UNIVERSITY OF ALBERTA

REGULATION OF HIPPOCAMPAL SYNAPTIC PLASTICITY BY cAMP-DEPENDENT PROTEIN KINASE AND PROTEIN SYNTHESIS

by

NEWTON H. WOO 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of *Doctor of Philosophy*

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Regulation of Hippocampal Synaptic Plasticity by cAMP-Dependent Protein Kinase and Protein Synthesis" submitted by Newton H. Woo in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Dedicated to my Mother for the love and support she has always given me.

ABSTRACT

Enduring changes in the physiological strength of synaptic transmission ("synaptic plasticity") are thought to underlie information storage in the mammalian brain. One particular form of hippocampal synaptic plasticity known as the late phase of long-term potentiation (L-LTP) has been correlated with hippocampal long-term memory (LTM). In light of this relationship, the present thesis identifies several critical factors that govern the induction and expression of L-LTP by using electrophysiological recording methods in mouse hippocampal slices.

Different patterns of stimulation can influence the expression of L-LTP in two important ways, by differentially recruiting distinct isoforms of PKA or by controlling the PKA-dependence of LTP. Thetaburst and spaced tetraburst stimulation both elicited PKA-dependent forms of L-LTP. Although, thetaburst LTP was attenuated by an isoform non-specific inhibitor of PKA, it was intact in slices taken from R(AB) mutant mice, which overexpress an inhibitory regulatory subunit. Thus, thetaburst stimulation, in contrast to spaced tetraburst stimulation, may recruit endogenous isoforms of PKA. In addition, when the spacing of the intertrain interval during tetraburst stimulation was compressed, a PKA-independent form of L-LTP was induced that was not sensitive to either genetic or pharmacological inhibition of PKA activity.

Secondly, protein phosphatases regulate L-LTP expression by acting as an inhibitory constraint. Slices from R(AB) mutant mice have impaired L-LTP because the protein phosphatase mediated constraint was enhanced by genetic suppression of PKA activity. When this inhibitory constraint was relieved by application of protein phosphatase

inhibitors, L-LTP induced electrically or chemically was fully rescued in mutant slices. Furthermore, it was found that this inhibitory constraint could also be regulated by synaptic activity. Prior activation of protein phosphatases by low frequency stimulation selectively impaired the *future* expression of L-LTP.

Finally, protein synthesis stabilizes L-LTP to a more permanent state. Multiple bursts of high-frequency stimulation changed the susceptibility of potentiated synapses to disruption by conferring resistance or "immunity" to depotentiation. This form of synaptic immunity was activity- and protein synthesis-dependent. Two forms of immunity were observed depending on the site of macromolecular synthesis. Local translation mediated a rapid, input specific form whereas transcription mediated a slowly generating, cell-wide form of synaptic immunity.

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AC	adenylyl cyclase
ACSF	artificial cerebrospinal fluid
AKAP	A-kinase anchoring protein
amn	*amnesiac*
AMPA/R	L-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid / receptor
ANOVA	analysis of variance between groups
APV	DL-2-amino-5-phosphonopentanoic acid
ARC	actin-binding cytoskeletal protein
BDNF	brain-derived neurotrophic factor
C	catalytic subunit of PKA
Ca^{+2}	calcium
CA	cornu ammonis
cal-A	calyculin A
cAMP	adenosine 3', 5'-cyclic monophosphate
CaM	calmodulin
CaMKII	calcium/calmodulin-dependent protein kinase II
CaMKIV	calcium/calmodulin-dependent protein kinase IV
CBP	CREB binding protein
CCAC	Canadian council on animal care guidelines
CRE	cyclic AMP response element
CREB	cyclic AMP response element binding protein
CS	conditioned stimulus
DG	dentate gyrus
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dnc	*dunce*
DPT	depotentiation
E-LTP	early phase of long-term potentiation
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
ERK	extracellular-signal regulated kinase
fEPSP	field excitatory postsynaptic potential
fyn	non-receptor tyrosine kinase
G-protein	guanine nucleotide-binding regulatory protein
Glu/R	glutamate / receptor

HFS	high-frequency stimulation
I-1	inhibitor 1
IACUC	Institutional Animal Care and Use Committee
IEG	immediate early gene
LFS	low-frequency stimulation
LTP	long-term potentiation
LTD	long-term depression
LTM	long-term memory
L-LTP	late phase of long-term potentiation
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated/extracellular signal-regulated kinase
MF	mossy fibre
Mg^{+2}	magnesium
mGLUR	metabotropic receptor
mRNA	messenger RNA
NMDA/R	N-methyl-D-aspartic acid / receptor
OA	okadaic acid
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PP1 / 2A	protein phosphatase 1 / 2A
PP2B	protein phosphatase 2B (calcineurin)
PPF	paired pulse facilitation
PSD	postsynaptic density
R	regulatory subunit of PKA
R(AB)	dominant negative inhibitor of PKA
RNA	ribonucleic acid
Rp-cAMPS	adenosine- 3', 5'-cyclic monophosphorothioate, Rp-isomer
rsh	*radish*
rut	*rutabaga*
S	stimulating electrode
SC	Schaeffer collateral
SEM	standard error of the mean
Sp-cAMPS	adenosine- 3', 5'-cyclic monophosphorothioate, Sp-isomer
TBS	thetaburst stimulation
US	unconditioned stimulus
WT	wildtype
5-HT	serotonin

*CHAPTER I:

GENERAL INTRODUCTION

*Portions of this chapter have been submitted for publication:

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Preface

Elucidating the mechanisms of learning and memory in the mammalian brain is one of the most difficult goals in science. Researchers are currently trying to understand how the central nervous system acquires (learning), stores (memory), and utilizes information derived from the external environment. These cognitive processes are crucial for survival and productivity in human society. Analysis of learning and memory has been achieved at many different levels: behavioral, systems, and more recently, cellular and molecular.

The neuron doctrine, which states that neurons are the functional units of the nervous system, was formulated by Santiago Ramon y Cajal. Shortly thereafter, a British neurophysiologist, Charles Sherrington, coined the term "synapse" to describe the connections between adjacent neurons. Cajal first proposed the notion that modifications of the synaptic interactions between neurons may underlie many cognitive processes, including learning and memory. In the 1940's, the Canadian psychologist, Donald Hebb, postulated that coincident activity between two connected neurons leads to strengthening of their synaptic connection (Hebb, 1949). This idea that changes in neural activity lead to persistent synaptic modifications, termed synaptic plasticity, is a central principle that has guided current research on cellular mechanisms of learning and memory.

Although much progress has been made in delineating various forms of synaptic plasticity, a number of significant challenges remain. In most cases, studies have primarily examined the functional significance of shorter-lasting forms of synaptic plasticity. However, the physiological functions of longer-lasting forms of synaptic plasticity, such as the late phase of long-term potentiation (L-LTP), have only recently been addressed.

The primary aim of this thesis is to identify some of the factors that critically regulate the expression of long-lasting forms of synaptic plasticity in the mammalian brain. Identifying these critical factors will provide important insights on the cellular events that may underlie certain forms of learning and memory.

Learning and Memory: Insights from Human Case Studies and Animal Models

Many early clinical studies conducted on amnesic patients with specific brain lesions have provided insights into the neural bases of learning and memory. Based on these studies, it became apparent that there exist different forms of memory that are mediated by distinct brain structures (*i.e.* memory systems) (Scoville and Milner, 1957; reviewed by Squire, 1992). Cognitive psychologists recognize two broad categories of memory: implicit and explicit memory also known as nondeclarative and declarative memory, respectively (Squire and Zola-Morgan 1991). Implicit memory refers to procedural information that is recalled without conscious effort. This form of memory usually involves recall of reflexive motor or perceptual skills. In contrast, explicit memory involves conscious recall of factual knowledge of people, places, and/or things. Over the past decade, remarkable progress has been made in characterizing these distinct forms of memory (Squire *et al.*, 1993).

Evidence for multiple forms of memory was highlighted in a study based on a patient named H.M (Scoville and Milner, 1957). This patient suffered from severe seizures that were not affected by large doses of anticonvulsant drugs. In hopes of reducing the severity and frequency of the seizures, he underwent surgery that bilaterally removed portions of the medial temporal lobe. This procedure selectively impaired his long-term

explicit memory (Scoville and Milner, 1957). More specifically, H.M possessed intact short-term memory that lasted for seconds to minutes, but he could not consolidate recently acquired information to long-term memory. Although explicit memory was impaired, H.M. could learn simple motor procedures, suggesting that his ability to form implicit memory was preserved. In support of this, H.M. showed consistent improvement in a mirror drawing test over several consecutive days. This task involves drawing a line within two concentric outlines of a star while looking through a mirror. After a series of trials, he made fewer errors, indicating that he successfully learned this sensorimotor skill (Milner *et al.*, 1965).

Extensive studies involving H.M. also confirmed the notion that multiple forms of memory are mediated by different parts of the brain. However, an important question that was not addressed was, "which brain region within the medial temporal lobe was responsible for the memory impairments observed?" Many inherent problems arise when trying to address this question in humans. The extent of human brain damage or lesions cannot be adequately controlled and assessed. As in the case of H.M., the removal of the medial temporal lobe included the amygdala, hippocampus, subiculum, entorhinal, perirhinal and parahippocampal cortices (Corkin *et al.*, 1997), and as a result the brain regions critical for memory function could not be identified. In addition, single cell activity monitored from particular brain structures *in vivo*, which is commonly measured in different animal models, cannot be applied to human research. Research conducted on animal models overcome inherent ethical issues associated with human research and permit better control of experimental conditions. Thus, animal models can provide

valuable avenues for incisive experimentation aimed at elucidating the anatomy and physiology of memory.

Primates

Shortly after the report on H.M.'s memory impairments, many attempts were made to replicate the amnesic characteristics seen after medial temporal lobe excision in primate models. Preliminary studies in which the hippocampus was lesioned reported a substantial deficit in a delayed-nonmatching-to-sample task (Gaffan, 1974; Mishkin, 1978). These findings were replicated and extended to other behavioral tasks to show that removal of the temporal medial lobe impaired memory (Zola-Morgan and Squire, 1985). Collectively, these studies confirmed the model of amnesia by showing that short-term memory, as well as information acquired prior to surgery, were intact. These studies also provided valuable behavioral assessment tools and techniques to probe and delineate the anatomical components of the medial temporal lobe memory system in animals.

Because of the versatility of animal models, application of restricted lesions clarified the contribution of various brain areas involved in memory formation. Ablation of the amygdala, using stereotaxic surgical methods, did not produce deficits in memory as assessed by delayed-nonmatching-to-sample tasks and object discrimination tasks (Zola-Morgan *et al.*, 1989a, 1991). In contrast, selective lesions of either the hippocampus (Zola-Morgan *et al.*, 1992; Alvarez *et al.*, 1995; Zola *et al.*, 2000) or the perirhinal and parahippocampal cortices (Murray and Mishkin, 1986; Zola-Morgan *et al.*, 1989b; Suzuki *et al.*, 1993; Meunier *et al.*, 1993) in monkeys produced severe impairment of explicit long-term memory.

Rodents

Research on rodents has also shed light on the function of the hippocampus in learning and memory. Multiple theories of hippocampal information processing in relation to learning and memory have been proposed by many neuroscientists (see Eichenbaum and Cohen, 2001 for a general discussion).

One proposed function of the hippocampus is for the formation of "cognitive maps" that represent spatial information (*e.g.* coordinates) in the environment. This is supported by the observation that certain principal neurons, termed place cells (O'Keefe, 1976), fire when an animal is in a particular location within its environment (O'Keefe and Dostrovsky, 1971; O'Keefe and Burgess, 1996). Animals with hippocampal damage were severely impaired in many forms of spatial exploration and learning (O'Keefe and Nadel, 1978; O'Keefe and Conway, 1980). In one particular study, spatial learning after transection of the fornix was assessed by an elevated plus maze. Rats were trained to find a food reward in one baited arm with a fixed number of either distributed-cues or clustered-cues. Both groups performed well in the clustered-cue task. However, rats that underwent fornix transection were severely impaired compared to normal control rats in the distributed-cue version (O'Keefe and Conway, 1980). Thus, the rats that underwent fornix transection could not use the spatial arrangement of cues to solve the maze problem in the distributed-cue version.

Another test widely used to assess spatial memory is the *Morris water maze* (Morris, 1984). This task involves finding a hidden escape platform in a large pool of opaque fluid (Morris, 1984). After repeated training trials, rats learn to use visible cues on the walls around the room to locate the hidden platform. This results in decreased latency

times for locating the hidden platform. Studies that have inactivated the hippocampus either by physical ablation (Morris *et al.*, 1982) or by using receptor antagonists to block synaptic transmission or long-term potentiation (Davis *et al.*, 1992; Riedel *et al.*, 1999), resulted in increased escape latencies and a lack of preference for the quadrant in which the platform was located during training (Morris *et al.*, 1982; Riedel *et al.*, 1999).

Although it is well established that the hippocampus is important for spatial learning, its role is not restricted to spatial learning. Studies have demonstrated that some hippocampus-dependent memory tasks emphasize learning of the spatial context as a whole, instead of individual spatial cues (Phillips and Ledoux, 1992). During *contextual fear conditioning*, a rat is trained to associate the environment with an unconditional stimulus such as a noxious shock (see section on Fear Conditioning). Upon re-exposure to the environment where it was shocked, the animal displays fear responses, such as freezing. Ablation of the hippocampus blocked the acquisition of context-elicited freezing (Selden *et al.*, 1991; Kim and Fanselow, 1992; Phillips and LeDoux, 1992). In this case, instead of the animal creating a cognitive spatial map that it uses to navigate, it is thought that during fear conditioning the animal forms an associative neural representation of the context (Fanselow, 2000; Maren *et al.*, 1997).

In a different memory task, known as *social transmission of food preference*, the preference for a particular type of food may be altered based on the experience of a demonstrator. This form of social learning is based on the notion that recently consumed food is viewed as safe. Thus, when an observer encounters a demonstrator that has recently eaten a distinct scented food that is not preferred, the observer will tend to select the same scented food over other foods when presented a choice (Galef and Wigmore, 1983; Galef,

1985). Although memory for this type of social transmission is classified as non-spatial, it is critically dependent on the hippocampal region (Winocur, 1990; Bunsey and Eichenbaum, 1995).

Memory Consolidation

Clearly, in addition to mediating the acquisition of new information, the hippocampal system is also important for the conversion of newly acquired short-term memory to longer-lasting forms of memory. This is also known as *memory consolidation* during which new memories are transformed from a labile state to a more resilient form (Squire *et al.*, 1984). Humans with damage to the medial temporal lobe typically have intact short-term memory but show a delay-dependent memory deficit (Scoville and Milner, 1957). Likewise, following hippocampal lesions, animals cannot perform in delayed-nonmatching-to-sample tasks if the delay is greater than a few minutes (Zola-Morgan and Squire, 1985). Collectively, these studies show that initial acquisition of declarative memory is labile and cannot be fully consolidated to a permanent form in the absence of hippocampal function.

Although important for consolidation, it is well established that the hippocampus is not the permanent site of memory storage. This fact stems from studies of *retrograde amnesia*, in which memory loss is temporally graded (Squire and Alvarez, 1995). In other words, the level of memory impairment is inversely related to the recency of an event. For example, patients who underwent electroconvulsive therapy displayed retrograde amnesia extending back from 1-3 years (Squire *et al.*, 1975; Squire and Cohen, 1979). In amnesic patients, remote memories for places learned long ago are consistently intact. For instance,

patient E.P. who experienced bilateral damage to his hippocampus was able to recall the spatial layout of the region where he grew up (Teng and Squire, 1999). His performance was comparable to aged matched controls who also grew up in the same region and moved away. Although he can recall his childhood neighborhood, he had no knowledge of his current neighborhood, to which he moved after he became amnesic (Teng and Squire, 1999). Thus, the hippocampus initiates a gradual process of reorganization and stabilization of learned information, after which it is not required for storage or retrieval of declarative memory (Squire and Alverez, 1995).

Hippocampal Memory System

The hippocampus receives processed information from various cortical areas (**Figure 1.1**). It has been proposed to act as a "gateway" that brings together and associates incoming information over time to encode explicit memory (Mishkin *et al.*, 1998). All association areas in the neocortex, including those in the parietal, temporal and frontal lobes converge to the hippocampus through the parahippocampal and entorhinal cortex (Suzuki and Amaral, 1994a,b). This information flow also occurs in the reverse direction, from hippocampus back to these same neocortical areas (Deacon *et al.*, 1983; Van Hoesen *et al.*, 1972; Amaral and Witter, 1989). Thus, information flow to and from the hippocampus is bidirectional (**Figure 1.1**).

Taken together, the function of the hippocampus covers a broad domain that includes spatial-, non-spatial-, and context dependent- forms of learning and memory. This allows the hippocampal system to support the formation of *relational* representations of information in memory (Eichenbaum *et al.*, 1992; Cohen and Eichenbaum, 1993), and that

permits the inferential manipulation of memories in novel situations (Eichenbaum and Cohen, 2001).

Once declarative memory is encoded in the hippocampus then a conversion of the labile state to a permanent form may be transferred (*i.e.* memory consolidation) to other areas of the brain (Squire *et al.*, 1984; Squire and Alvarez, 1995; Bontempi *et al.*, 1999). Anatomical and behavioral studies indicate that the functional organization of the medial temporal lobe system is similar across species (Squire, 1992; Mayford *et al.*, 1996). Moreover, the requirement for the hippocampus for memory formation is a common thread among humans, non-human primates, and rodents (Squire, 1992).

Neuronal Communication within the Mammalian Hippocampus

Chemical synaptic transmission within the mammalian brain is believed to underlie numerous cognitive processes. Because different behaviors arise from alterations at the connections between cells within various brain regions, an understanding of the properties of individual neurons is required for elucidating the cellular bases of behavioral plasticity (Kandel, 1976). *Synapses* are the site of chemical synaptic transmission, where signals are transmitted from the nerve terminal of a presynaptic cell to the postsynaptic cell.

Communication between neurons is initiated when an action potential invades the presynaptic terminal. Depolarization of the presynaptic cell causes an influx of Ca^{+2} and results in the release of a chemical neurotransmitter from the nerve terminal. The neurotransmitter then binds to its respective receptor on a postsynaptic cell. This results in a transient change in ion conductance of the postsynaptic cell, producing a graded change in membrane potential. Analysis of these postsynaptic responses has identified two distinct

forms of potentials, called excitatory and inhibitory postsynaptic potentials. When summation of these synaptic potentials reaches or exceeds a particular threshold, an action potential is initiated.

A remarkable property of synapses is its inherent degree of plasticity. The strength of a synapse often can be modified, such that synaptic transmission is enhanced or depressed. Such modifications in the efficacy of synaptic transmission are thought to underlie a number of important behaviors. For example, transient modifications of synaptic transmission have been associated with sensory adaptation and alteration in receptive fields (O'shea and Rowell, 1976; Furukawa *et al.*, 1982). In addition, transient synaptic depression at sensory terminals was found to underlie habituation of escape responses to repeated stimuli (Auerbach and Bennet, 1969; Zucker 1972; Zilber-Gachelin and Chartier, 1973).

Hippocampal Synaptic Plasticity

Donald Hebb formulated the notion that memory may be stored as a result of cellular activity (Hebb, 1949). His famous postulate stated that the strength of a synapse can be increased when firing of the presynaptic and postsynaptic neuron is closely correlated in time. Hebb proposed that this co-activation could lead to synaptic changes that outlast the initiating signal (*e.g.* a novel event). *Synaptic plasticity* (*i.e.* activity-dependent changes in synaptic efficacy) underlies many of the current models that attempt to explain how information is stored in the mammalian brain

In the hippocampus, a long lasting form of synaptic plasticity was first demonstrated by Timothy Bliss and Terje Lomo (1973) in the anesthetized rabbit. Brief

high-frequency stimulation (tetanus) of the entorhinal perforant pathway led to a long-lasting increase in synaptic transmission between the perforant path and their target cells, the dentate granule cells (Bliss and Lomo, 1973). They observed that a single pulse after tetanus elicited an excitatory synaptic potential with a significantly steeper slope. Because enhancement of the synaptic response persisted long after tetanus, they termed this *long-term potentiation (LTP)*. Consequently, LTP has been extensively studied in the mammalian hippocampus.

LTP has attracted a prolific amount of attention partly because a diverse range of signalling pathways, neurotransmitters, and neuromodulatory substances exists in the mammalian hippocampus that can regulate induction and expression of LTP (Sanes and Lichtman, 1999). There are numerous types of LTP, including those that appear to require tyrosine kinases (O'Dell *et al.*, 1991; Grant *et al.*, 1992; Kojima *et al.*, 1997; Lu *et al.*, 1998; Huang and Hsu, 1999), protein kinase C (Lovinger *et al.*, 1987; Hu *et al.*, 1987; Malinow *et al.*, 1989; Colley *et al.*, 1990; Wang and Feng, 1992; Abeliovich *et al.*, 1993; Hvalby *et al.*, 1994; Osten *et al.*, 1996; Ling *et al.*, 2002), nitric oxide (Schuman and Madison, 1991; O'Dell *et al.*, 1991, 1994; Izumi *et al.*, 1992; Zhuo *et al.*, 1993; Williams *et al.*, 1993; Lu *et al.*, 1999; Bon and Garthwaite, 2003), various neuropeptides (Wagner *et al.*, 1993; Derrick and Martinez, 1994; Terman *et al.*, 1994; Sakurai *et al.*, 1996; Manabe *et al.*, 1998; Whittaker *et al.*, 1999; Wang *et al.*, 2000; Coumis and Davies, 2002; Tomizawa *et al.*, 2003), metabotropic glutamate receptors (Bortolotto and Collingridge, 1993; Conquet *et al.*, 1994; Richter-Levin *et al.*, 1994; Bortolotto *et al.*, 1994, 1999; Lu *et al.*, 1997; Wilsch *et al.*, 1998), or voltage-gated calcium channels (Kullman *et al.*, 1992; Castillo *et al.*, 1994; Huber *et al.*, 1995; Cavus and Teyler, 1996; Izumi and Zorumski,

1998) for their induction or expression. However, the form of LTP most widely studied is the NMDA receptor dependent form, which will be discussed in more depth in subsequent sections (for reviews see Collingridge and Bliss, 1995; Malenka and Nicoll, 1993, 1999).

However, if only one predominant form of plasticity that increased synaptic efficacy existed, then saturation of synapses would severely limit the storage capacity of neurons. Thus, a mechanism for decreasing synaptic efficacy is required. Indeed, different forms of plasticity that decrease synaptic strength, *e.g. long-term depression (LTD) and depotentiation (DPT)*, have been discovered and are mediated by different mechanisms (Fujii *et al.*, 1991; Bashir and Collingridge, 1994; Dudek and Bear, 1992, 1993; O'Mara *et al.*, 1995; Bear and Abraham, 1996; Oliet *et al.*, 1997). One form of long-lasting hippocampal synaptic depression that has received attention is homosynaptic LTD. Prolonged low-frequency stimulation induces homosynaptic LTD that requires NMDA receptor activation as well as a rise in intracellular Ca^{+2} concentration (Dudek and Bear, 1992). Thus, bidirectional modification of synaptic transmission is evident in the hippocampus, and may function to increase the capacity for information storage.

Synaptic Organization of the Hippocampus

In the mammalian brain, the hippocampus is a C-shaped structure extending from the temporal lobe caudoventrally to the septal nuclei rostrodorsally (Amaral and Witter, 1989). The subdivisions of the hippocampus are well defined and consist of Ammon's horn, the dentate gyrus and the subiculum. Ammon's horn can be further subdivided into four subfields: cornu ammonis 1 – 4 (*i.e.* CA1 – CA4) (Lorente de No, 1934).

Anatomical (Blackstad *et al.*, 1970; Hjorth-Simonsen and Jeune, 1972; Hjorth-Simonsen *et al.*, 1973; Amaral and Witter, 1989) and electrophysiological studies (Andersen *et al.*, 1971) have described the hippocampus as a layered, or lamellar, structure. Because of this layered arrangement, the hippocampus is very amenable to cellular electrophysiology experimentation. The hippocampal slice, a commonly used *in vitro* preparation, contains a functional and intact circuit. This "tri-synaptic" circuit consists of the dentate gyrus, CA1, and CA3 connected in series (**Figure 1.2A**). The perforant pathway, originating in the entorhinal and perirhinal cortices, projects to the molecular layer of the dentate gyrus. The granule cells, the principal cell type receiving the majority of the input, project to the proximal dendrites of the CA3 pyramidal neurons, via the mossy fibres. A distinct property of the CA3 pyramidal neurons is their large number of collateral axons. Some of these axons terminate on other CA3 cells within the same field, whereas some give rise to the *Schaeffer collaterals* that project to the CA1 region.

A common theme of current research on learning and memory is that the extent of a memory deficit is proportional to the amount of damage to the medial temporal lobe (Zola-Morgan *et al.*, 1994; Rempel-Clower *et al.*, 1996; Zola and Squire, 2001). However, damage limited to a restricted area within the hippocampus is sufficient to elicit memory impairment (Zola-Morgan *et al.*, 1986). A patient known as R.B. suffered from cardiac arrest that selectively destroyed the CA1 region of his hippocampus. As a result, he had explicit memory deficits and exhibited retrograde amnesia (Zola-Morgan *et al.*, 1986). This proves that one particular subregion of Ammon's Horn is important for memory storage. Behavioral studies in animals with selective lesions to the CA1 region also show that memory deficits can occur after such lesions (Auer *et al.*, 1989). Furthermore, studies

using genetic approaches show synaptic plasticity in the CA1 region is required for certain forms of memory (Tsien *et al.*, 1996; Nakazawa *et al.*, 2002). Consequently, many studies have focused on the CA1 region of the hippocampus to probe the relationship between synaptic plasticity and behavioral learning and memory.

