groups.

Water maze (Tu et al., 1997)

Figure Appendix 3: Performance of Mice in the Water Maze was Impaired in the Appendix: Mu2 and Spatial Learning
Figure appendix 4 Contextual fear conditioning is impaired in mGluR5 mutants (Lu et al., 1997)

A) The duration of freezing during the training phase of fear conditioning. Mutant mGluR5 and control mice showed a comparable amount of freezing immediately after the footshock. A solid line indicates the duration of the tone (CS), while a square indicates the 2s. footshock (US). (B) The mice were tested for contextual conditioning 24 h after training. Mutant mice showed significantly less freezing than controls when returned to the training chamber. (C) Control Tone (CS) conditioning test was carried out in a new context 2 h after the context test. Both mutants and controls showed no freezing in a new context, and comparable amounts of freezing when a tone (CS) was presented in a new context.

** p <0.01, *** p<0.001.
SUMMARY OF MY WORK IN THIS THESIS

I have carried out extracellular recording and patch-clamp recording from hippocampal slices to clarify the roles of Src kinase, mGluR5 and Zn\(^{2+}\) in the induction of LTP in hippocampus. My major work in this thesis are as followings:

- In chapter three, I made use of patch-clamp recordings from the CA1 region of the hippocampus, and applied several Src functional blockers or augmentors during LTP. First, I found that the blockade of Src activity with Src(40-58) or anti-Src1 antibody, at single pyramidal cells, inhibited LTP induction, while the extracellular monitored neighbor cells showed normal LTP, indicating that endogenous Src is necessary for LTP induction. Second, application of a Src family activating peptide, EPQ(pY)EEPIA, caused synaptic potentiation, which was prevented by the prior administration of Src(40-58). Administration of exogenous pp60c-src into the pyramidal cells also enhanced EPSP. The activation of Src-induced synaptic potentiation and tetanus-induced LTP occluded each other, suggesting that the endogenous Src is sufficient for LTP. Furthermore, I found that like tetanus-induced LTP, Src-mediated synaptic potentiation required NMDARs and elevation of [Ca\(^{2+}\)]i.

In this chapter, Jonathan Davidow performed the Src activity assay.

- In chapter four, I carried out all the electrophysiological experiments. Zhenping Jia provided mGluR5 mutant mice. First, I studied the effects of mGluR agonists on excitatory synaptic transmission in hippocampus. I found that 1S,3R-ACPD at the concentration of 50 \(\mu\)M caused the reduction of EPSPs by 60% in control mice, but produced no change in recorded EPSPs in mGluR5 mutant mice at the same
concentration, suggesting that mGluR5 may be the dominant receptor of ACPD. Next, I analyzed field potentials (fEPSP) in mGluR5 mutants, and showed a small decrease in the induction of LTP in CA1 and dentate gyrus but normal LTP in the CA3 region of the hippocampus. The difference between genotypes (+/- or -/-) was abrogated by adding AP5 after the induction of LTP, indicating that the difference resulted from the NMDAR-mediated response. In further studies, I found that the expression phase of \( \text{LTP}_{\text{NMDA}} \) is completely lost, whereas \( \text{LTP}_{\text{AMPA}} \) is normal in mGluR5 mutants. Finally, I found that the impairment of \( \text{LTP}_{\text{NMDA}} \) in mGluR5 mutants was rescued by the stimulation of PKC with PDBu. My results in this chapter indicated that the mGluR5 coupled PKC signaling pathway is specifically responsible for the induction of \( \text{LTP}_{\text{NMDA}} \), but not for \( \text{LTP}_{\text{AMPA}} \). The finding raises the possibility that the underlying mechanism for the induction of \( \text{LTP}_{\text{NMDA}} \) and \( \text{LTP}_{\text{AMPA}} \) are distinct, and suggest a postsynaptic locus for LTP expression.

- In chapter five, I performed all the LTP recording. Franco Taverna carried out the \( \text{Zn}^{2+} \) fluorescence assay. I also generated \( \text{Zn}^{2+} \)-deficient rats. The chronic dietary deficiency of \( \text{Zn}^{2+} \) resulted in depletion of \( \text{Zn}^{2+} \)-fluorescence from the mossy fibers. I have shown here for the first time that one-to-one correlation between the endogenous \( \text{Zn}^{2+} \) depletion and LTP deficits at \( \text{Zn}^{2+} \)-containing mossy fiber-CA3 synapses, while \( \text{Zn}^{2+} \) depletion caused no change in LTP at non-\( \text{Zn}^{2+} \)-containing commisural fiber-CA3 and Schaffer collateral-CA1 synapses. A quantal analysis of mossy fiber unitary EPSCs showed that \( \text{Zn}^{2+} \) depletion abolished the increase in the quanta content with LTP. In addition, the administration of the cell membrane impermeable \( \text{Zn}^{2+} \) chelator, \( \text{Ca}^{2+}\text{EDTA} \), into the postsynaptic neurons produced no alteration of mossy fiber LTP.
Summary of my work in this thesis

My results in this chapter indicated for the first time that presynaptically localized Zn$^{2+}$ is specifically required for LTP induction at the mossy fiber input into CA3 neurons.
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