Glutamatergic Synaptic Transmission

Glutamatergic synapses are the most prevalent type of excitatory synapses in the mammalian hippocampus. L-glutamate binds to two distinct classes of glutamate receptors: the ligand-gated ion channels (ionotropic receptors) and the G-protein coupled receptors (metabotropic receptors) (**Figure 1.2B**). Much research has focused on the ionotropic class of receptors because they play an important role in synaptic plasticity. These will be discussed further in subsequent sections (**Figure 1.4B**). Two distinct types of ionotropic glutamate receptors were initially identified on the basis of specific agonists and antagonists (Watkins and Evans, 1981): N-methyl-D-aspartate (NMDA) and non-NMDA receptors. Developments in electrophysiological techniques, such as whole cell recording (Blanton *et al.*, 1989), have allowed the pharmacological properties of the excitatory postsynaptic current / potential (EPSC / EPSP) to be extensively characterized in the CA1 region. In CA1, the synaptic response at excitatory synapses consists of two components: a fast, rapidly decaying non-NMDA receptor mediated component and a late, slow onset NMDA receptor mediated component (Hablitz and Langmoen, 1982; Herron *et al.*, 1985; Collingridge *et al.*, 1988; Hestrin *et al.*, 1990). The kinetics and time course of these two components are markedly different, with the NMDA component of the EPSP lasting much longer than the non-NMDA component.

The NMDA receptor is a heteromeric receptor composed of two subunits, NMDAR1 and NMDAR2. The latter subunit exists in four isoforms termed, NMDAR2A-D (for review see Hollmann and Heinemann, 1994; Dingledine *et al.*, 1999). Studies demonstrate that recombinant heteromeric NMDA receptors display different properties depending on the subunit composition, with NMDAR1 serving as the fundamental subunit and NMDAR2 subunits having a modulatory role. NMDA receptors are important because they are the major Ca^{+2} conducting (MacDermott *et al.*, 1986; Mayer and Westbrook, 1987) glutamatergic channel. These receptors also function as a "coincidence detector" of conjoint presynaptic activity and postsynaptic depolarization. The convergence of these two signals, which displaces Mg^{+2} from the channel pore, is required for NMDA receptor gating (Mayer *et al.*, 1984; Nowak *et al*, 1984) and concomitant influx of Ca^{+2}.

The majority of fast excitatory synaptic transmission in the hippocampus is mediated by a glutamate receptor that is selectively activated by α-amino-3-hydroxy-5-methyl-4-isoxazol-proprionate (AMPA). These receptors are composed of four principal types of subunits, GluR1-4 or GluRA-D (**Figure 1.2B**), and they can be expressed as either homomeric or heteromeric oligomers of the four principal subunits (Hollmann and Heinemann, 1994; Dingledine *et al.*, 1999). Functional properties of native AMPA receptors are dictated by subunit composition. For example, coexpression of GluR2 with other subunits results in an AMPA receptor that is predominantly Ca^{+2} impermeable and outward rectifying (Jonas *et al.*, 1994; Liu and Cull-Candy, 2002). The properties of this ionotropic receptor can also be regulated by the phosphorylation of individual subunits by various protein kinases (Wang *et al.*, 1991; Greengard *et al.*, 1991; Roche *et al.*, 1996; Barria *et al.*, 1997; Mammen *et al.*, 1997).

More recently, an additional glutamate receptor subclass sensitive to kainate has been identified (Castillo *et al.*, 1997; Vignes and Collingridge, 1997; Frerking *et al.*, 1998; Cossart *et al.*, 1998). This receptor is encoded by two gene families, GluR5-7 and KA-1 and KA-2, which have significant structural homology to AMPA receptors (**Figure 1.2B**). Although there is evidence that kainate receptors are expressed in pyramidal neurons, they do not significantly contribute to the EPSC in the CA1 region (Frerking *et al.*, 1998; Bureau *et al.*, 1999).

The final class of glutamate receptors is known as the metabotropic glutamate receptors. These receptors are G-protein coupled receptors that can be divided into three broad categories based on their homology: Group 1-3 mGluR. So far, eight subtypes have been identified, mGluR1-8 (**Figure 1.2B**). Activation of mGluRs results in a number of diverse cellular actions, including inhibition of Ca^{+2} channels and K^{+} channels (for review see: Anwyl, 1999).

Synaptic Plasticity and Learning & Memory

Progress in elucidating the mechanisms of learning and memory has been greatly facilitated by research on invertebrates (for review see Burrell and Sahley, 2001). Invertebrates are useful experimental models because of several traits that facilitate genetic manipulations and intracellular recordings of synaptic activity from identifiable cells. In addition, cellular sparsity of the CNS of invertebrates allows easy mapping of neuronal networks that participate in mediating simple behaviors. Many of the earliest molecular insights were discovered by using this reductionist approach, and they support the notion

that activity-dependent changes in chemical transmission between synapses underlie certain forms of behavioral learning (Kandel, 1976).

Aplysia – Sensitization

Sensitization is a form of implicit learning that has been extensively characterized in *Aplysia* (**Figure 1.3**). *Aplysia* readily learns to increase its defensive reflexes to a neutral stimulus after it has been exposed to harmful or noxious stimulus, such as a shock (Pinsker *et al.*, 1973). By recording the amplitude and duration of gill withdrawal reflexes after shocking the tail, behavioral memory of sensitization can be monitored. The duration of sensitization was observed to be proportional to the number of shocks administered. A single training trial elicited a short lasting form of sensitization whereas multiple training trials elicited a much longer lasting form (Pinsker *et al.*, 1973; Frost *et al.*, 1985; Castellucci *et al.*, 1989).

Because the underlying neuronal circuitry mediating the gill withdrawal reflex is known, significant advances in understanding the cellular and molecular mechanisms involved in sensitization have been achieved (Castellucci *et al.*, 1970; Byrne *et al.*, 1974, 1978; Hawkins *et al.*, 1981a,b; Castellucci *et al.*, 1986). It was discovered that a noxious stimulus strongly activated interneurons, which synapse onto the sensory neurons and facilitate the efficacy of transmission between the sensory neurons and its targets (Hawkins *et al.*, 1981; Cleary *et al.*, 1995). Other studies examined the mechanism by which this facilitation occurred at the cellular level by using tissue culture methodology. Reconstitution of the neurons that make up the monosynaptic pathway in cultures allowed for a variety of mechanistic questions to be addressed (**Figure 1.3B**; Rayport and

Schacher, 1986). In this culture model, application of serotonin, one of the neuromodulators of the withdrawal reflex, mimicked the noxious stimulus induced release of endogenous serotonin from the interneurons to elicit an increase in synaptic efficacy (Mackey *et al.*, 1989; Glanzman *et al.*, 1989). This facilitatory effect of serotonin was mediated in part by an increase in transmitter release and an increase in excitability due to an inhibition of potassium channels (Klein and Kandel, 1980; Hochner *et al.*, 1986; Scholz and Byrne, 1987).

A single pulse of serotonin was found to elicit short-term synaptic facilitation effects that were not sensitive to protein synthesis inhibition (Schwartz *et al.*, 1971). However, when multiple pulses of serotonin were applied to the sensory neuron, a long-lasting form of facilitation was induced and this was blocked by inhibitors of protein synthesis (Montarolo *et al.*, 1986; Castellucci *et al.*, 1986). Similarly, application of protein synthesis inhibitors blocked long-term sensitization (Castellucci *et al.*, 1989). Collectively, these studies indicate that both long-term sensitization and long-term facilitation require protein synthesis, whereas short-term facilitation and short-term sensitization require covalent modification of pre-existing proteins. Furthermore, both behavioral sensitization and facilitation at the cellular level share many other similar requirements, including PKA activation and gene transcription (**Figure 1.3C**). This correlation between synaptic plasticity and behavior in *Aplysia* has provided a critical foundation for subsequent studies on mammalian behavior and synaptic plasticity (Goelet *et al.*, 1986; Kandel, 2001).

Links Between LTP, Learning and Memory

Although correlations between LTP and hippocampal-dependent memory have been observed in mammalian models, definitive evidence for cause and effect is still lacking. A direct answer to the question, "Does LTP equal memory?" (Stevens, 1998), has been elusive because of the many technical difficulties associated with the methodology required to directly address this question (for review see Martin *et al.*, 2000). For instance, memory of a previous experience should be detected as a change in synaptic efficacy in the hippocampus. However, storage of information may occur over a spatially distributed set of synapses, making the search for potentiated synapses analogous to searching for a needle in a haystack. Instead, other strategies, such as occlusion studies, have been developed to relate LTP with learning and memory. These occlusion studies entail preventing (occluding) learning after strong and repeated stimulation that induces synapse saturation, a state in which further LTP cannot be induced. Initially, this hypothesis was supported by studies that tested behavioral learning after artificially inducing synaptic saturation. Spatial learning assessed by either a circular maze (McNaughton *et al.*, 1986) or a Morris water maze (Castro *et al.*, 1989) was severely impaired. However, subsequent studies did not replicate these findings (Cain *et al.*, 1993; Jeffery and Morris, 1993; Korol *et al.*, 1993; Sutherland *et al.*, 1993). The issue of LTP saturation and memory impairment was at an impasse until a more recent study re-examined this issue using more sophisticated methods (Moser *et al.*, 1998). This clever study used an array of cross-bundle stimulation electrodes that was designed to maximally activate the perforant pathway. Control animals that received low-frequency stimulation learned the location of the hidden platform in the Morris water maze. Those animals that received high-frequency stimulation

of the angular bundle, thereby preventing further LTP, showed impaired learning (Moser *et al.*, 1998) and defective spatial memory (Brun *et al.*, 2001). Despite these positive findings, careful consideration of the secondary changes induced by high-frequency stimulation must be assessed. These secondary changes, such as alterations in inhibitory transmission, might account for the difficulties associated with inducing a saturated level of LTP (Moser and Moser, 1999).

Fear Conditioning

The strongest evidence for a link between LTP and memory has emerged from studies of conditioned fear in the amygdala. Pavlovian fear conditioning is widely used to study learning and memory because of the simpler neuronal network involved and the experimental control over the stimulus contingencies. Training consists of pairing a neutral conditioned stimulus (CS), such as a sound, with an unconditioned stimulus (US), such as an electric shock. The environment of the training trial, often referred to as the context, can also act as a conditioned stimulus. When animals are exposed to the CS alone during the testing phase, fear responses, manifested as increased breathing, increased heart rate and a higher incidence of freezing, are clearly evident. Freezing, the fear response most commonly used as an index of learning and memory, is defined as cessation or absence of movement.

The neural circuitry responsible for conditioned fear is well characterized (Davis, 1994; Fendt and Fanselow, 1999; Ledoux, 2000; Maren, 2001). The amygdala is required for the acquisition and expression of cued fear conditioning. It is comprised of several distinct nuclei, including the lateral, basolateral, basomedial, and central nucleus. These

anatomically distinct nuclei form the basolateral complex (BLA), which is a critical site important for acquiring the CS-US association during fear conditioning (Fanselow and LeDoux, 1999; Maren, 1999). Multiple inputs converge onto the BLA and provide the primary sensory interface that convey information about auditory and contextual CS, in addition to the aversive US (LeDoux *et al.*, 1991; Romanski *et al.*, 1993; Canteras and Swanson, 1992; Maren and Fanselow, 1995; Shi and Davis, 1999).

Physical or neurotoxic lesions of the BLA, which do not alter shock sensitivity or basal locomotor activity (Campeau and Davis, 1995; Maren, 1998), when administered before or shortly after training, can impair acquisition and expression of fear conditioning (Cousens and Otto, 1998; Sananes and Davis, 1992; Maren *et al*, 1996). Moreover, inactivating the BLA neurons by muscimol infusion to the amygdala before training impaired fear memory (Helmstteter and Bellgowan, 1994; Muller *et al.*, 1997; Wilensky *et al.*, 1999). Similarly, infusion of APV, a NMDA receptor antagonist, impaired both acquisition and expression of learned fear (Fanselow and Kim, 1994; Gewirtz and Davis, 1997; Lee and Kim, 1998; Lee *et al.*, 2001). Collectively, these results demonstrate that synaptic activity in BLA neurons is critically required for CS-US association during fear conditioning.

In addition, BLA neurons exhibit activity-dependent forms of synaptic plasticity. Several studies have compared the synaptic changes that accompany fear conditioning *in vivo*. Induction of LTP in the thalamo-amygdala pathway increased the magnitude of auditory (CS) evoked potentials in the LA (Rogan and LeDoux, 1995), which is comparable to the fear conditioning-induced increases in the magnitude of CS-evoked unit activity of these neurons (Quirk *et al.*, 1995, 1997). In addition, auditory-evoked responses

of amygdalal neurons after conditioning were significantly potentiated *in vivo* (Rogan *et al.*, 1997). Thus, LTP of thalamo-amygdaloid synaptic transmission is thought to contribute to the acquisition and expression of auditory fear conditioning. In a separate but similar study, fear conditioning enhanced synaptic currents in amygdalal neurons *in vitro* measured after behavioral testing (McKernan and Shinnick-Gallagher, 1997). Rats that received CS-US pairings exhibited an increase in synaptic efficacy in the amygdala, whereas those animals that did not receive CS-US pairings did not exhibit this increase. When synaptic transmission was monitored in the endopyriform nucleus, another part of the brain that does not play a role in fear conditioning, no changes were observed following the conditioning procedures (McKernan and Shinnick-Gallagher, 1997). Thus, induction of amygdalal LTP and acquisition of amygdalal fear conditioning produce parallel changes in the synaptic efficacy of BLA neurons. This represents an important step towards establishing causality between activity-dependent plasticity (*i.e.* LTP) and learning and memory in the mammalian brain.

Hippocampal Long-term Potentiation (LTP)

Modern neuroscience has made significant advances since Hebb's postulate in our comprehension of LTP and its role in memory. As mentioned earlier, an integral part of Hebb's rule is co-activation. Indeed, the hippocampus displays a Hebbian form of LTP that is associative in nature (Levy and Steward, 1979; Barrionneuvo and Brown, 1983; Wigstrom and Gustafsson, 1983). This associativity is characterized by the observation that when a synapse is activated by presynaptic activity and there is substantial postsynaptic depolarization, the synapse will be strengthened (**Figure 1.4A**; Wigstrom and

Gustafsson, 1985; Kelso *et al.*, 1986; Malinow and Miller, 1986; Sastry *et al.*, 1986; Wigstrom *et al.*, 1986).

Another prominent feature that has made hippocampal LTP a strong candidate mechanism for learning and memory is its characteristic persistence over time (Abraham, 2003). Rapidly induced LTP can last for many hours in brain slices, but more impressively, it can persist for several weeks (Bliss and Gardner-Medwin, 1973; Douglas and Goddard, 1975; Barnes, 1979; Barnes and McNaughton, 1980, 1985) and up to a year in intact animals (Abraham *et al.*, 2002). This persistence of LTP was statistically correlated with the degree of retention of spatial memory over time, and it has been confirmed in subsequent studies (Barnes, 1979; Barnes and McNaughton, 1985).

An additional property of hippocampal LTP is its synapse specificity. Hippocampal synapses can be modified independently of one another (Bliss and Lomo, 1973; Andersen *et al.*, 1977). When two sets of synapses that converge onto the same neuron are stimulated and tetanus is applied to one set, these synapses will be strengthened, but the synapses within the other unstimulated set will not be altered (**Figure 1.4A**). This parallels memory specificity in that an individual can remember many distinct episodes involving the same object or place. Because each synapse can theoretically be used to store information, there is a potential for storage of prolific amounts of information. Thus, synapse specificity may importantly contribute to the large storage capacity of the brain.

Mechanisms of LTP

Although there are several types of LTP found in the hippocampus, the NMDA receptor dependent form of LTP is the most widely studied. This form of LTP critically

depends on synaptic activation of postsynaptic NMDA receptors (Collingridge *et al.*, 1983; Wigstrom and Gustafsson, 1986). Activation of NMDA receptor channels requires depolarization by tetanic stimulation or direct depolarization of the postsynaptic cell. At resting potential, NMDA receptors are blocked by extracellular magnesium ions (Mayer *et al.*, 1984; Nowak *et al.*, 1984) and they contribute minimally to basal synaptic transmission. However, when a tetanus or postsynaptic depolarization pulse is administered to induce LTP, Mg^{+2} ions dissociate from their binding site within the NMDA receptor (**Figure 1.4B**). This allows for Ca^{+2} and Na^{+} influx into the dendritic spine. Ca^{+2} is a critical trigger for induction of LTP (**Figure 1.4B**). Specific NMDA receptor antagonists, which have minimal effect on basal synaptic transmission, block LTP induction (Collingridge *et al.*, 1983; Teyler and DiScenna, 1987). Similarly, inhibiting the rise in intracellular Ca^{+2} blocks LTP induction (Lynch *et al.*, 1983; Malenka *et al.*, 1988). By contrast, an increase in intracellular Ca^{+2}, by photolysis of caged Ca^{+2}, mimics LTP (Malenka *et al.*, 1988, 1992; Neveu and Zucker, 1996; Yang *et al.*, 1999).

Genetic Approaches to LTP

Developments in molecular biology (Jaenisch and Mintz, 1974; Costantini and Lacy, 1981; Evans and Kaufman, 1981; Palmiter *et al.*, 1982; Smithies *et al.*, 1985; Thomas and Capecchi, 1986, 1987; Capecchi, 1989; Bradley, 1993) have led to the creation of genetically modified mice. These mice have been used to define the function of signalling cascades in synaptic plasticity and behavioral learning and memory (**Figure 1.5**; Chen and Tonegawa, 1997). Specific genes encoding signal transduction enzymes or receptors can be overexpressed (*transgenic*) or deleted (*knockout*) within specific regions

of the brain (Mayford *et al.*, 1995). Thus, by combining electrophysiological, pharmacological and molecular biological techniques, a developing picture of the cellular mechanisms involved in LTP is beginning to emerge.

The first reports using a genetic approach to investigate the role of a particular molecule in LTP was based on a genetically engineered line of mice that lacked either αCaMKII (Silva *et al*, 1992) or tyrosine kinase fyn (Grant *et al.*, 1992). Null mutations of either signalling enzyme impaired LTP induction in the CA1 region of hippocampal slices. A subsequent study examined the effects of a point mutation that substituted threonine for alanine at position 286, which is the autophosphorylation site of CaMKII (T268A). This method was employed in an attempt to address the biochemical specificity of this key enzyme, since this mutation prevented this autophosphorylation event. Similar to the initial CaMKII knockout study, the T286A mutation also resulted in severe impairment of hippocampal LTP (Giese *et al.*, 1998). More importantly, this study demonstrated the importance of postsynaptic signalling, since presynaptic function was not affected by the point mutation (Giese *et al.*, 1998).

Many pharmacological findings have stimulated subsequent genetic studies of LTP, many of which are in agreement with each other. However, concerns arise from the lack of regional and/or temporal specificity of the genetic manipulations used in many of the earlier studies. The progression of genetic approaches employed to address these inherent problems is highlighted by a series of studies that examined the role NMDARs. As discussed earlier, the NMDAR acts as a molecular coincidence detector and is crucial for induction of LTP. Various studies have manipulated a subunit of the NMDAR to examine its functional significance. Deletion of the NR2 subunit attenuated hippocampal LTP and

impaired spatial learning (Sakimura *et al.*, 1995). In addition, a point mutation in the glycine site of the NR1 subunit also disrupted hippocampal LTP and learning (Kew *et al.*, 2000). However, when the NR1 subunit was completely abolished, severe developmental defects were observed, which resulted in neonatal lethality (Kutsuwada *et al.*, 1996). In addition to the developmental problems associated with the above studies, the regional specificity of the genetic manipulation is also a problem because both subunits are expressed in a wide range of cell types and brain regions. Thus, interpretation of the learning impairments becomes more complicated. Because NMDARs are also known to play a key role in synaptic plasticity expressed in the neocortex (Artola and Singer, 1987; Kirkwood *et al.*, 1993; Bear and Kirkwood, 1993; Castro-Alamancos *et al.*, 1995; Castro-Alamancos and Connors, 1996; Bear, 1996), impairments in spatial learning may be a result of defective cortical sensory processing.

To circumvent the lethality of NR1 deletion and regional specificity problems, a restricted knockout approach was employed. This involved deleting the subunit by using a Cre/LoxP system under the regulation of the αCaMKII promoter (Mayford *et al.*, 1996). Consequently, NR1 ablation was restricted to the CA1 region (Tsien *et al.*, 1996). It was shown that loss of NR1 did not affect the expression of other glutamate receptors. However, the absence of NR1 subunits resulted in selective deficits of LTP and LTD in area CA1 of hippocampal slices (Tsien *et al.*, 1996). Furthermore, spatial learning in the Morris water maze was impaired (Tsien *et al.*, 1996).

A similar approach was used to delete the NR1 subunit in CA3 pyramidal cells of the hippocampus (Nakazawa *et al.*, 2002). In contrast to mice with deletions in the CA1 region, mice that lacked NR1 in area CA3 exhibited intact spatial memory. However, when

only partial external cues were presented, these mice showed impaired spatial memory, suggesting that CA3 area is required for pattern completion (Nakazawa *et al.*, 2002). Collectively, these results show that different subregions of the hippocampus may have different functions related to memory, with CA1 playing a pivotal role. These studies also underscore the advantages of using genetically modified mice to probe the signalling mechanisms underlying LTP and memory.

Cellular Consolidation

As discussed earlier, memory consolidation refers to the notion that recently acquired memories may stabilize over time, rendering them less susceptible to disruption. However, this process of stabilization can be observed at different levels of the brain. Two distinct levels of consolidation have been classified: systems and cellular consolidation (Dudai, 1996). The first refers to processes that occur at a circuit level and may involve the progressive organization of memory traces to different areas (*i.e.* circuits) of the brain. The second refers to processes that take place locally in specific components of the circuit. "*Cellular consolidation*" is highlighted by studies that demonstrate that synaptic plasticity can consolidate into longer-lasting forms that require gene transcription and new protein synthesis (Krug *et al.*, 1984; Stanton and Sarvey, 1984; Frey *et al.*, 1988; Otani *et al.*, 1989; Nguyen *et al.*, 1994; for review see Huang *et al.*, 1996). This conversion, which takes place at the level of the synapse, allows short-term alterations in synaptic transmission to be transformed into a persistent form through the synthesis of new proteins, and may allow for the encoding of long-term memory in the hippocampus.

Long-lasting Forms of Hippocampal Synaptic Plasticity: Late Phase of LTP (L-LTP)

Much of our understanding of the mechanisms for LTP has come from studies that monitor this form of plasticity for periods of up to an hour (Malenka and Nicoll, 1999). But it is well known that LTP can persist for many hours in hippocampal slices, and for many months *in vivo*. Although relatively fewer studies have focused on longer lasting forms of synaptic plasticity, its importance to long-term memory formation has been highlighted in recent studies (Abel *et al.*, 1997; Wong *et al.*, 1999; Guzowski *et al.*, 2000; Jones *et al.*, 2001; Kang *et al.*, 2001; Miller 2002).

Like memory, LTP has at least two distinct temporal phases: an early phase (E-LTP) and a longer lasting late phase (L-LTP) (**Figure 1.6A**). These phases of LTP have been identified in each pathway of the trisynaptic circuit of the hippocampus (Huang and Kandel, 1994; Huang *et al.*, 1994; Nguyen *et al.*, 1994; Nguyen and Kandel, 1996) as well as the amygdala (Huang *et al.*, 2000). In area CA1 of hippocampal slices, E-LTP is usually induced by giving a single train of 100Hz stimulation and lasts approximately 1-3 hours (Huang *et al.*, 1994). In contrast, L-LTP is induced by giving three or more trains of 100Hz stimulation and lasts greater than 8 hours in slices (Frey *et al.*, 1993, Huang *et al.*, 1994; Nguyen *et al.*, 1994). In addition to their different temporal characteristics, L-LTP also differs from E-LTP in its requirements for protein kinase A (PKA) activity and protein synthesis (**Figure 1.6B**; Frey *et al.*, 1988, 1993; Huang *et al.*, 1994; Nguyen *et al.*, 1994; Huang *et al.*, 1996; Matsushita *et al.*, 2001).

cAMP Dependent Protein Kinase (PKA)

cAMP dependent protein kinase is ubiquitously expressed in neurons of the central nervous system, and it can regulate many important cellular processes such as growth, metabolism, development, and gene transcription (Krebs and Beavo, 1979; Montminy, 1997). Beginning with the pioneering work that identified cAMP as the first second messenger (Sutherland and Rall, 1957; Sutherland *et al.*, 1965), much progress has been made in understanding its activation of PKA, which mediates the majority of the effects elicited by cAMP. Preliminary characterization of PKA in muscle (Walsh et el., 1968) and in bovine brain (Miyamoto *et al.*, 1968, 1969) has progressed to the identification and characterization of the many isoforms of subunits of PKA (Cadd and McKnight, 1989)

In the absence of cAMP, PKA is an inactive tetrameric holoenzyme composed of two regulatory (R) subunits bound to two catalytic (C) subunits (**Figure 1.7**). The binding of numerous transmitters and hormones to membrane receptors that are linked by heterotrimeric G-protein complexes to adenylyl cyclase can increase the intracellular synthesis of cAMP (Tang and Gilman, 1991). Cooperative binding of four molecules of cAMP to sites located on the regulatory subunits results in the release of monomeric C subunits (Taylor *et al.*, 1990; Wang *et al.*, 1991; Gibbs *et al.*, 1992). These dissociated C subunits can then phosphorylate serine and threonine residues on numerous downstream proteins (**Figure 1.8A**). Recently, synaptic organizing molecules that target distinct pools of kinases to particular substrates have been identified and characterized (Pawson and Scott, 1997). One type of anchoring protein, known as *A-kinase anchoring protein (AKAP)*, binds to the regulatory subunit of PKA (RII) and confines the holoenzyme to distinct cellular subdomains (**Figure 1.8B**; Hausken *et al.*, 1994). This interaction allows

PKA to be exposed to isolated cAMP gradients and it permits selective regulation of different effectors at higher levels of enzymatic specificity and efficiency than would be possible with a freely diffusing signalling system.

Conserved Requirement of PKA in Synaptic Plasticity, Learning, and Memory

Both vertebrates and invertebrates utilize PKA to perform many evolutionarily conserved processes. Studies on *Aplysia* and *Drosophila* have consistently demonstrated the importance of PKA in various forms of synaptic plasticity and memory (Silva and Murphy, 1999). For instance, studies using *Drosophila* have demonstrated PKA's role in olfactory memory (DeZazzo and Tully, 1995; Davis, 1996; Waddell and Quinn, 2001).

Because *Drosophila* is very amenable to genetic manipulation, it has been intensively studied by neurobiologists. Mutagenesis by chemicals or transposable elements allows for the screening of progeny flies for a certain behavior or lack thereof. For example, screening of flies with learning and memory deficits can be accomplished by an olfactory aversion test, which requires a fly to integrate and associate an odor (CS) with an electric shock (US). During training, flies are exposed to a particular odor and experience an electric shock. After a predetermined time has elapsed, flies are subsequently tested by transporting them to a place where they are allowed to choose between environments having two different odors. The number of flies that avoid the shock associated odor provides an index of learning and memory (Quinn *et al.*, 1974; Tully and Quinn, 1985). The first genetic screening identified four different memory impaired mutant flies: dunce (*dnc*), rutabaga (*rut*), amnesiac (*amn*) and radish (*rsh*) (Quinn *et al.*, 1974, 1979; Dudai *et al.*, 1976; Livingstone *et al.*, 1984). Subsequent detailed analysis of the mutants showed

that three of the mutants were defective in one step of the cAMP cascade (reviewed by Davis, 1996). The gene products of *dnc*, *rut* and *amn* were characterized to be a cAMP specific phosphodiesterase (Byers *et al.*, 1981; Davis and Kiger, 1981; Chen *et al.*, 1986), Ca^{+2}/calmodulin-dependent adenylyl cyclase type I (Livingstone *et al.*, 1984; Levin *et al.*, 1992) and a neuropeptide that stimulates adenylyl cyclase activity (Quinn *et al.*, 1979; Feany and Quinn, 1995), respectively.

The importance of the cAMP/PKA pathway has also been identified in studies of mammalian synaptic plasticity and long-term memory. Application of a PKA activator, Sp-cAMPS, to the Schaeffer collateral - CA1 pathway induces a synaptic enhancement that occludes electrically- induced L-LTP (Frey *et al.*, 1993). In addition, PKA inhibitors, Rp-cAMPS and KT-5720, block L-LTP when applied to slices during multiple trains of high-frequency stimulation (Frey *et al.*, 1993; Huang and Kandel, 1994). The results of these pharmacological studies have been recently confirmed by genetic studies that have manipulated a step in the cAMP/PKA cascade (Brandon *et al.*, 1995; Huang *et al.*, 1995; Qi *et al.*, 1996; Abel *et al.*, 1997; Wong *et al.*, 1999).

In the mouse hippocampus, there are at least four different types of PKA regulatory subunits (RIα, RIβ, RIIα, RIIβ) and two different catalytic subunits (Cα, Cβ), each encoded by a unique gene (Cadd and McKnight, 1989). A comprehensive cDNA library of each subunit has been constructed and mutant mice have been developed to study the links between the subunits of PKA and the neurophysiological and behavioral processes (for review see Brandon *et al.*, 1997). Genetic ablation of the Cβ1 catalytic subunit of PKA attenuated the expression of L-LTP in the Schaeffer collateral pathway (Qi *et al.*, 1996) and in the mossy fibre pathway (Huang *et al.*, 1995). However, these knockout mice did

not show alterations in PKA activity or behavioral memory (Huang *et al.*, 1995), presumably because of compensatory changes in the levels of other PKA subunits (Brandon *et al.*, 1995; Qi *et al.*, 1996). To circumvent many of the problems associated with conventional knockout mice (see earlier section on Genetic approaches to LTP), Abel *et al.* (1997) employed a transgenic approach to reduce PKA activity (**Figure 1.5**). A transgenic mouse line that expresses R(AB), a dominant negative form of the RIα regulatory subunit of PKA, was created to examine the role of PKA in L-LTP and memory. R(AB) possesses mutations to both cAMP binding sites and acts as a dominant negative inhibitor of PKA (Clegg *et al.*, 1987; Ginty *et al.*, 1991). A CaMKIIα promoter was used to drive postnatal expression of R(AB) in the forebrain (Mayford *et al.*, 1996). As a result, these mice show a 40-50% reduction in hippocampal basal PKA activity (Abel *et al.*, 1997). The transgenic mice do not display any alterations of basal synaptic transmission or short-term plasticity when compared to wildtype littermates. However, when multiple trains of high-frequency stimulation were applied to mutant slices, a deficit in L-LTP was observed (Abel *et al.*, 1997). This was correlated with impaired long-term memory (LTM) as assessed by the Morris water maze and contextual fear conditioning. The deficits in LTM were selective to hippocampus-dependent processes because mutant mice exhibited normal LTM in a taste aversion task that is dependent on amygdalal but not hippocampal function (Yamamoto *et al.*, 1994). In a subsequent study, genes for adenylyl cyclase types 1 and 8 (AC1 & AC8) were deleted (Wong *et al.*, 1999). These genes encode calmodulin-stimulated adenylyl cyclases that are Ca^{+2} sensitive. Mice that lacked both forms of adenylyl cylcase showed selective impairments of L-LTP and were also impaired in LTM for passive avoidance and contextual learning (Wong *et al.*, 1999). Collectively,

these studies directly demonstrate that the cAMP/PKA signalling pathway has a critical role in consolidating both short-term memory and short-term synaptic plasticity to more enduring forms in the mammalian brain.

Downstream Effectors

Recent studies have examined the downstream effectors of PKA to determine how transient increases in cAMP caused by repeated trains of tetanic stimulation (Chetkovich *et al.*, 1991; Frey *et al.*, 1993) lead to long-term synaptic changes such as L-LTP. Although the precise underlying molecular mechanisms have not been fully elucidated, many downstream proteins, including some that contribute to other signalling cascades, have been demonstrated to be important mediators of synaptic plasticity (**Figure 1.8A**). Phosphorylation of these proteins by PKA controls a diverse range of synaptic processes, including gene transcription, transmitter receptor function, and transmitter release. In addition, PKA interacts with several other important signal transduction enzymes.

Interactions with Other Signalling Cascades

Numerous signalling cascades have been implicated in the expression of hippocampal synaptic plasticity (Micheau and Riedel *et al.*, 1999; Sanes and Lichtman, 1999; Soderling and Derkach, 2000). Many of these cascades interact extensively to create an intricate network of signalling cascades that can be regulated at various levels, thus enhancing the processing capabilities of neurons (Weng *et al.*, 1999; Bhalla and Iyengar, 1999). cAMP/PKA can interact with, and affect the functions of, many different signalling molecules. Protein phosphatases are of prime interest because they have been strongly

implicated in the expression of hippocampal synaptic plasticity (Mulkey *et al.*, 1993, 1994; O'Dell and Kandel, 1994; Allen *et al.*, 2000; Brown *et al.*, 2000; Malleret *et al.*, 2001; Zeng *et al.*, 2001; for review see Winder and Sweatt, 2001).

Protein Phosphatases

Three principal types of protein phosphatases are found in the hippocampus: protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A) and calcineurin (PP2B). Collectively, these phosphatases antagonize kinases by dephosphorylating several different substrates (for review see Price and Mumby; 1999; Rusnak and Mertz, 2000; Cohen, 2002. One important modulator of phosphatase activity is the cAMP signalling pathway. Activation of dopaminergic receptors, which are coupled to cAMP production, leads to phophorylation of DARPP-32 (dopamine and cAMP-regulated phosphoprotein, 32 KDa), an isoform of I-1 (inhibitor-1) that is an endogenous inhibitor of PP1 (Hemmings *et al.*, 1984; reviewed by Greengard *et al.*, 1999). This interaction between PKA / I-1 / PP1 has been implicated in E-LTP by studies suggesting that activation of the cAMP/PKA pathway may "gate" expression of E-LTP by suppressing PP1's inhibitory constraint on E-LTP (Blitzer *et al.*, 1995, 1998). PKA is thought to phosphorylate I-1, thereby enabling I-1 to inactivate PP1 and allow E-LTP expression (Blitzer *et al.*, 1995, 1998). Conversely, dephosphorylation of I-1 by calcineurin (PP2B; Ca^{+2}/calmodulin-dependent protein phosphatase) is thought to enable PP1/2A activation to mediate LTD (Mulkey *et al.*, 1994). One key downstream regulatory event mediated by phosphatases is the dephosphorylation of glutamate receptors. In addition, both AMPA/kainate- and NMDA- receptors are subject

to modulation by protein phosphatases (Wang *et al.*, 1994; Lieberman and Mody, 1994; Lee *et al.*, 2000).

cAMP Responsive Element Binding Protein (CREB)

A critical downstream effector of PKA is the cAMP responsive element binding protein (CREB). This nuclear protein modulates the transcription of genes with cAMP responsive elements (CRE) in their promoters (see review Lee and Masson, 1993; Montminy, 1997; Lonze and Ginty, 2002). Studies have identified Ser-133 as a key regulatory residue that must be phosphorylated for CREB to function as a stimulus-dependent transcriptional activator. Although it was initially discovered that PKA critically regulates the phosphorylation of this key regulatory site (Gonzalez and Montminy, 1989), many other signalling cascades and molecules, including ERK/MAPK, CaMKIV, CREB binding-protein (CBP), and protein phosphatases, can also regulate CREB phosporylation (**Figure 1.9A**; Bito *et al.*, 1996; Ho *et al.*, 2000; Kang *et al.*, 2001; Xing *et al.*, 1996; Impey *et al.*, 1998, 2002; for review see Lonze and Ginty, 2002).

Preliminary studies in *Aplysia* and *Drosophila* have demonstrated that members of the CREB family of transcription factors are necessary for long-lasting forms of synaptic plasticity and long-term memory (Dash *et al.*, 1990; Tully, 1991; Yin *et al*, 1994, 1995; Bartsch *et al.*, 1995; Martin *et al.*, 1997). In the reconstituted cell culture *Aplysia* model, many mechanisms of activity dependent gene expression were discovered, one of which was the requirement of cAMP/PKA - CREB pathway (Dash *et al.*, 1990). Subsequent studies have extended this finding and demonstrated the necessity and sufficiency of the CREB transcriptional pathway for long-term facilitation (LTF) (Kaang *et al.*, 1993;

Alberini *et al.*, 1994; Bartsch *et al.*, 1995, 1998). Likewise, studies using *Drosophilae* have demonstrated a critical role for CREB in learning and memory. Inducible expression of a CREB activator or repressor was found to enhance or block the formation of long-term memory in the olfactory aversion task, respectively (Yin *et al.*, 1994, 1995).

Several studies have implicated CREB in synaptic plasticity in mammals. Increased CRE-mediated gene expression in CA1 was induced by electrical stimulation that normally elicits L-LTP but not E-LTP (Impey *et al.*, 1996), as well as by contextual and passive avoidance learning (Impey *et al.*, 1998). Monitoring CRE-mediated gene expression was accomplished by producing transgenic mice that expressed a CRE-β-galactosidase reporter (CRE-LacZ). Application of forskolin or glutamate resulted in a detectable increase in CRE-driven LacZ expression (Bading *et al.*, 1993; Impey *et al.*, 1996). More importantly, CREB mediated transcription was also dependent on PKA. Inhibition of PKA by two different inhibitors significantly attenuated tetanus-induced upregulation of CRE-LacZ expression. In addition, prior application of multiple trains of high-frequency stimulation that increased CRE-LacZ expression significantly occluded forskolin-induced expression of CRE-lacZ expression (Impey *et al.*, 1996). Additional evidence that PKA is a critical trigger for CREB-mediated gene expression necessary for L-LTP comes from a study by Matsushita *et al.* (2001), in which specific blockade of *nuclear* PKA activity resulted in deficient CA1 L-LTP. Moreover, selective attenuation of L-LTP was also observed with a corresponding reduction in CREB phosphorylation (Matsushita *et al.*, 2001).

These findings are complemented by genetic studies that have manipulated CREB function. One of the first genetic studies regarding the role of CREB used a targeted mutation of the CREB gene, producing mice lacking the α and Δ isoforms of CREB

(Bourtchouladze *et al.*, 1994; Hummler *et al.*, 1994). These mice displayed attenuated LTP and had defective long-term retention of several learning tasks. However, interpretation of the results can be complicated due to compensatory effects of other isoforms of CREB and developmental complications (Hummler *et al.*, 1994; Blendy *et al.*, 1996). A recent study that bypasses these complications involved the use of a dominant negative inhibitor, KCREB, which inhibits CREB and its closely related transcription factors (Walton *et al.*, 1992; Jean *et al.*, 1998). A deficit in spatial learning (assessed by the Morris water maze) and long-term memory (assessed by an object recognition test) were observed (Pittenger *et al.*, 2002). Similarly, several long-lasting forms of synaptic plasticity were impaired (Pittenger *et al.*, 2002). These studies confirm the requirement of CREB-dependent gene transcription for mammalian synaptic plasticity and long-term memory (Bourtchouladze *et al.*, 1994).

Conserved Requirement of Protein Synthesis in Synaptic Plasticity, Learning, and Memory

The importance of protein synthesis in learning and memory was demonstrated in numerous early studies (Flexner and Flexner, 1966; Agranoff, 1967; Barondes and Cohen, 1967; Barondes, 1970; Squire and Barondes, 1970; for review see Davis and Squire, 1984). Various inhibitors of protein synthesis block long-term memory exclusively, leaving short-term memory intact (Bourtchouladze *et al.*, 1998; Schafe *et al.*, 1999; refer to section on Aplysia - Sensitization). Similarly, at the neuronal level, L-LTP also shares the requirements of protein synthesis, as well as gene transcription (Krug *et al.*, 1984; Stanton and Sarvey, 1984; Frey *et al.*, 1988; Otani *et al.*, 1989; Nguyen *et al.*, 1994). Although

many studies have examined the sequence of events leading to gene transcription, the *specific roles* of protein synthesis in L-LTP remain unclear (Duffy *et al.*, 1981).

Synaptic Tagging

As discussed earlier, one of the hallmarks of LTP is input specificity. Likewise, protein synthesis dependent L-LTP also displays input-specific characteristics (Impey *et al.*, 1996; Frey and Morris, 1997). However, an inherent problem that arises is the issue of how newly synthesized plasticity related proteins reach the activated synapse to support input specific L-LTP. Only recently has this issue been addressed by using two pathway experiments, which allows one to record the synaptic responses of two independent population of synapses converging on the same postsynaptic neurons. The terms "synaptic tagging" or "synaptic capture" were introduced in studies designed to address this problem (Frey and Morris, 1997, 1998). The idea here is that plasticity-related proteins, presumably synthesized at the nucleus, are widely distributed and are preferentially captured at recently tagged (activated) synapses (**Figure 1.9B**). In support of this, it was found that L-LTP induced in one pathway can convert a short-lasting form of potentiation in another pathway (induced either by a weak or a strong stimulus) to a more enduring form in the presence of a protein synthesis inhibitor (Frey and Morris, 1997, 1998; for single cell data and analysis in *Aplysia* see Martin *et al.*, 1997; Casadio *et al.*, 1999). It was later demonstrated that generation of this synaptic tag involves NMDA receptor and PKA activation (Barco *et al.*, 2002).

Local Protein Synthesis

Synapse-specific changes in synaptic efficacy may also be mediated by local protein synthesis. Early evidence for dendritic translation came from electron microscopy studies that noted the presence of messenger RNA (mRNA) and polyribosomes in dendrites (Steward and Levy, 1982; Steward and Falk, 1986). mRNA was found to colocalize with polyribosomes found at the base of spines (Martone *et al.*, 1996). Subsequent studies showed that these polyribosomes located in or near dendritic spines could support translation (Torre and Steward, 1992; Crino and Eberwine, 1996; Aakalu *et al.*, 2001).

Thus, in addition to the classical view of protein synthesis mediated by the nucleus, it is now recognized that protein synthesis can occur locally at synapses. Local translation may simplify the task of targetting newly synthesized proteins, and it may endow synapses with a mechanism by which they can independently modify their properties. Recently, studies have demonstrated that local protein synthesis (independent of the soma) may mediate certain forms of synaptic plasticity (Kang and Schuman, 1996; Huber *et al.*, 2000; Miller *et al.*, 2002). Brain-derived neurotrophic factor (BDNF), when applied to isolated neuropil, induced a long-lasting potentiation in hippocampal slices that was blocked by anisomycin, a translational inhibitor (Kang and Schuman, 1996). In addition, it was shown that mGluR activation in hippocampal slices could elicit LTD that is dependent on dendritic translation (Huber *et al.*, 2000). Thus, local protein synthesis provides key factors for the modification of activated synapses in the hippocampus.

Objectives of the Present Thesis

A current theme that has emerged is the requirement of protein synthesis and PKA for both long-lasting LTP and long-term memory. Understanding the critical factors that regulate long-lasting forms of synaptic plasticity, such as L-LTP, and their relation to PKA activation and protein synthesis, should advance our understanding regarding the cellular and molecular processes that underlie certain forms of learning and memory. Consequently, the primary objective of my research is to identify the factors that regulate the induction and expression of L-LTP, with particular emphasis on the putative roles of PKA and protein synthesis.

The first objective of the present thesis was to assess the importance of stimulation parameters used to elicit long-lasting forms of synaptic plasticity (*Chapters 3 & 4*). The second objective was to examine the role of protein phosphatases, a downstream target of PKA, in the expression of L-LTP (*Chapters 5 & 6)*. Finally the third objective was to explore specific aspects of L-LTP, including its requirement of protein synthesis and its reversibility (*Chapter 7*). Briefly, I have addressed the following questions in my thesis:

1. *Are distinct PKA isoforms needed for different types of L-LTP?*
2. *What effect does changing the temporal spacing of stimulation have on L-LTP expression?*
3. *Do PKA and protein phosphatases interact to regulate expression of L-LTP?*
4. *Can protein phosphatases modulate the expression of future L-LTP by their early activation?*
5. *What specific role does protein synthesis play in regulating expression of L-LTP*

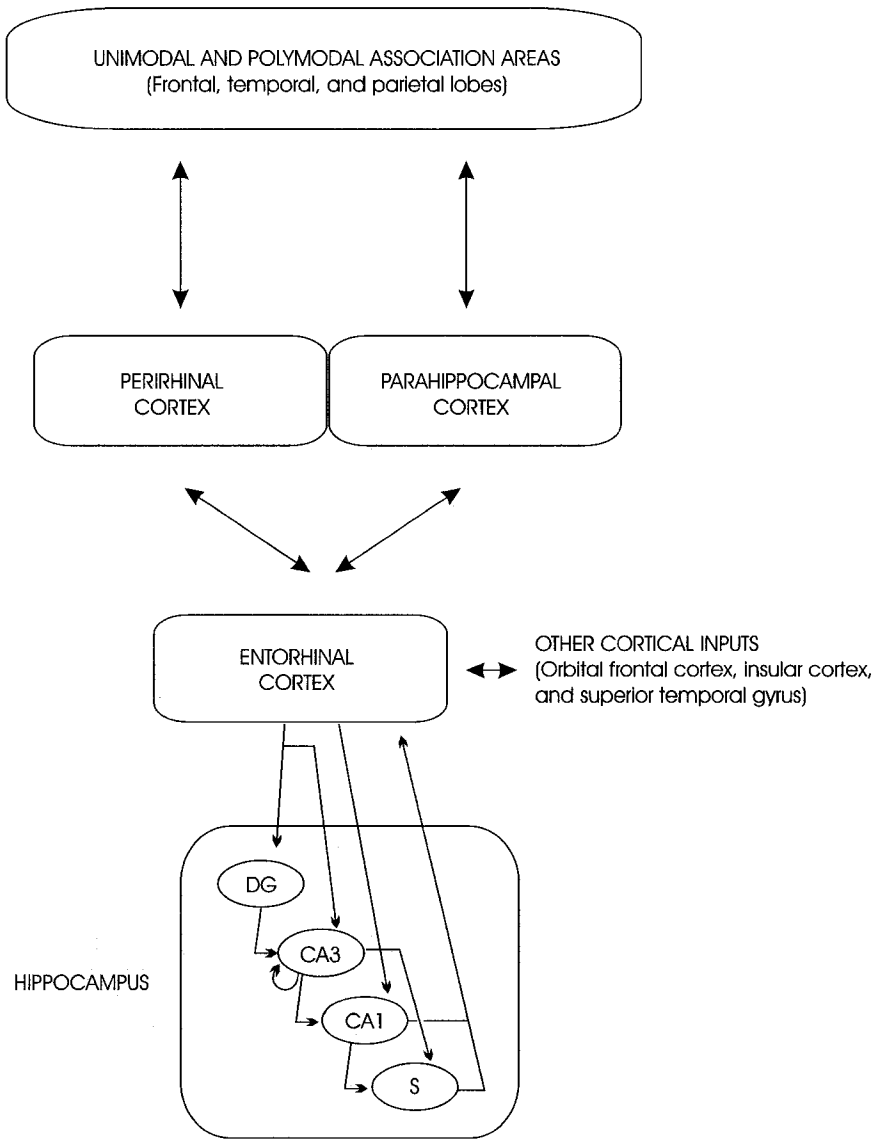

Figure 1.1: Organization of the medial temporal lobe memory system. Incoming information derived from unimodal and polymodal association areas project to the perirhinal cortex, parahippocampal cortex and the entorhinal cortex. The entorhinal cortex is a major source of projections to the hippocampus, which includes the dentate gyrus (DG), the cell fields of the hippocampus (CA1 and CA3), and the subiculum (S). Within the hippocampus, the large network of connections, which support plasticity mechanisms, is thought to underlie the rapid encoding of information. [Adapted from Squire and Zola, 1996.]

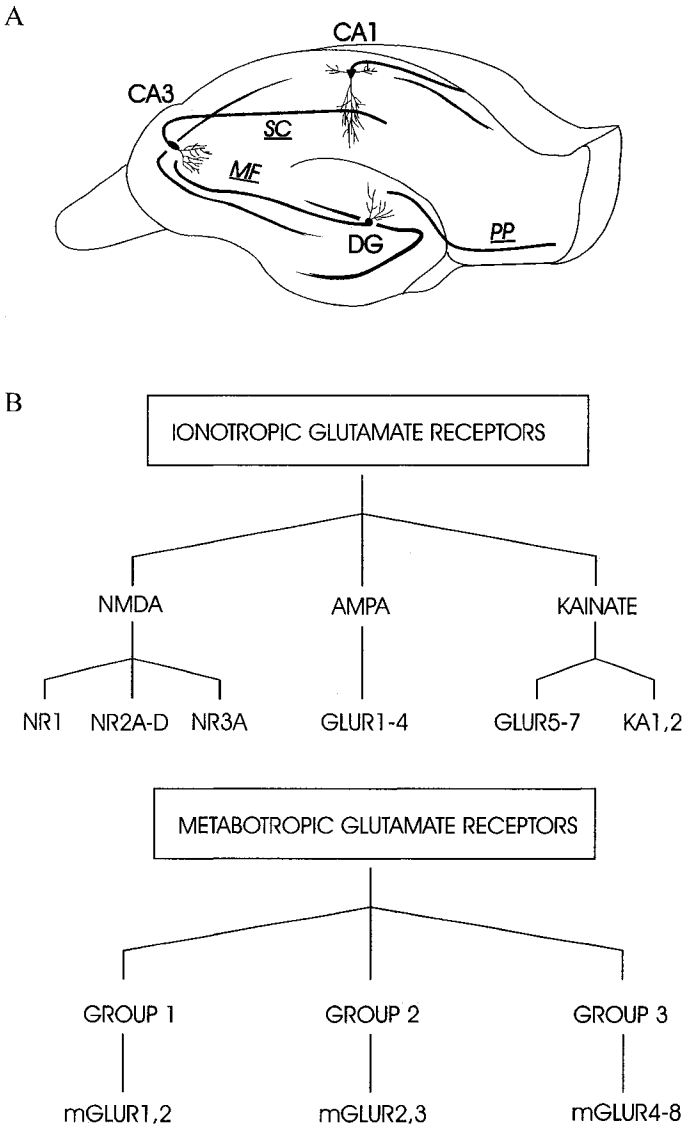

Figure 1.2: Glutamatergic synapses of the mammalian hippocampus. **(A)** The three principal pathways of the hippocampus. i) The *PP* - perforant pathway projects from the entorhinal cortex to the granule cells of the dendate gyrus (DG). ii) The *MF* - mossy fibre pathway is composed of the axons of the granule cells and project to the CA3 region of the hippocampus. iii) The *SC* - Schaeffer collaterals consists of the axon collaterals of the CA3 region and project to the CA1 region. [Adapted from O'Keefe and Nadel, 1978.] **(B)** Classification of glutamatergic receptors found in the hippocampus. Ionotropic and metabotropic receptors are the major types of glutamate receptors. Within the ionotropic or ligand binding classification, three principal receptor subtypes exist: AMPA, NMDA and kainate receptors. In the other division, the metabotropic or G-protein coupled receptors are subdivided into three subdivisions based on sequence similarity, pharmacology and intracellular signaling mechanism.

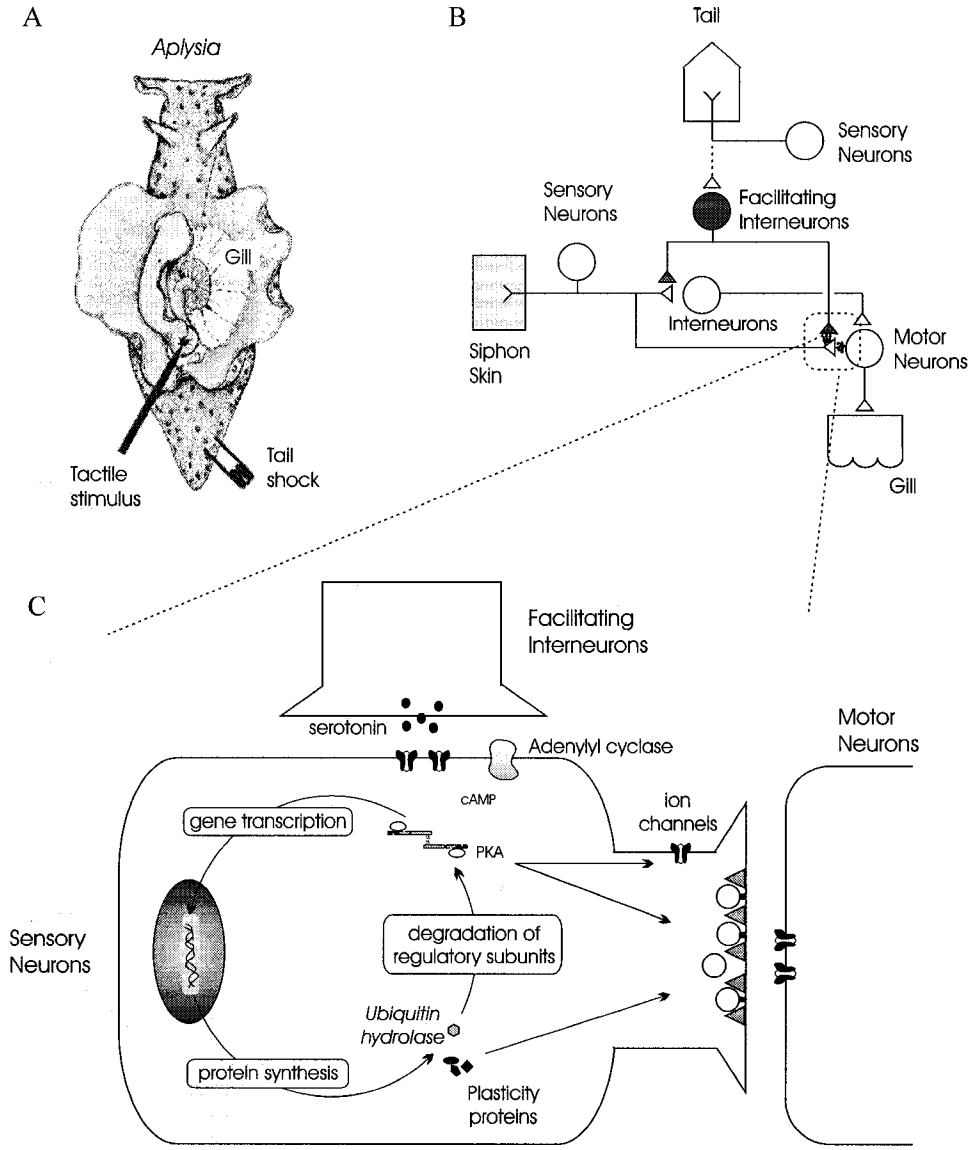

Figure 1.3: Sensitization- a simple learned behavior in the marine snail, *Aplysia*. [Adapted from Kandel, 2001.] **(A)** In *Aplysia* (dorsal view), a light touch to the siphon with a fine probe causes the gill to withdraw. Applying a noxious stimulus to the tail enhances or sensitizes the withdrawal reflex of the gill. **(B)** A simplified diagram depicting the neural circuit responsible for the *Aplysia* gill-withdrawal reflex. Noxious stimulation to the tail activates sensory neurons that excite facilitating serotonergic interneurons. These interneurons synapse onto the synaptic terminals of the sensory neurons from the siphon where they enhance neurotransmitter release to motor neurons innervating the gills. **(C)** Cellular mechanisms and loci of long-term sensitization. Repeated stimulation of the tail induces the release of serotonin from the interneuron. 5-HT then acts on a transmembrane serotonin receptor to activate the cAMP/PKA cascade. The catalytic subunits of PKA can then translocate to the nucleus, and induce gene transcription. As a result, levels of ubiquitin hydrolase are increased and lead to proteolysis of the regulatory subunit of PKA. Cleavage of the (inhibitory) regulatory subunit results in persistent activity of PKA, leading to persistent phosphorylation of the substrate proteins of PKA.

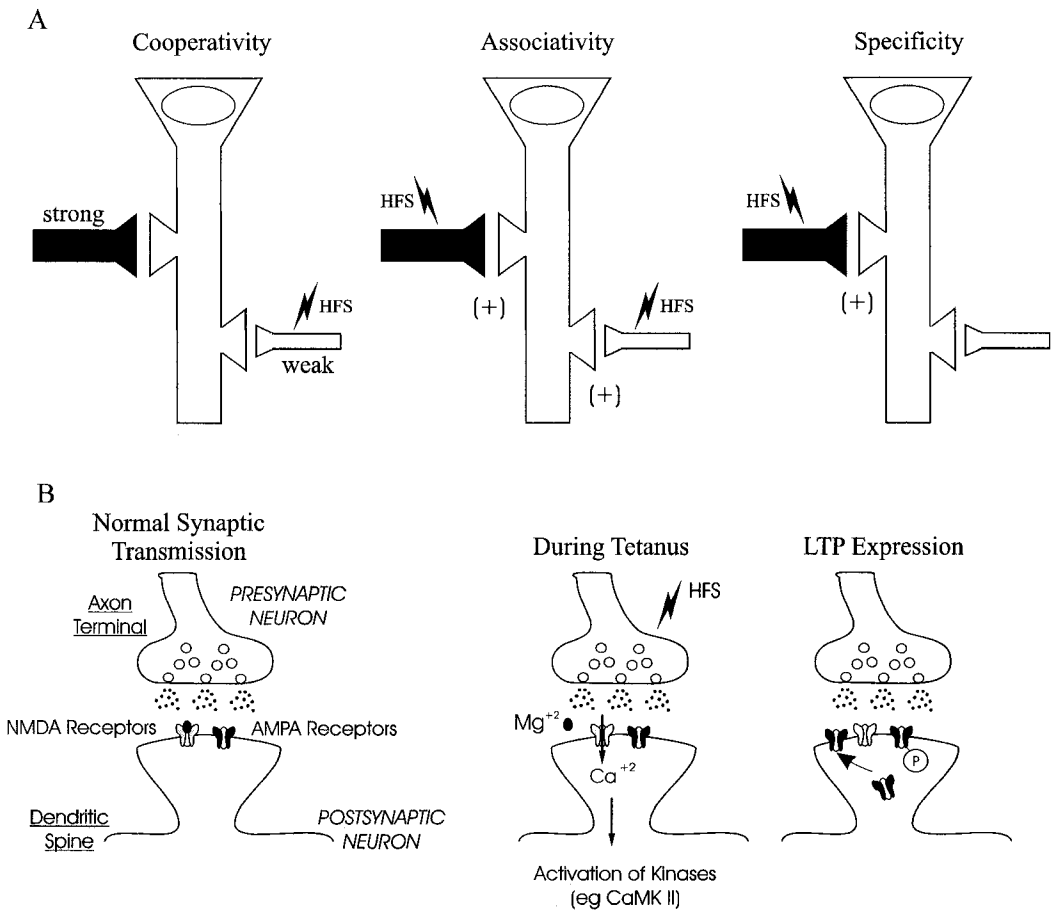

Figure 1.4: Long-term potentiation (LTP) in the mammalian hippocampus. **(A)** LTP in area CA1 of the hippocampus shows cooperativitiy, associativity, and specificity. Cooperativity: high frequency stimulation (HFS) applied to the weak input alone does not induce LTP. A minimum number of axons must be recruited for LTP induction (top left). Associativity: HFS applied to both the strong and weak inputs simultaneously induces LTP in both pathways (middle). Specificity: HFS applied to the strong input alone induces LTP in only the strong but not weak input. [Adapted from Nicoll et al., 1988.] **(B)** Cellular mechanism of LTP. During basal synaptic transmission, glutamate released by the presynaptic terminal will activate postsynaptic glutamate receptors. However, when a tetanus is delivered to the presynaptic terminals, the postsynaptic membrane is depolarized and this relieves the Mg^{+2} block of NMDA receptors. Subsequent influx of Ca^{+2} activates various kinases and initiates the induction of LTP. These kinases then phosphorylate different proteins that cause sustained enhancement of synaptic transmission. One example is the ability of kinases to phosphorylate AMPA receptors and change their efficacy or affect their cycling kinetics during receptor trafficking. [Adapted from Malenka and Nicoll, 1999.]

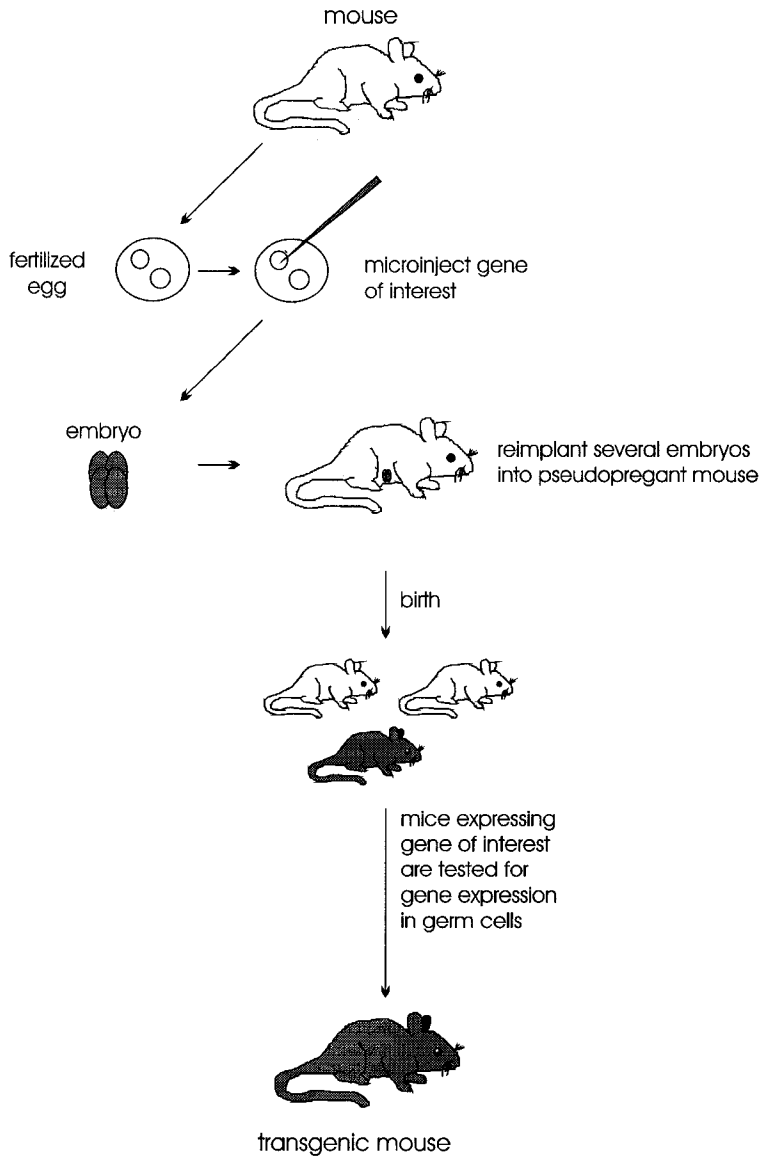

Figure 1.5: Generation of transgenic mice. Fertilized eggs are microinjected with the modified gene of interest. Several embryos are reimplanted into the pseudopregnant female and allowed to develop to term. Offspring are genotyped and those carrying the transgene are bred. [Adapted from Picciotto and Wickman, 1998.]

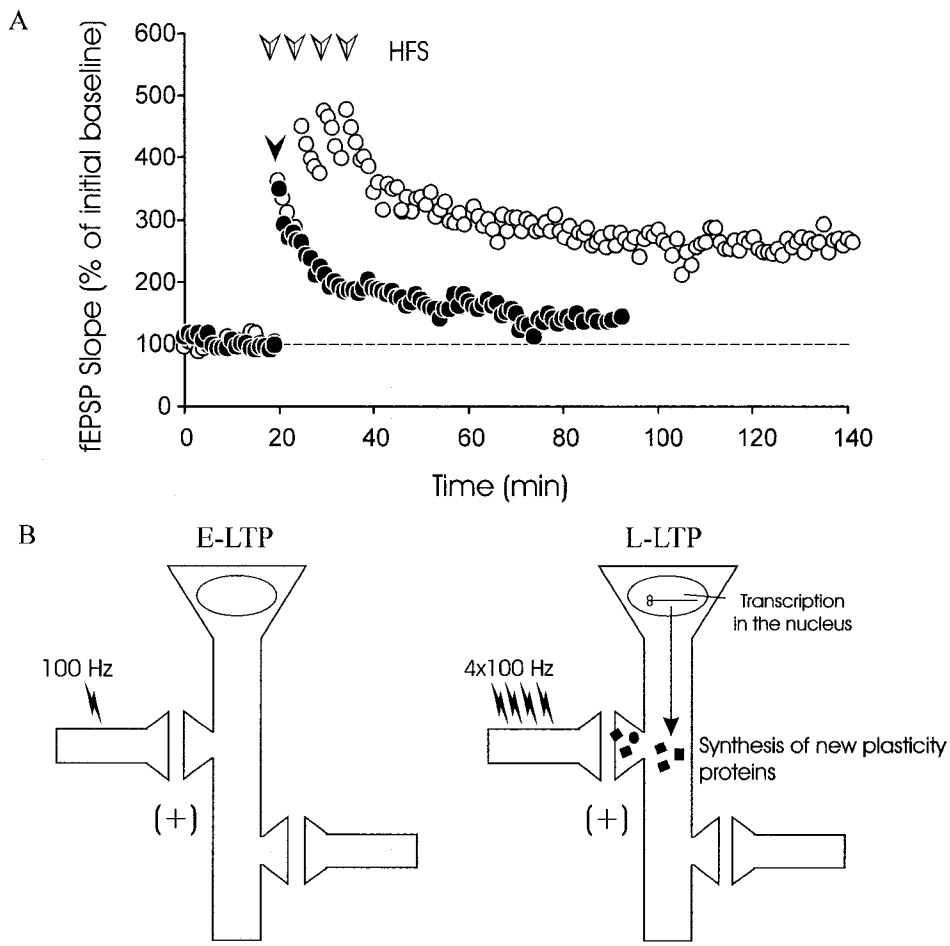

Figure 1.6: Different phases of long-term potentiation (LTP). **(A)** The early phase of LTP (E-LTP) is usually induced by a single train of high frequency stimulation (HFS) and lasts typically 1-2 hours. The more enduring form of LTP, the late phase of LTP (L-LTP) is induced by three or more trains of HFS and is associated with larger amplitudes of field EPSPs for a longer period of time. **(B)** Different phases of LTP possess distinct properties. The expression of E-LTP involves the covalent modifications of pre-existing molecules, whereas expression for L-LTP requires *de novo* protein synthesis.

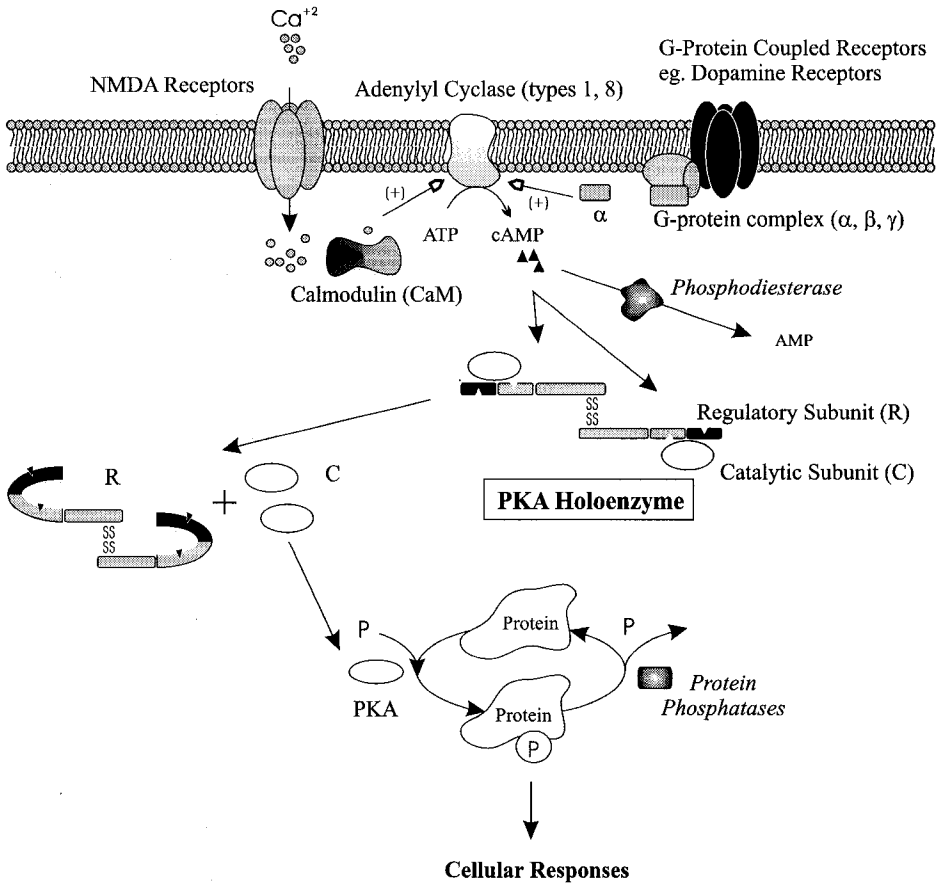

Figure 1.7: Simplified schematic diagram of the cAMP-PKA signal transduction pathway. Adenylyl cyclase activation through G-coupled receptors results in cAMP production. Upon binding of cAMP to the regulatory subunits, the catalytic subunits dissociate from the regulatory subunit. Activation of PKA leads to phosphorylation of various proteins that may mediate synaptic plasticity and memory. P = phosphate

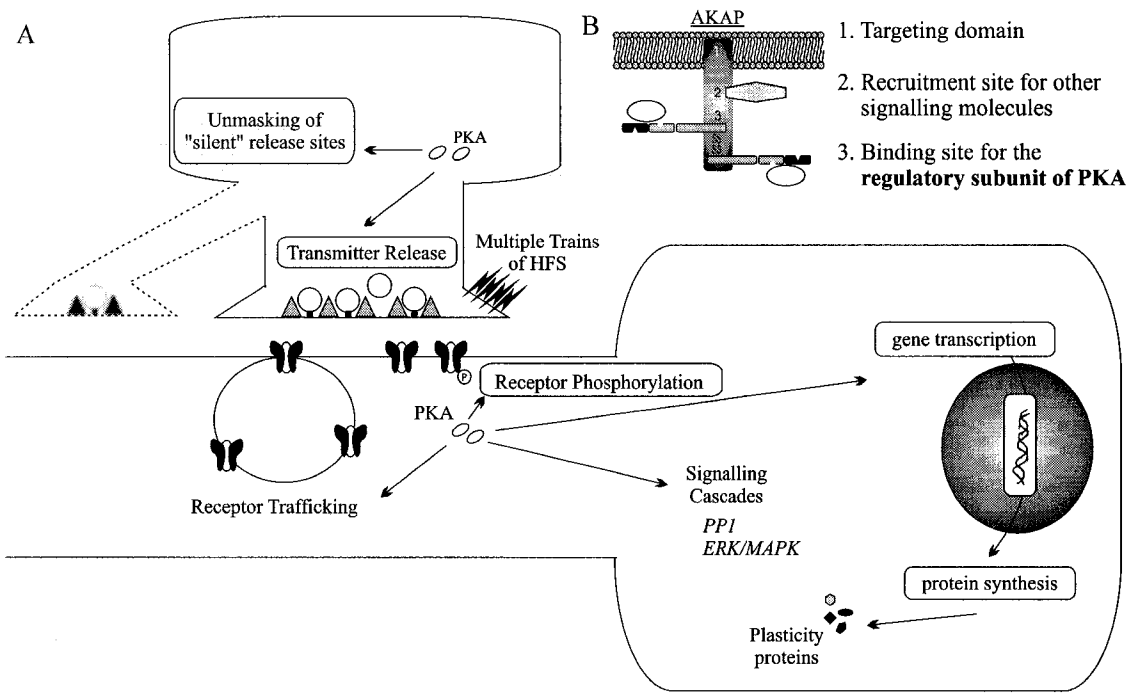

Figure 1.8: Potential mechanisms and sites of action for PKA during hippocampal synaptic plasticity. **(A)** Phosphorylation of transmitter receptors, receptor trafficking, regulation of gene expression and protein synthesis, modulation of transmitter release, and unmasking of "silent" release sites are some of the ways by which PKA may regulate activity-dependent synaptic modifications in the hippocampus. These processes may occur in both presynaptic and postsynaptic neurons. [From Nguyen and Woo, 2003, *submitted*] **(B)** Schematic diagram of an A-kinase anchoring protein (AKAP), showing three distinct functional domains. [Adapted from Michel and Scott, 2000.]

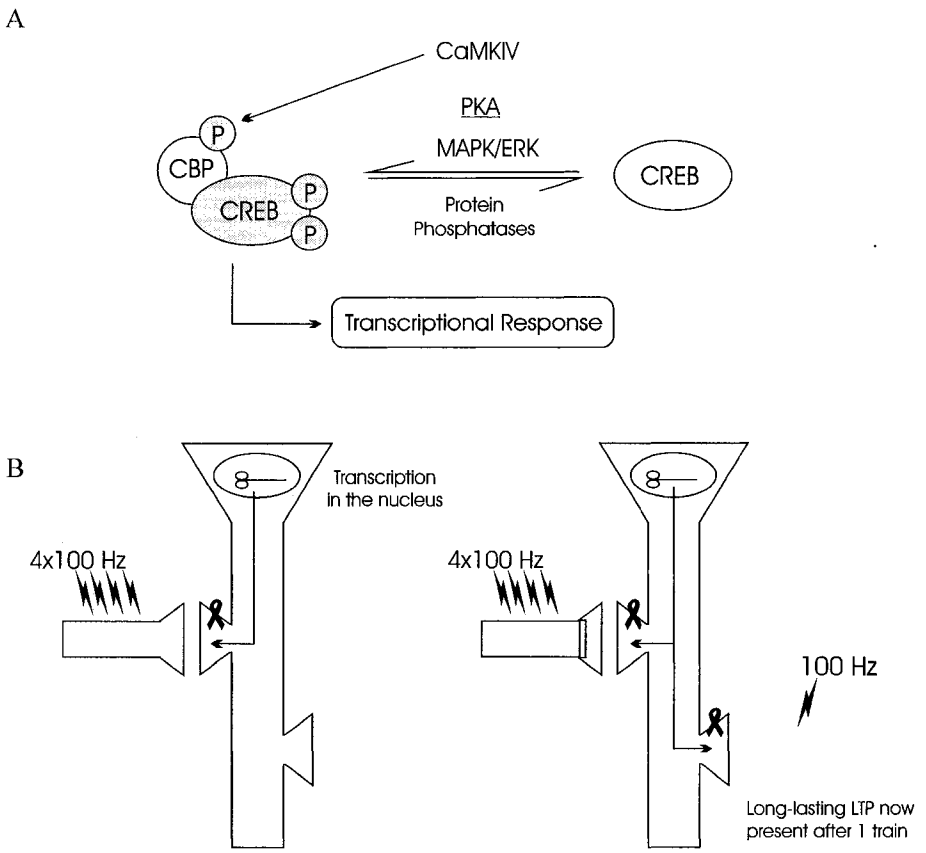

Figure 1.9: Gene transcription and protein synthesis are required for the expression of L-LTP. **(A)** cAMP-response element binding protein (CREB) transcription factor is required for long-lasting forms of synaptic plasticity. Phosphorylation of CREB is regulated by numerous kinases including PKA, CaMKIV, and MAPK/ERK. Upon phosphorylation, CREB initiates gene transcription of those genes containing the cAMP responsive element (CRE) promoter. **(B)** Schematic diagram of synaptic tagging. Multiple trains of HFS can induce input specific L-LTP that requires gene transcription and protein synthesis (bottom left). Because the products of macromolecular synthesis are distributed cell wide, an adjacent synapse can also utilize them. When a synaptic tag (*ribbon*) is created by HFS that normally does not elicit L-LTP, the tagged synapse can capture or sequester newly synthesized plasticity proteins to convert temporary synaptic modifications to a longer-lasting form. [Adapted from Frey and Morris, 1997.]

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CHAPTER II:

GENERAL METHODOLOGY

Animals

R(AB) Mice

R(AB) transgenic mice were generously provided by Dr. Ted Abel from the University of Pennsylvania. They were derived from two independent lines that were previously characterized for neural expression of the R(AB) transgene, hippocampal PKA activity, and hippocampal synaptic physiology (Abel *et al.*, 1997). A CaMKIIα promoter was used to drive the postnatal expression of R(AB), a dominant negative form of the RIα regulatory subunit of PKA, in the hippocampus, neocortex and amygdala and striatum (Mayford *et al.*, 1996). As a result, these mice show a 40-50% reduction in hippocampal basal PKA activity (Abel *et al.*, 1997). Transgenic mice were maintained in the hemizygous state on a C57BL/6J background. The R(AB) transgenic colony was at N8 to N10 on a C57BL/6J background. For genotyping, tail DNA was prepared and analyzed by Southern blotting using a transgene-specific probe (Abel *et al.*, 1997). Male and female mice between the ages of 4 and 6 months from the two transgenic lines, R(AB)-1 and R(AB)-2, were used. Mice were maintained at the University of Pennsylvania and at the University of Alberta consistent with Institutional Animal Care and Use Committee (IACUC) and Canadian Council on Animal Care (CCAC) guidelines. Controls were wildtype littermates of the mutants. Experimenters were blind to genotypes.

C57BL/6 Mice

C57BL/6 is the most widely used inbred strain. It is commonly used as a background strain for breeding congenic and transgenic mice. Female C57BL/6 mice, aged

8-12 weeks (Charles River, Canada) were used for all experiments. Animals were housed at the University of Alberta using guidelines approved by the CCAC.

Slice Preparation and Electrophysiology

Mice were euthanized by rapid cervical dislocation followed by decapitation. The isolated brain (**Figure 2.1A**) was transferred to a beaker of ice-cold artificial cerebrospinal fluid (ACSF; composition in mM: 124 NaCl, 4.4 KCl, 1.3 $MgSO_4$, 1.0 NaH_2PO_4, 26.2 $NaHCO_3$, 2.5 $CaCl_2$, 10 Glucose). Following a brief recovery period, the hippocampus was isolated free from the adjoining tissue using two spatulas. The long axis of the hippocampus was placed parallel to the long axis of the acrylic tissue block on a piece of filter paper. Once properly oriented, the hippocampus was thinly sectioned using a manual tissue chopper (Stoelting). A drop of ACSF was placed on the hippocampus immediately prior to sectioning so that slices would adhere to the blade. By using a fine paintbrush, slices were gently transferred from the blade to a small glass Petri dish containing ice-cold oxygenated ACSF. Transversely sectioned hippocampal slices (400μM) were then transferred to an interface recording chamber (**Figure 2.1B**). This interface chamber allowed slices to be maintained at 28°C and continuously perfused with ACSF (1mL/min) aerated with carbogen (95% CO_2 and 5% O_2) in a humidified atmosphere.

After a minimum recovery period of at least one hour, extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded with an ACSF filled glass microelectrode (1-3 MΩ) positioned in stratum radiatum of area CA1. Evoked fEPSPs were elicited by stimulation of the Schaeffer collateral fibres using an extracellular bipolar nickel-chromium electrode (diameter 130μm) (**Figure 2.1C**). The stimulation intensity (0.08 ms

pulse width) was adjusted to give fEPSP amplitudes that were approximately 40% of maximal fEPSP sizes. Control "baseline" responses were elicited once per minute at this intensity. Slices that showed maximal fEPSP sizes < 3 mV were rejected. In some cases, synaptic input output curves were generated by plotting fEPSP slopes and their corresponding presynaptic fibre volley sizes, elicited at different stimulus strengths.

For two-pathway experiments, two stimulating electrodes (S1 & S2) were placed in the stratum radiatum on opposite sides of the recording electrode to stimulate two separate groups of Schaffer collateral fibres (**Figure 2.1C**). The independence of the two pathways was demonstrated by the absence of paired-pulse facilitation of fEPSP when two sets of orthodromic stimuli were delivered to the two pathways at 50ms interval (in either order, S1-S2 or S2-S1); however, paired-pulse facilitation of fEPSP was observed when the stimuli were applied to the same stimulating electrode.

Evoked fEPSPs were low pass filtered (1-3kHz) and amplified by an intracellular amplifier (Warner Instruments; IE-210). These evoked responses were digitized at a rate of 20kHz by the Digidata 1200 acquisition system (Axon Instruments), and were recorded with pCLAMP 7.0 software (Axon Instruments). Offline data analysis was performed in Clampex 7.0 software (Axon Instruments) by measuring the initial slope of the evoked fEPSPs.

Electrodes

Bipolar stimulating electrodes were created by twisting two fine nickel chromium wires (A-M Systems) together and placing them within a glass electrode. Because the thin wires are coated with formvar, one end of the thin wires was flamed and connected via an

isolator to a dual channel stimulator (Grass S88). The final diameter of the stimulation electrode was approximately 130μm.

Glass recording electrodes were pulled in a P-47 flaming brown micropipette puller (Sutter Instruments). The recording electrode was filled with ACSF and had a final electrode resistance of approximately 1 – 3 MΩ.

Stimulation Protocols

LTP was induced by applying either a single one-second train (100Hz, at test strength), four one-second trains (100Hz, test strength) spaced at various intervals, or by using theta-burst stimulation. The thetaburst stimulation protocol consisted of 15 bursts of four pulses at 100Hz, delivered at a 200-ms interburst interval. Depotentiation was induced by applying low-frequency stimulation (LFS) after LTP induction. The following protocols for LFS were used: 5Hz for 3min and 5Hz for 6min. In another set of experiments, LFS was applied prior to LTP induction to induce "metaplasticity." LFS protocols used were as follows: 5Hz for 3min, 5Hz for 30s, 1Hz for 15min or 1Hz for 3min (Table 2.1 summarizes the various protocols described). In most cases, a second independent pathway was monitored to gauge the effects of applied drugs on basal synaptic strength. This "control" pathway received only stimulation at the rate of once/min.

Drugs

Several drugs were used to address specific hypothesis (Table 2.2). Drugs were dissolved in the appropriate solvents to make concentrated stock solutions. They were aliquoted into smaller volumes and frozen at –20°C. Prior to an experiment, the stock

solution was thawed and diluted with ACSF to the desired final concentration. Each drug was then bath-applied to hippocampal slices. In the case of calyculin A, slices were incubated in the drug for 40min prior to start of baseline recordings. Those drugs that used DMSO as the solvent did not have a final concentration of DMSO exceeding 0.1%. At this concentration, DMSO did not affect basal synaptic transmission or LTP (data not shown). Table 2.2 summarizes the concentrations, solvents and pharmacological actions of the drugs used. All experiments using light-sensitive drugs were performed under dimmed-light conditions. Drug experiments were interleaved with drug-free controls.

Data and Statistical Analysis

The initial slope of the fEPSP was measured as an index of synaptic strength (Johnston and Wu, 1995). Average "baseline" slope values were measured over a period of 20min. For each experiment, fEPSP slopes were expressed as a percentage of average pre-tetanus baseline slope values. Student's t-test (2 groups) or an ANOVA with a Tukey-Kramer post-test (3 or more groups) was used for statistical comparisons of mean fEPSP slopes using Instat software (GraphPad). All comparisons were performed using a two-tailed analysis with a significance level of $\mathbf{p < 0.05}$. All values shown are mean ± standard error (SEM), with n = number of slices. In the case of R(AB) transgenic mice, the results from both lines of transgenic mice and from both sexes were combined because an analysis of variance did not reveal significant differences between these transgenic lines or between males and females of the same genotype.

Table 2.1: Summary of stimulation protocols employed in the present thesis

Stimulation Protocol Frequency	Inter-train Interval	Duration of Single Train	Type of Synaptic Plasticity Elicited	References
2 x 100Hz	20s	1s	E-LTP	Winder et al., 1998
4 x 100Hz	5min	1s	L-LTP	Huang and Kandel, 1994; Nguyen et al., 1994;
4 x 100Hz	20s	1s	*L-LTP	*Appendix A; Scharf et al., 2002
4 x 100Hz	3s	1s	*L-LTP	*Appendix A
15 x 100Hz	200ms	40ms	L-LTP	Nguyen and Kandel, 1997
1Hz	--	15min	LTD / DPT	Fujii et al., 1991; Dudek and Bear, 1992, 1993; Brandon et al., 1995; Kauderer and Kandel, 2000
5Hz	--	3min	LTD / DPT	Fujii et al., 1991; O'Dell and Kandel, 1994; Brandon et al., 1995; Thomas et al., 1996; Zhuo et al., 1999; Kauderer and Kandel, 2000

Abbreviations: LTP – long-term potentiation; E-LTP – early phase LTP; L-LTP – late phase LTP; LTD – long term depression; DPT – depotentiation
* Observed to be protein synthesis dependent in present study (Appendix A)

Table 2.2: Summary of pharmacological tools used in the present thesis

Name of Drug	Distributor	Solvent	Concentration (μM)	Mode of Action	Chapter(s)
Actinomycin D (ACT-D)	SIGMA/RBI	DMSO	25	Transcriptional inhibitor	7
2-amino-5-phosphonopentanoic acid (APV)	SIGMA/RBI	Distilled water	25-50	NMDAR antagonist	4 & 6
Anisomycin (ANISO)	SIGMA/RBI	DMSO	25	Translational inhibitor	7 & Appendix A
Calyculin A (CAL-A)	BIOMOL	DMSO	1	PP1/2A inhibitor	5 & 6
Emetine	SIGMA/RBI	Distilled water	20	Translational inhibitor	7
FK-506	Calbiochem		1	PP2B inhibitor	5
Forskolin	SIGMA/RBI	DMSO	50	Adenylyl cyclase activator	5
IBMX	SIGMA/RBI	DMSO	30	Phosphodiesterase inhibitor	5
KT-5720	BIOMOL	DMSO	1	Inhibitor of the catalytic subunit of PKA	4
Okadaic Acid (OA)	SIGMA/RBI	Distilled water	1	PP1/2A inhibitor	5 & 6
Rp-cAMPS (RP)	SIGMA/RBI	DMSO	30-100	PKA inhibitor	3 & 5

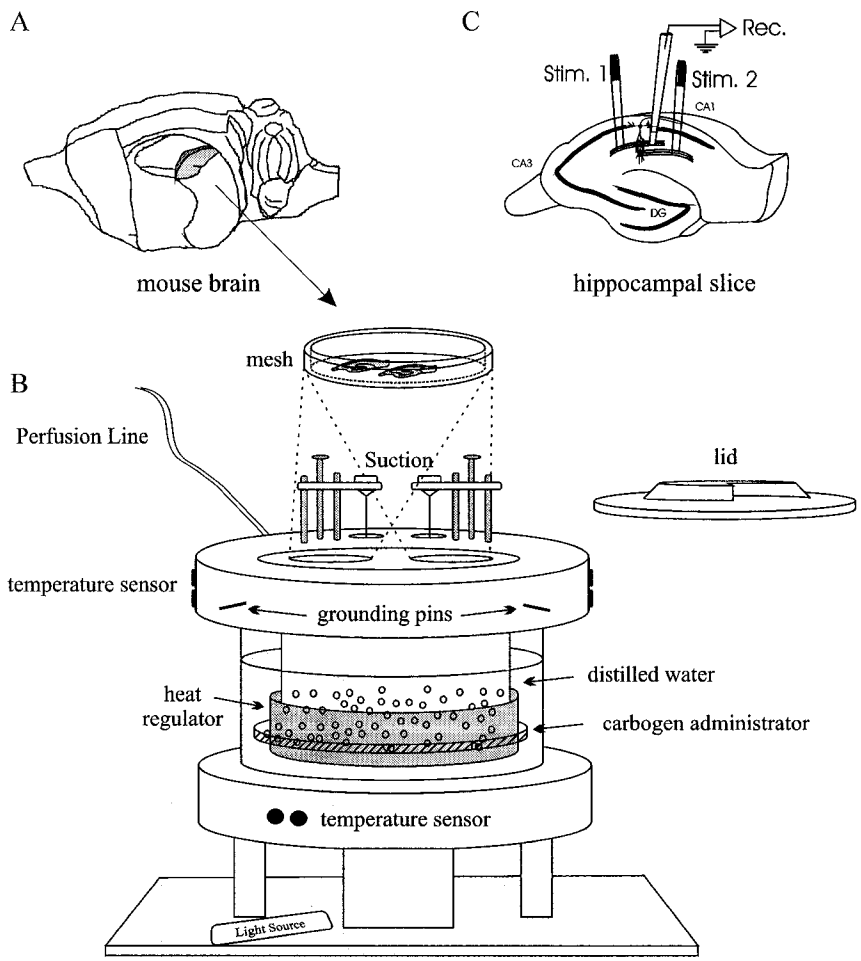

Figure 2.1: Recording evoked field potentials (fEPSPs) from hippocampal slices in an interface chamber. **(A)** Sketch of the mouse brain showing the position of the hippocampus (shaded in grey). **(B)** Schematic of the recording interface chamber. Hippocampal slices are placed on meshes that are positioned at the top of the meniscus of the ACSF bath in the interface chamber. This temperature-regulated chamber allows slices to be perfused with ACSF while exposed to a humidified carbogen atmosphere. **(C)** Positioning of recording and stimulating electrodes. After slices are allowed to recover, a bipolar stimulating electrode and a glass recording electrode filled with ACSF are placed in the stratum radiatum of area CA1 of hippocampal slices.

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*CHAPTER III:

DIFFERENTIAL RECRUITMENT OF CAMP-DEPENDENT PROTEIN KINASES DURING LTP

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Introduction

Hippocampal long-term potentiation (LTP) is an activity-dependent enhancement of synaptic transmission believed to be a cellular mechanism for some forms of learning and memory in the mammalian brain (Bliss and Collingridge, 1993; Martin *et al.*, 2000). One particular protein kinase that is critical for hippocampal LTP is cAMP-dependent protein kinase (PKA) (Abel *et al.*, 1997; Blitzer *et al.*, 1995; Frey *et al.*, 1993; Huang and Kandel, 1994; Weisskopf *et al.*, 1994). In area CA1 of hippocampal slices, strong, repeated stimulation using temporally spaced bursts of activity (*e.g.* 4x100Hz, 5min intervals) induces long-lasting LTP that requires PKA (Huang and Kandel, 1994). Transgenic mice with genetically reduced hippocampal PKA activity show impairment of synaptic potentiation induced by this temporally spaced tetraburst stimulation (Abel *et al.*, 1997). Although the PKA dependence of hippocampal LTP has been characterized, an unresolved question is whether various forms of LTP induced by different patterns of synaptic stimulation require distinct types of PKA holoenzyme.

PKA is a tetrameric enzyme composed of two catalytic and two regulatory subunits (Reimann *et al.*, 1971). At least two distinct types of catalytic subunits and four different types of regulatory subunits are expressed in the mouse hippocampus (Cadd and McKnight, 1989). Thus in principle, there may exist as many as 30 different tetrameric subunit combinations within the PKA holoenzyme *in situ* (*i.e.* 30 different "isoforms"). Mice with genetic deletions of the $C\beta_1$ catalytic subunit of hippocampal PKA showed perturbed LTP and defective long-term depression (LTD) in area CA1 of hippocampal slices (Qi *et al.*, 1996), whereas genetic deletion of the RIβ regulatory subunit impaired LTD but did not affect LTP (Brandon *et al.*, 1995). These results suggest that different

forms of hippocampal synaptic plasticity may be regulated by distinct isoforms of PKA subunits, but it should be noted that in both of these mouse models, measurable levels of hippocampal PKA activity were unaffected by the genetic manipulations employed to delete these particular subunits (Brandon *et al.*, 1995; Qi *et al.*, 1996). Hence, these two "knock-out" mouse models (Brandon *et al.*, 1995; Qi *et al.*, 1996) cannot provide evidence as to whether the dependence of LTP expression on PKA *enzymatic* activity involves differential recruitment of distinct isoforms of PKA. An essential requirement for testing the hypothesis that the amount and temporal pattern of synaptic activity may regulate the PKA dependence of LTP by recruiting different PKA isoforms is to look for LTP expression in the presence of substantial reductions in the enzymatic activity of hippocampal PKA.

Are different isoforms of PKA involved in types of LTP induced by distinct temporal patterns of synaptic stimulation? One way of addressing this question is to use pharmacological and genetic approaches to examine the dependence of different types of LTP on PKA. Transgenic mice expressing an inhibitory form of the RIα regulatory subunit of PKA [termed R(AB) mice] show significantly reduced levels of hippocampal PKA activity (approximately 40–50% of basal activity) (Abel *et al.*, 1997). This reduction of PKA activity results from the suppression of the enzymatic activity of tetrameric holoenzymes containing the mutant inhibitory form of the RIα subunit of PKA (Abel *et al.*, 1997; Woodford *et al.*, 1989). R(AB) transgenic mice therefore provide an appropriate and effective means of exploring the hypothesis that different patterns of synaptic activity may induce forms of LTP that require distinct isoforms of PKA holoenzyme (or more specifically, different isoforms of regulatory subunits). Additionally, pharmacological

inhibition of PKA can be used to establish the PKA dependence of forms of LTP induced by different patterns of synaptic activity. Together these two approaches, one genetic and the other pharmacological, can be combined to explore the roles of different patterns of synaptic activity in regulating the PKA dependence of LTP through putative recruitment of particular isoforms of PKA.

The present study has used R(AB) mice and an inhibitor of PKA, Rp-cAMPS, to test the hypothesis that different forms of LTP may require distinct isoforms of PKA. It should be noted that at the concentration used here (100μM), Rp-cAMPS does not preferentially inhibit specific isoforms of PKA (Cadd *et al.*, 1990; Dostmann, 1995; Woodford *et al.*, 1989), but it does suppress total PKA activation by binding to regulatory subunits (Dostmann, 1995). LTP induced by temporally spaced, repeated stimulation (a tetraburst protocol) was defective in area CA1 of hippocampal slices from R(AB) mice, and this form of LTP was blocked in wildtype slices by Rp-cAMPS. This same drug also blocked LTP induced by a compressed, thetaburst pattern of synaptic stimulation in R(AB) mutant slices. Thus both forms of LTP, induced by distinct patterns of synaptic stimulation, are PKA dependent as defined by their sensitivity to the pharmacological inhibitor of PKA. However, thetaburst LTP was intact in hippocampal slices from R(AB) mutant mice as compared with control slices from wildtype mice. These findings suggest that thetaburst and tetraburst stimulation may engage distinct isoforms of PKA. In particular, although thetaburst LTP was shown to be PKA dependent, normal expression of thetaburst LTP in R(AB) mutant slices indicates that this pattern of stimulation may significantly recruit isoforms of PKA holoenzyme that do not contain the mutant RIα subunit. The results from this study provide evidence to support the idea that distinct

patterns of synaptic activity can regulate the PKA dependence of LTP by engaging distinct isoforms of PKA.

Materials and Methods

Experiments were performed on R(AB) transgenic mice (aged 6–8 mo) that were previously characterized for hippocampal PKA activity, synaptic plasticity, learning, and memory (Abel *et al.*, 1997). Transgenic mice were maintained in the hemizygous state on a C57BL/6J background. Currently the R(AB) transgenic colony is at the 8th–10th backcross generation onto a C57BL/6J background. Age-matched wildtype littermates of the mutants were used for controls.

Transverse hippocampal slices (400μm thickness) were prepared and maintained in an interface chamber at 28°C (for details, see *Chapter II*). Slices were perfused with standard artificial cerebrospinal fluid (ACSF, 1 ml/min flow rate) with ionic composition as described in *Chapter II*. A microelectrode filled with ACSF was used to record field excitatory postsynaptic potentials (fEPSPs) from stratum radiatum of area CA1 during extracellular stimulation with a bipolar nickel-chromium electrode that was also positioned in stratum radiatum. Stimulation intensity was adjusted to give fEPSP amplitudes approximately 40% of maximum sizes, and baseline responses were elicited once per minute at this intensity.

LTP was induced by applying four 100Hz bursts (1s duration) spaced 5min apart (tetraburst protocol) or by using thetaburst stimulation (Table 2.1). In some experiments, 100μM Rp-cAMPS, an inhibitor of PKA (Dostmann, 1995), was bath applied to wildtype and mutant slices to test for the PKA dependence of tetraburst and thetaburst LTP.

Results

LTP induced by temporally spaced stimulation is defective in R(AB) mutant mice

LTP induced by four 100Hz bursts of stimulation (5min interburst interval) in R(AB) mutant and wildtype slices were compared (**Figure 3.1**). In wildtype slices, this stimulation regimen induced a robust and long-lasting form of LTP in area CA1: the mean fEPSP slope measured 2h after LTP induction was 169±22% (mean ± SEM) of pre-LTP baseline. In contrast, the same amount and pattern of stimulation elicited a gradually decaying LTP in R(AB) mutant slices: the mean fEPSP slope measured 2h after LTP induction was only 106±9% of pre-LTP baseline ($p<0.02$ for comparison with wildtypes, **Figure 3.1**). These data replicate the previous observations reported by Abel *et al.* (1997). A noteworthy difference between the present data and those in Abel *et al.* (1997) is the consistently reduced levels of potentiation observed in R(AB) mutants here at all time points following LTP induction. This may be the result of the higher levels of potentiation observed in slices from wildtype mice in the present study. In the original study of Abel *et al.* (1997), the early stage of potentiation measured minutes after induction was unaffected in mutants. The larger potentiation seen here may result from the fact that the genetic background of the mice used in the current study is more completely C57BL/6J, a mouse strain known to exhibit robust LTP (Nguyen *et al.*, 2000). Regardless of the actual reason for the present study's stronger inhibition of LTP expression in mutants, these results are consistent with those reported previously by Abel *et al.* (1997). The data also support the idea that PKA may indeed play important roles in both early and late phases of LTP in area CA1 (Blitzer *et al.*, 1995; Huang and Kandel, 1994; see also Otmakhova *et al.*, 2000, for cAMP data).

Thetaburst stimulation induces robust LTP in R(AB) mutants

Previous studies on R(AB) mice have used temporally spaced, repeated bursts of stimulation to induce persistent forms of hippocampal LTP (Abel *et al.*, 1997). This strong regimen of stimulation is reliable in eliciting long-lasting forms of LTP that are PKA dependent (Abel *et al.*, 1997; Huang and Kandel, 1994), but it is not clear whether altering the amount and temporal spacing of stimulation can modulate the PKA dependence of hippocampal LTP by recruiting different isoforms of PKA. To address this hypothesis, LTP was induced by reducing both the amount and the temporal spacing of imposed synaptic activity in R(AB) and wildtype slices (**Figure 3.2**). A thetaburst pattern of stimulation (15 bursts of 4 pulses at 100Hz, delivered at a 200ms interburst interval) elicited a substantial amount of synaptic potentiation in wildtype slices: the mean fEPSP slope measured 2h after thetaburst stimulation was 142±8% of pre-LTP baseline (**Figure 3.2A**, □). In R(AB) mutants, thetaburst LTP was almost identical in time course and amplitude to that seen in wildtypes (**Figure 3.2A**, ●). The mean fEPSP slope measured in mutant slices 2h post induction was 147±12% of pre-LTP baseline. This was not significantly different from the value observed in wildtypes ($p>0.2$). Hence unlike the temporally spaced protocol that yielded an LTP deficit in mutants, the sharply reduced amount of imposed synaptic activity (total of 60 pulses of stimulation vs. 400 pulses in the spaced tetraburst protocol) and the decreased temporal spacing between bursts (200ms interburst interval vs. 5min) in the theta-pattern protocol fully rescued LTP in R(AB) mutants.

These data, obtained from experiments performed on PKA mutant mice, suggest that the amount and temporal pattern of synaptic activity can modulate the PKA

dependence of LTP in animals with a genetic reduction of hippocampal PKA activity. However, these results alone do not provide evidence for activity-dependent recruitment of different isoforms of PKA following synaptic stimulation leading to LTP. The thetaburst LTP described here may still be PKA dependent because the R(AB) mutation predominantly affects activation of PKA tetramers that contain the mutant RIα isoform of the regulatory subunit. In other words, thetaburst stimulation may recruit primarily PKA tetramers that contain regulatory subunit isoforms other than the mutant RIα subunit. If this is true, then LTP induced by thetaburst stimulation should be blocked by generic pharmacological inhibition of PKA (there are presently no pharmacological inhibitors available that can preferentially block specific isoforms of PKA). Hence, the aim of the next experiment was to determine whether thetaburst LTP in R(AB) mutants is PKA dependent as defined by sensitivity to block by a specific pharmacological inhibitor of PKA.

Inhibition of PKA blocks expression of LTP induced by both tetraburst and thetaburst stimulation

The observation that thetaburst LTP was intact in R(AB) mutant slices prompted further investigation to try to establish whether this form of LTP was PKA dependent in R(AB) mutant mice. Previous studies, performed on area CA1 of hippocampal slices from C57BL/6J mice, have shown that a thetaburst stimulation protocol (identical to that used in the present study) induced LTP that was blocked by inhibitors of PKA (Nguyen and Kandel, 1997). Thus, the normal thetaburst LTP seen in R(AB) mutant slices could still be PKA dependent as defined by its putative sensitivity to block by a specific

pharmacological inhibitor of PKA. Also the PKA dependence of tetraburst LTP, which was defective in R(AB) mutant slices, needs to be confirmed in wildtype slices. A specific inhibitor of PKA, Rp-cAMPS (Dostmann, 1995), blocked expression of tetraburst LTP in wildtype slices without affecting synaptic transmission in an adjacent, untetanized pathway (**Figure 3.3A**). The mean fEPSP slope measured 2h after the end of tetraburst stimulation was 227±18% of pre-LTP baseline values in wildtype control slices, whereas the corresponding mean fEPSP slope measured in slices treated with 100μM Rp-cAMPS was only 100±2% ($p<0.01$, Student's t-test). In an adjacent untetanized pathway, the mean fEPSP slope 2h after tetraburst stimulation of the neighboring pathway was 92±9% (**Figure 3.3A**). Hence these results establish that tetraburst stimulation produces a form of LTP that is blocked by a specific pharmacological inhibitor of PKA.

The next set of experiments tested the hypothesis that intact thetaburst LTP in R(AB) mutant slices may still be PKA dependent. In R(AB) mutant slices, thetaburst stimulation induced robust LTP: the mean fEPSP slope measured 2h after the end of thetaburst stimulation was 155±12% in drug-free slices (**Figure 3.3B**). In contrast, Rp-cAMPS blocked expression of thetaburst LTP in R(AB) mutant slices: the mean fEPSP slope measured 2h after thetaburst stimulation in drug-treated slices was only 97± 8% of pre-LTP baseline values ($p<0.02$, **Figure 3.3B**). Rp-cAMPS had no significant effect on fEPSP slopes recorded during stimulation of an adjacent untetanized pathway (**Figure 3.3B**, □).

These data suggest that thetaburst LTP in R(AB) mutant slices is indeed PKA dependent (see also Nguyen and Kandel, 1997 for wildtype data) as defined by its block by

Rp-cAMPS. When considered alongside the finding that thetaburst LTP was normal in R(AB) mutant slices that express an inhibitory form of the RIα regulatory subunit, these pharmacological data suggest that although both tetraburst and thetaburst patterns of stimulation elicit PKA-dependent forms of LTP in R(AB) slices, each may recruit different isoforms of PKA. In particular it appears that thetaburst LTP requires significant amounts of PKA isoforms that contain regulatory subunits other than the mutant RIα subunit, whereas tetraburst LTP requires isoforms of PKA that contain the mutant RIα subunit.

Discussion

Synaptic activity is an important regulator of plastic changes in hippocampal synaptic transmission (Madison *et al.*, 1991; Martin *et al.*, 2000). Protein kinases are also key effectors of synaptic plasticity, and it is generally believed that the activation of particular kinases may be modulated by specific patterns of electrical activity (Chapman *et al.*, 1995; Lisman, 1989; Micheau and Riedel, 1999). There is biochemical evidence that some kinases may be tuned to discrete patterns of impulse activity for optimal activation (see De Koninck and Schulman, 1998, for CaMKII data). However, *in vitro* biochemical assays of kinase activation kinetics cannot address how synaptic activity influences kinase mediated synaptoplastic processes, such as LTP, in living neurons. In contrast, genetic modification of the hippocampal activities of specific kinases in mice can be used to study the *in situ* kinase dependence of different forms of LTP that are induced by distinct temporal patterns of synaptic activity (Abel *et al.*, 1997; Mayford *et al.*, 1995).

Decreasing both the amount and the temporal spacing of imposed synaptic activity (thetaburst stimulation) in CA1 neurons in hippocampal slices can fully restore LTP in

R(AB) mutant mice that have genetically reduced levels of hippocampal PKA activity. The simplest explanation for this result is that thetaburst stimulation rescued LTP by recruiting other subcellular processes or signalling pathways that are not dependent on, or not linked to, a significant activation of the cAMP-PKA signalling pathway. However, it is clear that thetaburst LTP is critically dependent on the activation of PKA as evidenced by the block of thetaburst LTP by Rp-cAMPS in R(AB) slices (present study) and in slices from wildtype mice (Nguyen and Kandel, 1997).

R(AB) mice have hippocampal basal PKA activity approximately 40–50% less than that of wildtype mice (Abel *et al.*, 1997). This reduced PKA activity resulted from expression of a dominant negative form of the RIα regulatory subunit of PKA (Clegg *et al.*, 1987). One possible mechanism for the observed rescue of LTP in R(AB) mutants is that thetaburst stimulation may have produced modest increases in cAMP, not enough to substantially recruit isoforms of PKA containing the mutant RIα regulatory subunit (see following text for discussion of cAMP activation constants of regulatory subunits). However, the block of thetaburst LTP by Rp-cAMPS in these mutants suggests that thetaburst stimulation still activated significant amounts of PKA isoforms that were critical for expression of this form of LTP. These isoforms likely did not contain mutant RIα subunits because thetaburst LTP was normal in R(AB) mutants. Thetaburst stimulation might recruit tetramers of PKA-containing RII subunits, the subcellular localization of which is mediated by interactions with A-kinase anchoring proteins (Carr *et al.*, 1991; Scott *et al.*, 1990). Although hypothetical, these considerations underscore the need for further research, using molecular biological and biochemical techniques, to specifically

identify and measure the amounts of particular isoforms of PKA that are recruited by these different patterns of synaptic activity in the slice preparation.

It is noteworthy that another isoform of PKA, containing a different type of regulatory subunit, RIβ, is 2.5-fold more sensitive to activation by cAMP than RIα (Cadd *et al.*, 1990). It is therefore plausible that the thetaburst rescue of LTP seen in R(AB) mutants may have resulted from the activation of PKA isoforms containing RIβ following a modest increase in intracellular cAMP elicited by thetaburst stimulation. Strong stimulation protocols, such as the tetraburst regimen used here, produced an LTP deficit in R(AB) mutant slices probably because such stimulation engaged significant amounts of PKA isoforms containing the mutant RIα subunit (in addition to tetramers containing RIβ) or because the amount of PKA activated by tetraburst stimulation is not sufficient to support this type of LTP in mutant slices. Levels of expression of PKA tetramers containing the mutated form of RIα are likely higher than the expression levels of tetramers containing RIβ. This notion is supported by the observation that tetraburst stimulation, which should activate PKA more strongly than the weaker thetaburst protocol, still produces defective LTP in R(AB) mutant slices. Tetraburst stimulation should still activate isomers containing the RIβ subunit, but the presence of isomers containing mutant RIα in high or saturating concentrations may buffer PKA activation sufficiently to block tetraburst LTP in R(AB) mice. Also the markedly lower sensitivity to activation by cAMP of tetramers containing the mutant RIα subunit (Woodford *et al.*, 1989) would prevent significant activation of these isoforms by the more modest increases in cAMP that may result from weaker thetaburst stimulation. Hence tetraburst, but not thetaburst, LTP likely

requires PKA tetramers containing the mutant form of RIα regulatory subunit (**Figure 3.4**).

It is interesting that RIβ knockout mice (Brandon *et al.*, 1995) showed normal LTP induced by a spaced stimulation protocol similar to the regimen that was used here to demonstrate deficient tetraburst LTP in R(AB) mutants. This may have resulted from selective, or substantial, recruitment of PKA isoforms containing native RIα subunits in these RIβ knockout mice. Indeed the expression of RIα subunits is believed to increase in compensation for the genetic knockout of the RIβ subunit (Brandon *et al.*, 1995). Thus LTP induced by spaced 100Hz stimulation in these RIβ knockout mice does not appear to require tetramers containing the RIβ subunit. In R(AB) mice, no compensatory changes in the hippocampal expression of other PKA subunits have been detected (T. Abel, unpublished observations). It would be interesting to see whether thetaburst LTP is affected by the knockout of the RIβ subunit. However, it should be noted that the knockout mice of Brandon *et al.* (1995) did not show measurable changes in hippocampal PKA activity unlike the R(AB) mice used in the present study, which showed reductions in hippocampal PKA activity (Abel *et al.*, 1997).

The observed block of both forms of LTP by Rp-cAMPS lends credence to the idea that although these distinct patterns of stimulation elicited forms of LTP that are PKA dependent (as defined by their sensitivities to Rp-cAMPS), this PKA dependence may be subtly regulated by the activity-dependent recruitment of different tetramers of PKA. Rp-cAMPS inhibits PKA activity by binding to the regulatory subunit of PKA, thereby preventing the cAMP-induced release of catalytic subunits (Dostmann, 1995). At the

concentration of Rp-cAMPS used here (100μM), inhibition of PKA by this drug should show no isoform specificity, as the half-maximal activation constants for cAMP activation of wildtype and mutant regulatory subunits are in the range of 40nM to 5μM (Cadd *et al.*, 1990; Woodford *et al.*, 1989). However, genetic reduction of hippocampal PKA activity in R(AB) mice, through directed expression of an inhibitory form of just one type of regulatory subunit, was sufficient to impair LTP induced by spaced tetraburst, but not by compressed thetaburst, stimulation. Hence the results, obtained by combining pharmacological, electrophysiological, and genetic approaches, support the notion that different patterns of synaptic activity may induce PKA-dependent forms of LTP that require distinct PKA isoforms (**Figure 3.4**).

Another interpretation of the results is that tetraburst and thetaburst stimulation require different amounts of the same isoform(s) of PKA in R(AB) mutant mice. Because R(AB) mutant mice show less hippocampal PKA activity, tetraburst LTP might require activation of larger quantities of PKA isoforms identical to those needed for thetaburst LTP. In contrast, thetaburst stimulation still elicits normal LTP in mutant slices because the amount of PKA present in mutant mice may be sufficient to support theta LTP. This scenario may be improbable for the following reasons. Tetraburst stimulation should lead to a larger increase in intracellular cAMP and stronger activation of *existing* PKA isoforms in mutant slices than thetaburst stimulation (consisting of only 20 pulses in the 1st second of the 3s theta protocol). Thus, one would have to explain how stronger activation of the same isoform(s) of available PKA following tetraburst stimulation can lead to defective LTP in mutant slices, whereas less robust activation of the same isoform(s) of PKA resulting from weaker thetaburst stimulation produces normal LTP in mutant slices. It

should be noted that the cAMP activation constants (which reflect the concentration of cAMP needed to bind to and activate PKA) of tetramers containing mutant RIα subunits are considerably higher than those measured from tetramers containing wildtype subunits (Cadd *et al.*, 1990; Woodford *et al.*, 1989). Hence tetraburst stimulation should more readily engage larger quantities of isoforms containing mutant RIα subunits than thetaburst stimulation. This scenario may explain why tetraburst, but not thetaburst, stimulation leads to defective LTP in mutant slices. Further research, involving exact measurements of *synaptic* PKA isoform expression and activities following defined patterns of stimulation, is required to resolve this issue.

A key question that needs to be examined is: what proportion of the total complement of hippocampal PKA is contributed by isoforms containing the RIα regulatory subunit? PKA is a tetrameric holoenzyme composed of two catalytic and two regulatory subunits. At least two distinct types of catalytic subunits and four different types of regulatory subunits are known to be expressed in the mouse hippocampus *in situ* and, particularly, in area CA1 (Cadd and McKnight, 1989). In principle, therefore, there may be as many as 30 different tetrameric combinations of these wildtype catalytic and regulatory subunits in situ. It is not known whether all 30 tetrameric isoforms actually exist in area CA1, and it is unclear whether there are significant constraints on the formation of particular tetrameric isoforms (*e.g.* spatial anchoring of regulatory subunits) (see Colledge and Scott, 1999). Nonetheless one can calculate a theoretical estimate of the proportion of the total complement of PKA holoenzyme that is contributed by tetrameric isoforms containing at least one RIα regulatory subunit. There are 12 possible tetrameric forms of PKA that contain at least one RIα subunit. Thus, of 30 possible tetrameric combinations of

catalytic and regulatory subunits, the contribution of tetramers containing the RIα subunit is, in principle, 12/30 or 40%. This figure assumes that all 30 possible tetrameric isoforms are present. How does this theoretical calculation compare to the measured level of basal PKA activity in R(AB) mutant mice? In these mice, basal PKA activity in hippocampal extracts is reduced by approximately 40–50% (Abel *et al.*, 1997) as a direct result of genetic expression of an inhibitory form of the RIα regulatory subunit. Furthermore, no compensatory changes in the hippocampal expression of other PKA subunits have been detected in R(AB) mice (T. Abel, unpublished observations). Hence, there is a close correspondence between the theoretical calculated proportion of the total PKA complement contributed by isoforms containing the RIα subunit and the observed levels of inhibition of hippocampal PKA activity produced by genetic mutation of RIα in R(AB) mice. This supports the notion that a significant amount (about 50%) of the total complement of hippocampal PKA available for recruitment in R(AB) mice consists of isoforms containing regulatory subunits other than RIα.

In summary, altering the amount and temporal spacing of synaptic stimulation fully restores LTP in hippocampal slices of R(AB) mutant mice that have genetically reduced levels of hippocampal PKA activity. Spaced tetraburst, but not thetaburst, LTP was impaired in R(AB) mutant slices. Together with the finding that Rp-cAMPS, an inhibitor of PKA, blocked thetaburst LTP in R(AB) slices, these experiments provide evidence for the notion that different amounts and patterns of synaptic activity may elicit PKA-dependent forms of LTP that require distinct isoforms of PKA.

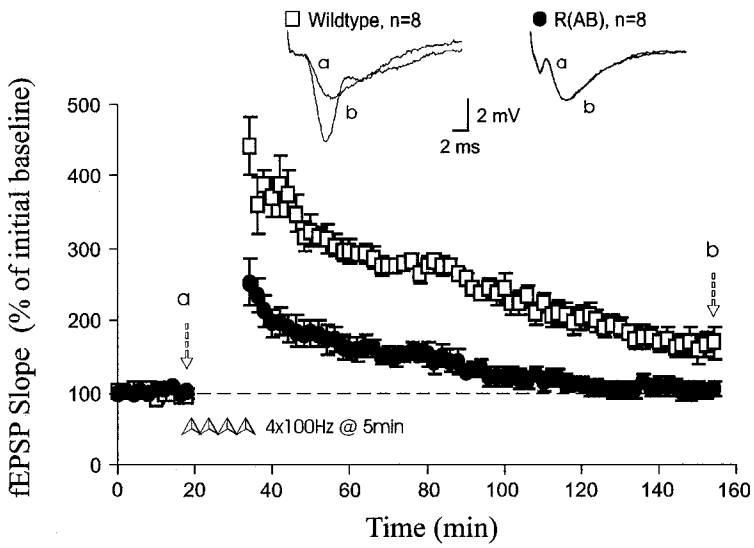


Figure 3.1: Strong, spaced stimulation reveals a deficit in long-term potentiation (LTP) in R(AB) mutant slices. A tetraburst stimulation protocol, consisting of four 100Hz bursts (each 1s in duration) with an interburst interval of 5min, elicited robust LTP in wildtypes (□), but not in R(AB) slices (●). Sample field excitatory postsynaptic potential (fEPSP) sweeps were recorded at the time points indicated as a and b on the graph.

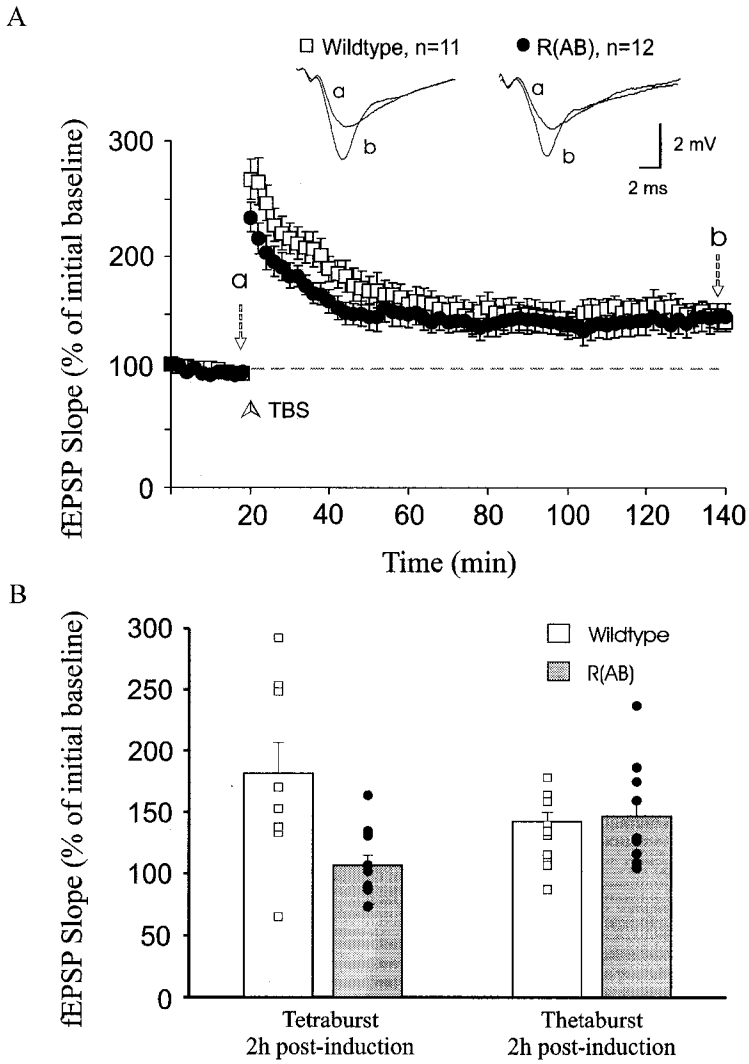
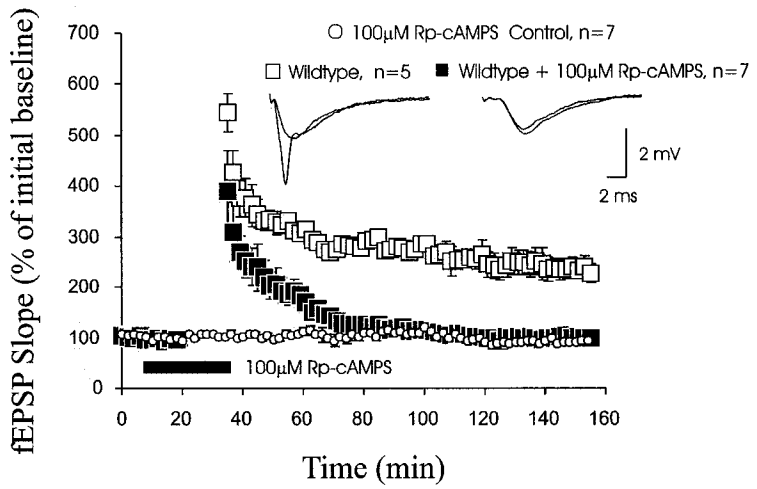


Figure 3.2: Reducing the amount and temporal spacing of imposed activity rescues LTP in R(AB) mutant slices. **(A)** Plot of mean fEPSP slopes recorded from mutant and wildtype slices. A 3s period of thetaburst stimulation (TBS) rescued LTP in R(AB) slices. Sample fEPSP traces were recorded at times a and b marked on the graph. **(B)** Summary histogram of cumulative fEPSP slopes recorded 2h after LTP induction by thetaburst and by tetraburst ("spaced") stimulation. Individual points superimposed on bars represent measurements from single slices. Many of these points overlap each other within a bar. Shapiro-Wilk's test for normality of the distribution of sampled data yielded $p>0.1$ for these data points, indicating that a parametric Student's t-test for statistical significance is appropriate.

A Wildtype - 4x100Hz @ 5min



B R(AB) - 3s Theta

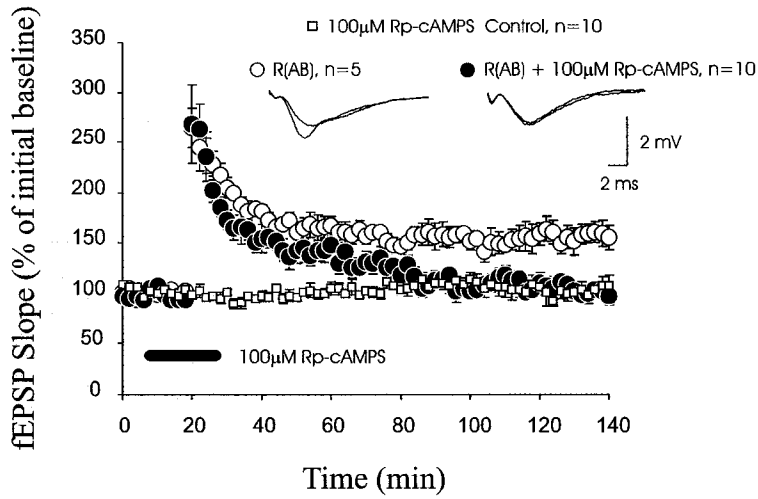


Figure 3.3: A pharmacological inhibitor of protein kinase A (PKA) blocks LTP induced by both tetraburst and thetaburst stimulation. **(A)** Rp-cAMPS blocked LTP in wildtype slices following tetraburst stimulation (■). In an adjacent untetanized pathway in drug-treated slices, fEPSP slopes were unaffected during stimulation at once per minute (○). **(B)** Thetaburst stimulation elicits a form of LTP in R(AB) mutant slices that is PKA dependent. Rp-cAMPS blocked expression of LTP in mutant slices (●), whereas baseline fEPSPs, measured in a 2nd adjacent untetanized pathway in drug-treated slices (□), were unaffected. Sample fEPSP traces were recorded at 5min before the start of LTP induction and 2h after the end of tetraburst or thetaburst stimulation.

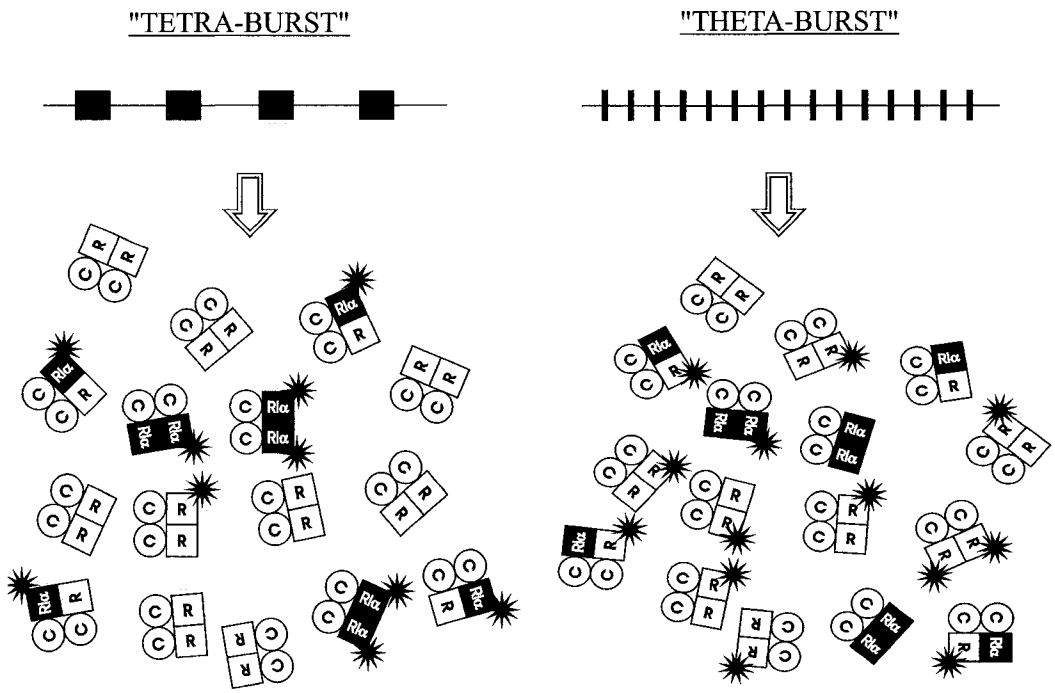


Figure 3.4: Model for activity-dependent recruitment of different PKA isoforms. Tetraburst stimulation (four 100Hz trains spaced 5min apart) induced deficient L-LTP in area CA1 of slices from R(AB) mutant mice. In contrast, weaker thetaburst stimulation elicited robust L-LTP in mutant slices that was still blocked by Rp-cAMPS, an isoform nonspecific inhibitor of PKA. Although both forms of L-LTP are PKA-dependent, thetaburst stimulation likely recruited endogenous isoforms of PKA that did not contain mutant RIα subunits and therefore was not sensitive to R(AB) overexpression. On the other hand, tetraburst stimulation likely engaged larger amounts of isoforms that contained mutant subunits (along with some wildtype native isoforms). Star symbols indicate isoforms that are recruited by stimulation. Mutant subunits are black, endogenous subunits are white.

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*CHAPTER IV:

TEMPORAL SPACING OF SYNAPTIC STIMULATION MODULATES THE DEPENDENCE OF LTP ON CAMP-DEPENDENT PROTEIN KINASE

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Introduction

Hippocampal long-term potentiation (LTP) is an activity-dependent enhancement of synaptic transmission that is an important regulator of some forms of learning and memory in the mammalian brain (Bliss and Lomo, 1973; Bliss and Collingridge, 1993; Moser *et al.*, 1998; Martin *et al.*, 2000; Brun *et al.*, 2001). Induction and expression of hippocampal LTP involves numerous protein kinases (Madison *et al.*, 1991; Micheau and Riedel, 1999; Martin *et al.*, 2000), which are believed to be critical for the translation of electrical activity into persistent subcellular alterations that may constitute neural representations of information storage in the brain. Electrical activity may control which kinases are critical for the expression of particular forms of synaptic plasticity (Lisman, 1989; Chapman *et al.*, 1995; Elgersma and Silva, 1999; Micheau and Riedel, 1999; Martin *et al.*, 2000). There is biochemical evidence that some kinases may be tuned to discrete patterns of stimulation for optimal activation (De Koninck and Schulman, 1998). However, *in vitro* biochemical assays of kinase activities cannot elucidate which particular features of synaptic stimulation patterns are critical for modulating the dependence of synaptic plasticity on specific kinases. In contrast, genetic modification of the hippocampal activities of specific kinases in mice can be used to identify specific characteristics of stimulation that are critical for modulating the dependence of synaptic plasticity on these particular kinases. Identifying the critical features of synaptic stimulation that can alter the kinase-dependence of particular forms of synaptic plasticity is an essential step towards establishing causality between the activity-induced recruitment of kinase-dependent neuronal processes and the expression of a certain type of synaptic plasticity.

A bewildering range of stimulation protocols has been used to induce kinase-dependent forms of LTP in rodent hippocampal slices (for reviews, see Madison *et al.*, 1991; and Martin *et al.*, 2000). However, it remains unclear which specific characteristics of imposed stimulation are critical for modulating the requirement for specific kinases during induction and expression of particular forms of LTP. The temporal spacing between successive trains of stimuli, or the total amount of imposed stimulation, could importantly regulate the kinase-dependence of some forms of hippocampal LTP. Identifying which of these two characteristics of stimulation protocols can critically alter the dependence of LTP on particular kinases is an important step towards elucidating the molecular bases of synaptic plasticity, as it will shed light on the experience-dependent mechanisms by which particular patterns of electrical activity can engage identifiable signalling pathways leading up to expression of key forms of synaptic modifications.

In rodent hippocampal slices, one kinase that is critical for expression of LTP in the Schaeffer collateral pathway of area CA1 is cAMP-dependent protein kinase (PKA). Pharmacological inhibition and genetic modification of PKA attenuate LTP in area CA1 of mouse and rat hippocampal slices (Frey *et al.*, 1993; Huang and Kandel, 1994; Blitzer *et al.*, 1995; Qi *et al.*, 1996; Abel *et al.*, 1997; Nguyen and Kandel, 1997; Woo *et al.*, 2000; Otmakhova *et al.*, 2000). Multi-train stimulation, consisting of 3-4 successive trains of 100Hz, spaced 5-10min apart, is a predominant stimulation pattern that has been widely used for inducing PKA-dependent forms of LTP in area CA1 of rodent hippocampal slices (rat data: Frey *et al.*, 1993, Huang and Kandel, 1994, Blitzer *et al.*, 1995; mouse data: Qi *et al.*, 1996, Abel *et al.*, 1997, Nguyen and Kandel, 1997, Woo *et al.*, 2000). In many of these studies, the requirement for PKA (or PKA-dependent processes) during multi-train LTP

was inferred from the disruption of LTP induction and/or maintenance by genetic inhibition of PKA or by acute application of pharmacological inhibitors of PKA to hippocampal slices. These types of experiments do not, however, provide any clue as to which specific characteristics of the stimulation protocol (*e.g.* temporal spacing, total number of pulses) are critical for shaping the PKA-dependence of LTP.

One direct approach to addressing this issue is to genetically reduce hippocampal PKA activity and probe the expression of LTP induced by distinct temporal patterns of stimulation with fixed amounts of stimulus pulses. The present study employed genetically modified mice expressing R(AB), a dominant negative inhibitor of PKA, which results in substantial inhibition of hippocampal PKA activity (Abel *et al.*, 1997). The findings of the study provide direct evidence that the temporal spacing of synaptic stimulation is a critical characteristic of multi-train stimulation that can modulate the PKA-dependence of LTP in area CA1. This activity-regulated plasticity of the PKA-dependence of hippocampal LTP may be important for gating long-lasting synaptic modifications and for regulation of neural information processing.

Materials and Methods

R(AB) Mice

Transgenic mice were derived from two independent lines that were previously characterized for neural expression of the R(AB) transgene, hippocampal PKA activity, hippocampal synaptic physiology, and hippocampus-dependent long-term memory (Abel *et al.*, 1997). Male and female mice between the ages of 4 and 6 months from the two transgenic lines, R(AB)-1 and R(AB)-2, were used.

Electrophysiology

Transverse hippocampal slices (400μm thickness) were prepared from R(AB) transgenic and wildtype mice as described in *Chapter II.* Slices were maintained in an interface chamber at 28°C and were perfused (1 mL/min) with artificial cerebrospinal fluid (ACSF; composition as described in *Chapter II*) that was aerated with a gaseous mixture of 95% O_2 and 5% CO_2. Extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded with a glass microelectrode that was positioned in stratum radiatum of area CA1. Evoked fEPSPs were elicited by stimulation of the Schaeffer collateral fibres using an extracellular bipolar nickel-chromium electrode.

LTP was induced by applying four trains (100Hz, each 1s duration, test strength), spaced 5min, 20s, or 3s apart (**Figure 4.1**; Table 2.1). 2-amino-5-phosphonopentanoic acid (D,L-APV), was prepared as a concentrated stock solution in distilled water, and it was bath-applied (after dilution to 50μM in ACSF) to slices in some experiments. KT-5720 was dissolved as a stock solution in DMSO and diluted in ACSF to a final perfusate concentration of 1μM (0.1% DMSO; this concentration of DMSO had no effect on basal fEPSPs). A second, independent pathway was monitored (Nguyen *et al.*, 1994) in area CA1 during experiments with KT-5720, in order to assess the effects of this drug on basal synaptic transmission. Drug experiments were interleaved with drug-free controls.

Results

LTP induction by diverse temporal patterns of stimulation requires NMDA receptors

Synaptic efficacy in the hippocampus is critically modulated by the temporal pattern of synaptic stimulation (see reviews by: Madison *et al.*, 1991, Hawkins *et al.*, 1993,

Bear and Abraham, 1996, Bito *et al.*, 1996). Temporally compressed patterns of high-frequency stimulation can elicit robust and long-lasting LTP in mouse hippocampal slices (Nguyen and Kandel, 1997), and these compressed patterns of stimulation more closely resemble the spike firing profiles of hippocampal neurons *in vivo* (Kandel and Spencer, 1961). As an initial step towards defining which particular aspects of imposed activity are critical for regulating the expression of PKA-dependent forms of LTP, the effects of varying the temporal spacing of synaptic stimulation on the NMDA receptor-dependence of LTP in area CA1 was explored.

Three different stimulation regimens, each containing the same number of bursts (four) and electrical impulses (400 pulses), were used in this study. However, the temporal spacing between successive bursts (**Figure 4.1**) was varied. In wildtype slices, four bursts of 100Hz stimulation, with interburst intervals of 5min, 20s, or 3s, induced robust and long-lasting LTP in area CA1 (**Figure 4.2,** □). In the presence of 50μM APV, induction of LTP in wildtype slices by these three stimulation regimens was significantly attenuated (**Figure 4.2,** ●). In drug-treated slices, the mean fEPSP slopes (measured 40min post-induction) for each of the 5min, 20s, and 3s interburst intervals, were 91±10%, 101±13%, and 116±29%, respectively, of pre-LTP control values ($p<0.01$, **Figure 4.2A-C**). These data show that multiple forms of long-lasting potentiation can be induced in area CA1 by temporally spaced and compressed patterns of stimulation, and that these types of LTP all require NMDA receptor activation for their induction.

LTP induced by temporally spaced stimulation is defective in slices from R(AB) mice

Can the temporal spacing of synaptic activity critically modulate the expression of PKA-dependent forms of LTP? To address this question, spaced and compressed stimulation regimens, as in the experiments of **Figure 4.2**, were used to induce LTP in hippocampal slices from R(AB) transgenic and wildtype mice.

As previously reported (see Abel *et al.*, 1997), four 100Hz bursts, each 1s in duration and spaced 5min apart, produced robust LTP in area CA1 of slices from wildtype mice (**Figure 4.3A,** □). Slices from R(AB) mice showed significantly less potentiation than wildtypes ($p<0.02$ at all time points after LTP induction; **Figure 4.3A,** ●). Mean values for fEPSP slopes measured 5min and 2h post-induction in wildtype slices were 372±24% and 182±25%, respectively. In slices from R(AB) mice, the corresponding slope values were 225±21% and 106±8%, respectively (**Figure 4.3B**). The fEPSP slopes observed in R(AB) slices decayed to pre-LTP baseline values within 100min post-induction. These results confirm previously published data (Abel *et al.*, 1997) and show that tetraburst stimulation using a temporally spaced pattern of imposed activity elicits PKA-dependent LTP.

Intact LTP induced by compressed patterns of stimulation in slices from R(AB) mice

Since slices from R(AB) mice showed defective LTP induced by a temporally spaced pattern of stimulation (**Figure 4.3**), the effects of temporally compressing the four 100Hz bursts of stimulation on LTP in hippocampal area CA1 of mutant and wildtype slices was explored next. Compressed stimulation can significantly increase cAMP levels in hippocampal slices (Chetkovich *et al.*, 1991), but it is not known whether compressed

tetraburst stimulation can elicit PKA-dependent forms of LTP. To address this issue, the stimulation patterns were compressed by shortening the interburst interval from 5min to either 20s or 3s, without changing the total amount of imposed activity (**Figure 4.1**). As shown in **Figure 4.4A, C**, both compressed patterns of stimulation produced robust synaptic potentiation that persisted for at least 2h in wildtype slices: mean fEPSP slopes were 267±18% and 132±14% for the 20s interburst interval (**Figure 4.4B**, white bars), and 298±28% and 169±15% for the 3s interburst interval (**Figure 4.4D**, white bars), measured at 5min and 2h post-induction, respectively. In contrast to the 5min interburst interval protocol, persistent LTP was observed in slices from R(AB) mice following compressed stimulation. Mean fEPSP slopes for R(AB) slices were 247±20% and 153±16% for the 20s interburst interval (**Figure 4.4B**, shaded bars), and 253±21% and 163±23% for the 3s interburst interval (**Figure 4.4D**, shaded bars), measured at 5min and 2h post-induction, respectively. These values were not significantly different from the time-matched measurements made from wildtype slices ($p>0.5$, **Figure 4.4B, D**, white bars). These results show that LTP induced by temporally compressed patterns of stimulation was not significantly impaired by genetic reduction of hippocampal PKA activity.

The PKA-dependence of LTP is critically modulated by the temporal spacing of stimulation

R(AB) mice express a partial reduction of hippocampal PKA activity (Abel *et al.*, 1997). As such, the intact LTP observed in mutant slices following compressed patterns of synaptic stimulation may represent either PKA-independent forms of LTP or types of LTP that still require recruitment of particular isoforms of PKA that are still expressed in

hippocampal tissue from R(AB) mice. Indeed, a previous study has shown that altering the total amount *and* temporal spacing of stimulation engages distinct isoforms of hippocampal PKA that are important for LTP (Woo *et al.*, 2000). To distinguish between these two interpretations of the present data, and to test the idea that temporal spacing of stimulation *per se* may critically modulate the PKA-dependence of LTP, the sensitivity of LTP to a pharmacological inhibitor of PKA catalytic subunits (KT-5720) was assessed. At the concentration used here (1μM), KT-5720 has no preferential affinity for blocking specific isoforms of PKA catalytic subunit (Kase *et al.*, 1987), so that intact LTP in the presence of this drug will indicate that LTP does not require PKA activation.

Acute bath application of KT-5720 to slices from R(AB) mutant mice did not significantly alter the induction or maintenance of LTP induced by compressed tetraburst stimulation (**Figure 4.5A, B**). With a 20s interburst interval (400 total pulses), the mean fEPSP slopes measured in control and drug-treated mutant slices at 2h post-tetanus were 174±20% and 162±10%, respectively ($p>0.5$, **Figure 4.5A**). For a 3s interburst interval, the mean fEPSP slopes measured in control and drug-treated mutant slices at 2h post-tetanus were 162±12% and 176±12%, respectively ($p>0.5$, **Figure 4.5B**). In contrast, KT-5720 significantly blocked maintenance of LTP in wildtype slices following the same tetraburst stimulation applied with a 5min interburst spacing: mean fEPSP slopes measured 2h post-tetanus in control and drug-treated slices were 207±26% and 99±6%, respectively ($p<0.01$, **Figure 4.5C**). KT-5720 did not significantly perturb synaptic transmission in a second, independent pathway that did not undergo high-frequency tetraburst stimulation (**Figure 4.5A, C**). The data suggest that compressing the temporal spacing of stimulation

per se in slices from mutant mice does not appear to differentially engage distinct isoforms of PKA, as LTP was not affected by a pharmacological inhibitor of PKA in mutant slices.

Discussion

Many different stimulation protocols have been used to induce different forms of LTP in rodent hippocampal slices. However, it remains unclear which specific characteristics of imposed stimulation (*i.e.* temporal vs. amount) are critical for modulating the requirement for PKA during induction and expression of particular forms of LTP. Suppressive effects of PKA inhibitors on LTP are most clearly evident when stronger stimulation regimens, such as multiple trains rather than a single train of 100Hz stimulation, are used (Frey *et al.*, 1993; Huang and Kandel, 1994; Blitzer *et al.*, 1995; Qi *et al.*, 1996; Abel *et al.*, 1997; Nguyen and Kandel, 1997; Abel *et al.*, 1997; Duffy *et al.*, 2000; but see Otmahkhova *et al.*, 2000). These results suggest that amount of stimulation can modulate the PKA-dependence of LTP. However, the present data show that the temporal spacing of tetraburst stimulation can also regulate the PKA-dependence of LTP. Interestingly a recent study has shown that the protein synthesis-dependence of tetraburst LTP is insensitive to the temporal spacing of stimulation (Scharf *et al.*, 2002). Thus, the temporal spacing of synaptic stimulation may selectively modulate the PKA-dependence, but not the protein synthesis- dependence, of LTP.

The nature of the signal transduction pathway activated can be influenced by the frequency and intensity of the stimulus used to induce LTP (Cavus *et al.*, 1996). Activation of NMDA receptors, which is coupled to downstream activation of PKA (Roberson and Sweatt, 1996) and adenylate cyclase (Chetkovich *et al.*, 1991; Chetkovich and Sweatt,

1993), is critical for induction of LTP by 100Hz trains in area CA1 (Collingridge *et al.*, 1983). As such, manipulations of the temporal spacing of stimulation may modulate the expression of LTP by altering the efficacy of NMDA receptor activation during high-frequency tetanic stimulation leading to LTP induction. Here both temporal and compressed tetraburst stimulation protocols elicit NMDA receptor dependent forms of LTP. Changing the temporal spacing of the tetani did not alter the receptor dependence of LTP induced.

Although PKA enzyme activity in these experiments were not measured, it is likely that all of the stimulus protocols (spaced and compressed) increased basal PKA activity, as PKA activity is sensitive to fine alterations in intracellular levels of calcium and cAMP (Beavo *et al.*, 1974; Chetkovich and Sweatt, 1993; Roberson and Sweatt, 1996). However, an increase in basal PKA activity does not necessarily entail, and is not equivalent to, a *critical requirement* for PKA during LTP. Hippocampal PKA may be activated by a wide range of stimulation frequencies, but events, occurring downstream from enzymatic activation, are the crucial elements in determining whether a particular type of LTP is critically dependent on PKA. Inhibitory synaptic inputs may be engaged more strongly by compressed stimulation, and they may play a role in dampening the amount of excitation experienced by these neurons that, in turn, might attenuate the PKA-dependence of LTP. Further studies are needed to identify the cellular mechanisms that permit LTP induced by compressed tetraburst stimulation to "bypass" the requirement for PKA normally seen following spaced tetraburst stimulation. Indeed, the PKA pathway may act as a "low-pass filter," such that temporally spaced patterns of electrical activity engage PKA-dependent

processes that are critical for LTP more strongly than compressed patterns (*i.e.* higher frequencies) of stimulation.

Another way by which the compressed burst stimulation generated long-lasting LTP in slices from R(AB) mutant mice is recruitment of other signalling pathways. Other signalling molecules known to be critical for LTP include MAP kinases (Orban *et al.*, 1999; Winder *et al.*, 1999; Davis *et al.*, 2000) and CREB (Bourtchouladze *et al.*, 1994; Deisseroth *et al.*, 1996). Indeed, a compressed thetaburst pattern of stimulation can engage MAP kinases during LTP (Winder *et al.*, 1999). Furthermore, activation of CREB is under the control of numerous kinases other than PKA, including CaMKII, CaMKIV, and the MAP kinases, ERK1 and ERK2 (Shaywitz and Greenberg, 1999; Ahn *et al.*, 2000). Therefore, LTP elicited by closely spaced tetani may selectively activate or upregulate MAPK or other kinases that lead to long-lasting potentiation. Further experiments examining the signalling transduction profiles engaged by different stimulation regimen are required. This will help us understand the nature and potential interactions between signalling cascades activated by different protocols of synaptic stimulation.

The results from the present study are important for understanding the molecular bases of synaptic plasticity because they show that modifications in the temporal pattern (interburst spacing) of synaptic stimulation, without changing the total amount of stimulus pulses, can recruit different signalling molecules for the expression of LTP in the hippocampus. It should be noted that the tetraburst stimulation used here is not unique in its ability to elicit PKA-dependent forms of LTP. In an earlier study by Nguyen and Kandel (1997), a single one-second train of 60 pulses induced LTP that was unaffected by an inhibitor of PKA, Rp-cAMPS. In contrast, the same total number of 60 pulses, when

delivered in a thetaburst pattern with more compressed temporal spacing between pulses, elicited a form of LTP that was attenuated by two different inhibitors of PKA (Nguyen and Kandel, 1997). However, in those experiments, the amount of pulses per burst *and* the temporal spacing between pulses and bursts were *both* substantially altered in the transition from a single 60Hz train (1s duration) to three seconds of thetaburst stimulation (15 bursts of 4 pulses at 100Hz, delivered at a burst frequency of 5Hz: see Nguyen and Kandel, 1997). The present study has clarified and extended these earlier findings by Nguyen and Kandel (1997), by showing that it is the interburst temporal spacing of tetraburst stimulation that is critical for modulating the PKA-dependence of LTP in the mouse hippocampus. Also, the present data suggest that recruitment of distinct isoforms of PKA probably does not account for the differences in LTP observed following manipulations of the temporal spacing of stimulation *per se* (see Woo *et al.*, 2000). Altogether, these findings, highlight the fact that multiple forms of LTP, that vary in their sensitivity to disruption by genetic or pharmacological inhibition of PKA, can be induced by altering only the temporal spacing of stimulation in mouse hippocampal slices.

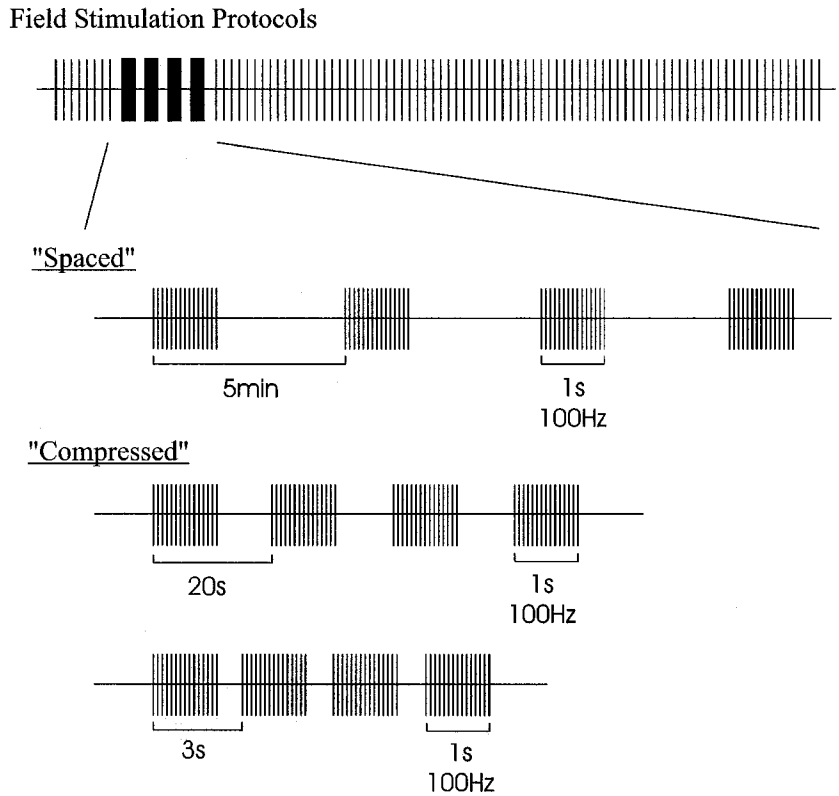

Figure 4.1: Experimental protocols used for synaptic stimulation. Different temporal patterns of synaptic stimulation were used to induce LTP. A "spaced" pattern consisted of four 100Hz bursts (each 1s duration) delivered with a 5min interburst interval. "Compressed" patterns included the same tetraburst regimen, but delivered with 20s or 3s interburst intervals. These stimulation protocols were applied after an initial 20min period of recording baseline fEPSP responses (stimulus rate of once/min). Following LTP induction by one of these protocols, fEPSP responses were sampled for another 2h at a stimulus rate of once/min. Time intervals shown are not drawn to scale.

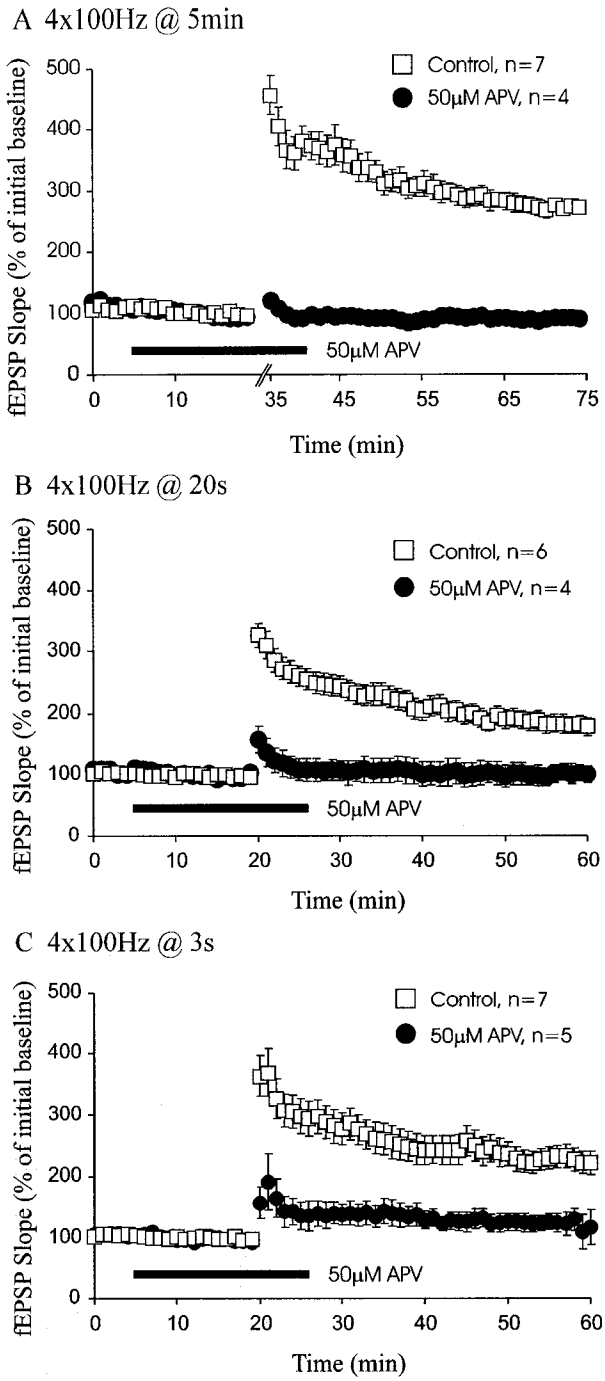

Figure 4.2: NMDA receptor-dependence of LTP induced by temporally spaced and compressed stimulation. **(A)** Spaced stimulation (4 bursts of 100Hz, delivered at a 5min interburst interval) induced LTP in wildtype slices (□) that was blocked by APV, an antagonist of NMDA receptors (●). **(B & C)** Two temporally compressed stimulation protocols (4 bursts of 100Hz, delivered at 20s or 3s interburst intervals) elicited LTP in wildtype slices (□) that was blocked by APV (●).

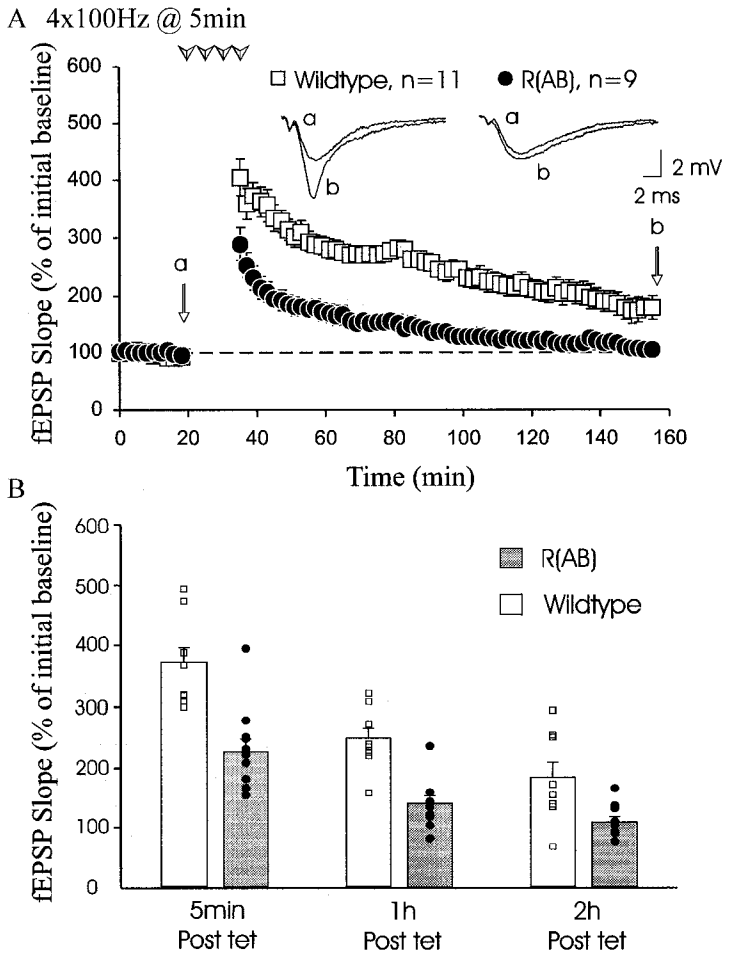

Figure 4.3: Defective LTP in R(AB) mutants following temporally-spaced stimulation. **(A)** LTP in area CA1 of hippocampal slices from R(AB) mutant mice (●) was significantly attenuated compared to wildtype slices (□). The stimulation protocol consisted of four 100Hz bursts (inverted arrowheads; 1s duration) spaced apart at a 5min interburst interval. Sample fEPSP traces were recorded at the times indicated by 'a' and 'b' on the graph. **(B)** Summary histogram of data in A. fEPSP slopes were measured at indicated times, after tetanic stimulation, from mutant (shaded bars) and wildtype (open bars) slices. Each point represents a measurement from one slice. Several points overlap each other within a bar.

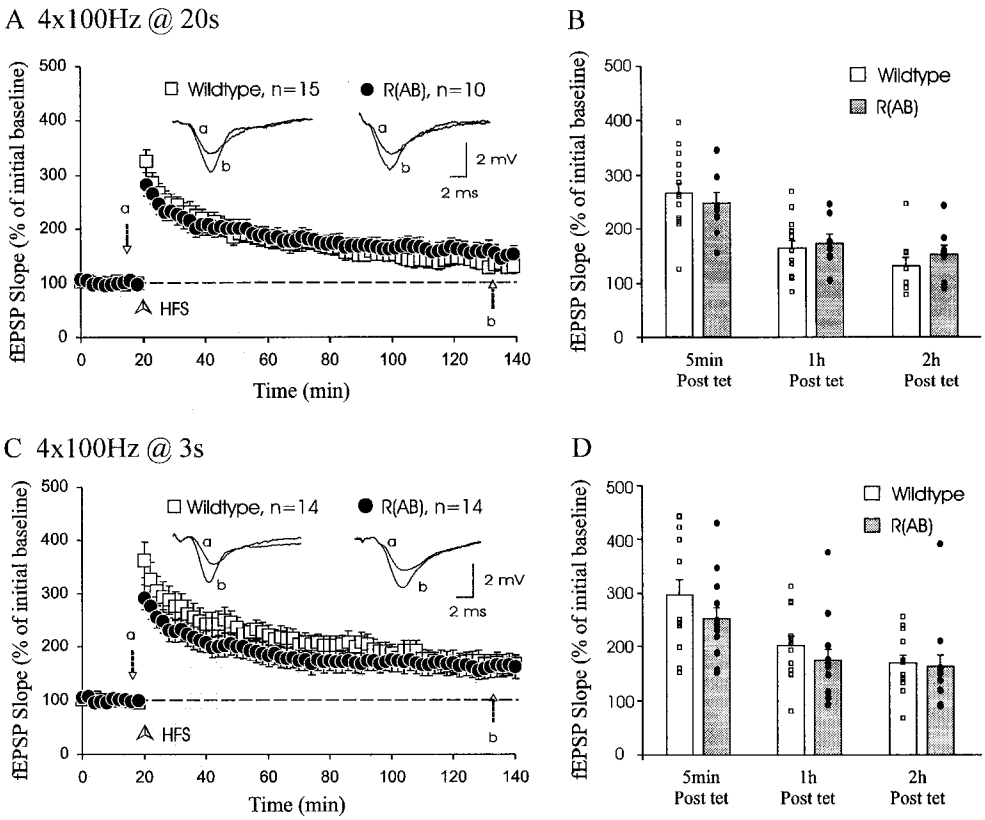

Figure 4.4: Rescue of LTP in R(AB) mutants by temporally compressed patterns of stimulation. **(A)** LTP observed in slices from R(AB) mutant mice (●) was not different than slices from wildtype mice (□) following tetraburst stimulation with a 20s interburst interval. Sample fEPSP traces were recorded at indicated times 'a' and 'b'. **(B)** Summary histogram of data in A. fEPSP slopes were measured from wildtype (open bars) and mutant (shaded bars) slices. Each point represents a measurement from one slice at the indicated times after tetanic stimulation. Numerous points overlap each other within a bar. **(C)** Tetraburst stimulation using a 3s interburst interval induced robust LTP in R(AB) slices (●) that was not significantly different from LTP in wildtype slices (□). Sample traces were recorded at times 'a' and 'b' on graph. **(D)** Summary histogram of data in C. Each point is a measurement from one mutant (shaded bars) or wildtype (open bars) slice.

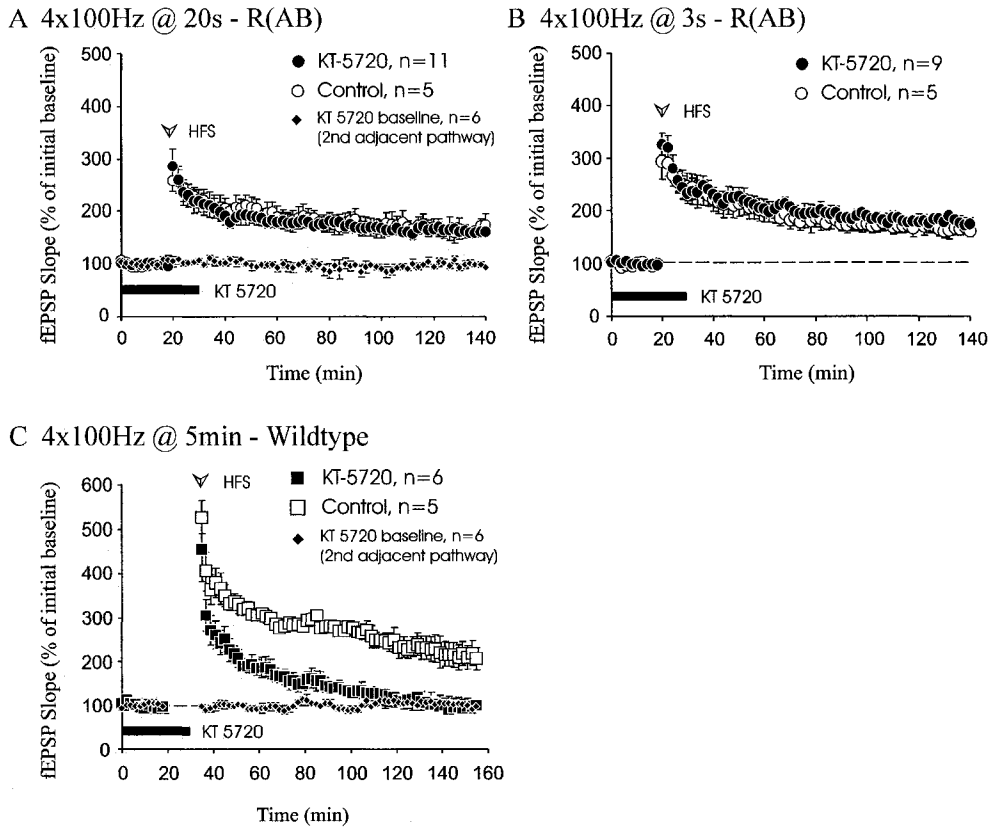

Figure 4.5: Modulation of the PKA-dependence of LTP by the temporal spacing of high-frequency stimulation. In mutant R(AB) slices, tetraburst stimulation using a **(A)** 20s or a **(B)** 3s interburst interval induced LTP that was not significantly affected by bath application (solid horizontal bars) of KT-5720 (●), an inhibitor of PKA, as compared to control drug-free slices (○). Field EPSPs measured in an adjacent pathway that did not experience tetraburst stimulation were not affected by drug application (◆ in A). **(C)** In contrast, KT-5720 significantly attenuated LTP following spaced tetraburst stimulation (5min interburst interval) in slices from wildtype mice (■). Field EPSPs were not significantly affected in a second, adjacent pathway that received only test stimulation at the rate of once/min (◆ in C).

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*CHAPTER V:

A ROLE FOR CYCLIC AMP-DEPENDENT PROTEIN KINASE-MEDIATED SUPPRESSION OF PROTEIN PHOSPHATASES IN GATING THE EXPRESSION OF LATE LTP.

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Introduction

Activity-dependent changes in the physiological strength of synaptic transmission ("synaptic plasticity") are thought to underlie certain types of learning and memory (Bliss and Collingridge, 1993; Martin *et al.*, 2000). Long-term potentiation (LTP), a persistent enhancement of synaptic transmission elicited by brief high-frequency stimulation (Bliss and Lomo, 1973), is one type of synaptic plasticity that may regulate information storage in the mammalian brain (reviewed by Martin *et al.*, 2000). LTP consists of at least two mechanistically distinct temporal phases, an early phase (E-LTP) that lasts for about one hour after induction, and a longer-lasting late phase (L-LTP) that can persist for more than 6 hours (Bliss and Lomo, 1973; Andersen *et al.*, 1977; Matthies and Reymann, 1993; Huang and Kandel, 1994). A hallmark of L-LTP is its requirement for PKA activation. Genetic and pharmacological manipulation of hippocampal PKA activity alters expression of L-LTP in the Schaeffer collateral pathway of area CA1 of hippocampal slices (Matthies and Reymann, 1993; Frey *et al.*, 1993; Huang and Kandel, 1994; Nguyen *et al.*, 1997; Abel *et al.*, 1997; Barad *et al.*, 1998; Bach *et al.*, 1999).

How does PKA modulate the expression of L-LTP? Protein phosphatases are key regulators of hippocampal synaptic plasticity, and their actions can impede expression of LTP (see review by Winder and Sweatt, 2001). Specific phosphatases, such as protein phosphatase-1 (PP1) and/or protein phosphatase–2A (PP2A), may inhibit expression of LTP (Blitzer *et al.*, 1995, 1998; Allen *et al.*, 2000; Brown *et al.*, 2000), and the cAMP-PKA signalling pathway may "gate" the expression of LTP by suppressing the inhibitory constraint exerted by PP1/2A (Blitzer *et al.*, 1995, 1998; see also Hemmings *et al.*, 1984). If this "gating" hypothesis of LTP were correct, then one would predict that *genetic*

reduction of hippocampal PKA activity in neurons should impair L-LTP by relieving PKA-mediated inhibition of PP1/2A.

To address this hypothesis, the present study uses PKA transgenic mice R(AB) transgenic mice that express reduced hippocampal PKA activity in neurons and show impaired L-LTP (Abel *et al.*, 1997). If PKA-mediated inhibition of PP1/2A critically "gates" the expression of L-LTP, then hippocampal slices from mutant mice should show deficient L-LTP that is rescued by pharmacological inhibition of PP1/2A.

Materials and Methods

Animals

Transgenic mice were derived from two independent lines that were previously characterized for neural expression of the R(AB) transgene, hippocampal PKA activity, and hippocampal synaptic physiology (Abel *et al.*, 1997). Male and female mice between the ages 9-12 weeks from the two transgenic lines, R(AB)-1 and R(AB)-2, were used. These mice were maintained in the hemizygous state on a C57BL/6J background.

Electrophysiology

Transverse hippocampal slices (400μm thickness) from R(AB) transgenic and wildtype mice were prepared and maintained as described previously (*Chapter II*). Extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded with a glass microelectrode, filled with artificial cerebrospinal fluid (ionic composition as described in *Chapter II*), and positioned in stratum radiatum of area CA1. fEPSPs were elicited by an extracellular bipolar nickel-chromium electrode positioned in stratum radiatum. Test

stimuli were given once per minute at this intensity. LTP was induced by applying four one-second trains (100Hz, test strength; "tetraburst" stimulation) spaced 5min apart. For some experiments, a second independent pathway that did not receive tetraburst stimulation was recorded to monitor the effects of applied drugs.

Sodium okadaic acid and Rp-cAMPS were prepared as concentrated stock solutions in distilled water, whereas calyculin A, FK-506, forskolin, and IBMX were dissolved in DMSO (Table 2.2). Application of DMSO alone, at a concentration used to solubilize these drugs (0.01%), did not affect basal synaptic transmission or L-LTP (data not shown). Drugs were diluted with ACSF to the desired concentration prior to bath application. For experiments using calyculin A, slices were incubated in drug for a minimum period of 40min prior to stimulation. Drug experiments were interleaved with control experiments.

Results

Inhibition of PP1/2A rescues L-LTP in R(AB) mutant slices

PKA is believed to modulate ("gate") LTP by its interactions with PP1/2A (Blitzer *et al.*, 1995, 1998). Previous studies emphasized the role of this interaction in the expression of E-LTP (Blitzer *et al.*, 1995, 1998). However, many of the experiments done by Blitzer *et al.* (1995) did not explore the contribution of the gating mechanism to later stages of LTP, beyond 30min post-induction. PKA-mediated inhibition of PP1/2A may also modulate L-LTP. To address this hypothesis, a tetraburst stimulation regimen that elicits L-LTP in wildtype slices was tested in hippocampal slices from R(AB) mutant mice (Abel *et al*, 1997; Woo *et al.*, 2000).

Mutant slices showed defective L-LTP that decayed to pre-tetanization fEPSP slope values within 100min after induction (**Figure 5.1A,** ○). Mean fEPSP slope values measured immediately after tetanization, and at 1h and 2h post-tetanization, were 341±26%, 166±11%, and 110±6%, respectively. These findings are consistent with previously published data (Abel *et al.*, 1997; Woo *et al.*, 2000).

To assess whether PKA-mediated inhibition of PP1/2A importantly contributes to the attenuation of L-LTP seen in R(AB) mutant slices, two inhibitors of PP1/PP2A, okadaic acid (OA) and calyculin A (cal-A), were applied. In contrast to the decay of L-LTP observed in R(AB) mutant slices, acute application of OA or prior incubation of slices in cal-A rescued L-LTP in mutant slices (**Figure 5.1A**). Mean fEPSP slopes measured immediately after tetanization, and at 1h and 2h post-tetanization, were: for OA: 407±41%, 191±18%, and 186±17%; for cal-A: 390±35%, 214±20%, and 185±24%, respectively ($p<0.05$ for OA and cal-A at 2h time point, $p>0.05$ for other cited times, as compared to drug-free slices; see **Figure 5.1C**). OA did not affect basal synaptic transmission when a second independent pathway was monitored (**Figure 5.1A**).

When OA or cal-A was applied to *wildtype* slices, the magnitude of L-LTP in drug-treated slices was not significantly different from that seen in control slices (**Figure 5.1B**, $p>0.05$ at all time points). Mean fEPSP values for control wildtype slices were 439±42%, 220±10% and 190±9% immediately after tetanization, and at 1h and 2h post-tetanization, respectively. Corresponding slope values were 384±28%, 198±11%, and 194±8% for OA-treated slices ($p>0.05$ for these time points). Slices incubated in cal-A showed

corresponding values of 386±24%, 217±25% and 203±33% ($p>0.05$ for these time points). **Figure 5.1C** summarizes these data.

Overall, these results show that pharmacological inhibition of PP1/2A rescues L-LTP in PKA mutant slices. The results extend the findings of Blitzer *et al.* (1995) by showing that PKA-mediated inhibition ("gating") of PP1/2A is critical for expression of later stages of LTP (measured 2h post-induction). Genetic inhibition of hippocampal PKA impairs L-LTP by enabling a PP1/2A-mediated inhibitory constraint on L-LTP.

Inhibition of PP1/2A does not significantly alter E-LTP in mutant and wildtype slices

Slices from R(AB) mice show normal LTP induced by one train of 100Hz stimulation (Abel *et al.*, 1997). This stimulation protocol elicits E-LTP that is less dependent on PKA (but see Otmakhova *et al.*, 2000). Acute application of OA did not enhance E-LTP in R(AB) or wildtype slices (**Figure 5.1D**, $p>0.05$ at all time points). Mean fEPSP slopes at 1h post-tetanization were 159±16% and 157±9% for R(AB) and wildtype slices, respectively. Thus, inhibition of PP1/2A does not enhance E-LTP.

Relief of the inhibitory constraint imposed by protein phosphatases permits cAMP-induced synaptic facilitation that is normally impaired by genetic inhibition of PKA

Chemical activation of adenylyl cyclase, by co-application of forskolin and IBMX, can induce robust synaptic facilitation in area CA1 by increasing cAMP levels (Chavez-Noriega and Stevens, 1992; Huang and Kandel, 1994). Are interactions between PKA and PP1/2A important for expression of forskolin/IBMX-induced facilitation? Co-application of forskolin and IBMX, a phosphodiesterase inhibitor, induced robust facilitation in

wildtype slices: the mean slope value was 285±26% 1h after application (**Figure 5.2A, ■**). This facilitation was blocked by Rp-cAMPS (**Figure 5.2A,** □), a membrane-permeant inhibitor of PKA: the corresponding mean fEPSP slope value was only 108±3% in Rp-cAMPS-treated slices. When IBMX was applied alone, no effect on transmission was seen (**Figure 5.2A, ◆**). However, when forskolin and IBMX were co-applied to R(AB) transgenic slices, a marked reduction in the magnitude of the facilitation was observed (**Figure 5.2B, ●**). The mean fEPSP slope value 1h post-drug was 129±9% ($p<0.05$ compared to wildtype values in **Figure 5.3A**). This attenuated facilitation was also blocked by Rp-cAMPS (**Figure 5.2B,** ○). When OA was applied to mutant R(AB) slices before and during application of forskolin/IBMX, robust facilitation, similar in magnitude to that seen in wildtype slices, was now induced in mutant slices: the mean slope value at 1h post-drug was 269±44% (**Figure 5.2C, ●**, $p>0.05$ compared to wildtype value in **Figure 5.3A**). These results directly show that forskolin-induced synaptic facilitation requires PKA, and they provide strong evidence that PP1/2A can suppress cAMP-induced facilitation when PKA activity is genetically reduced.

Characterization of the protein phosphatase gate

To determine whether engagement of the PP1/2A gate is persistent, OA was applied immediately after tetanization. A gate that remained closed after the end of tetraburst stimulation would allow L-LTP to be rescued by pharmacological inhibition of PP1/2A *after* the end of tetraburst stimulation. However, when OA was applied immediately after the end of tetraburst stimulation (at a concentration that rescued L-LTP

in R(AB) slices), it did not prevent the decay of L-LTP in R(AB) mutant slices (**Figure 5.3A,** ■). Thus, PKA-mediated inhibition of PP1/2A is likely short-lasting.

There are three well-characterized neuronal PPs: PP1, PP2A, and calcineurin (PP2B). To distinguish between PP1 and PP2A, their differential sensitivity to inhibition by OA was exploited: PP2A, but not PP1 and PP2B, is blocked by 0.1μM OA (Bialojan and Takai, 1988). Lowering the concentration of OA to 0.1μM eliminated rescue of L-LTP in mutant slices (**Figure 5.3A,** *grey squares*), suggesting that PP1 (and not PP2A or PP2B) constitutes the gate. In addition, application of FK-506, a specific inhibitor of PP2B, prior to tetraburst stimulation did not rescue L-LTP in R(AB) slices (**Figure 5.3B,** ■). Mean fEPSP slopes measured immediately post-tetanus, and at 1h and 2h post-tetanus for FK-506 treated R(AB) mutant slices, were 312±19%, 129±13%, and 105±8%, respectively. Corresponding slope values for untreated R(AB) mutant slices were: 348±17%, 140±12, and 108±13% ($p>0.05$ for all time points cited). FK-506 alone, without tetanization, did not affect fEPSP slopes in a second pathway (**Figure 5.3B,** ●). Furthermore, inhibition of calcineurin did not significantly alter the magnitude of LTP in *wildtype* slices (**Figure 5.3C**). Mean fEPSP slope values in drug-treated wildtype slices were 387±40%, 226±38% and 206±45% immediately after tetanus, and at 1h and 2h post-tetanus, respectively. Corresponding values in control untreated wildtype slices were 428±42%, 258±24% and 208±26% ($p>0.05$). These results suggest that gating of L-LTP is not persistent and is primarily mediated by PP1. These characteristics have not previously been defined by combining genetic and pharmacological approaches.

Discussion

The cAMP-PKA signalling cascade is important for the expression of LTP in hippocampal area CA1 (Frey *et al.*, 1993; Huang and Kandel, 1994; Blitzer *et al.*, 1995; Abel *et al.*, 1997; Nguyen *et al.*, 1997; Wong *et al.*, 1999; Woo *et al.*, 2000). Interactions between PKA and protein phosphatases are one way by which PKA may critically modulate expression of LTP (Blitzer *et al.*, 1995, 1998). By using a combination of genetic, pharmacological, and electrophysiological approaches, the findings of the present study show that genetic inhibition of PKA impairs L-LTP by enabling protein phosphatases to act as inhibitory constraints on L-LTP expression. The involvement of PP2A and calcineurin (PP2B) as key players in the gating model for L-LTP in PKA transgenic mice cannot be ruled out. However, the pharmacological data suggest that it is PP1, and not PP2A or PP2B, which primarily constitutes the "gate" that regulates L-LTP expression following tetraburst stimulation. Lowering the concentration of OA to 0.1μM, which should inhibit PP2A and not PP1 (Bialojan and Takai, 1988), failed to rescue L-LTP in mutant R(AB) slices. In contrast, 1μM OA, which should inhibit both PP1 and PP2A (Bialojan and Takai, 1988), rescued L-LTP in mutant slices. Cal-A, another inhibitor of PP1/2A, also rescued L-LTP in mutant slices. In contrast, a specific inhibitor of PP2B (FK-506) did not affect L-LTP in either mutant or wildtype slices. These collective results suggest that PP1 has a critical role in gating the expression of L-LTP through its interactions with PKA. This is consistent with previous studies that utilized pharmacological approaches to suggest PP1-PKA interactions in gating E-LTP (Blitzer *et al.*, 1995, 1998; Brown *et al.*, 2000). By using PKA transgenic mice, this study extends these earlier findings by providing direct evidence that L-LTP deficits, caused by specific,

genetic reduction of hippocampal PKA activity in neurons, can be eliminated by pharmacological inhibition of PP1/2A. The data also confirm a crucial prediction arising from the gating model: specifically, *genetic* reduction of hippocampal PKA activity impairs L-LTP by relieving PKA-mediated inhibition of PP1/2A. Furthermore, the data suggest that this PKA-mediated gating of L-LTP is limited in its duration of action, a conclusion not previously established.

PP2B plays an important role in synaptic plasticity (Winder *et al.*, 1998; Malleret *et al.*, 2001; Zeng *et al.*, 2001; reviewed by Winder and Sweatt, 2001). Genetic reduction of PP2B activity enhances one-train LTP without altering tetraburst LTP (Malleret *et al.*, 2001). Similarly, pharmacological inhibition of PP2B did not enhance tetraburst LTP, suggesting that PP2B critically modulates the induction threshold, but not the maintenance, of LTP. In addition, the results from the present study suggest that PP2B is not a downstream target of PKA that is critical for tetraburst L-LTP, as assessed by genetic inhibition of PKA.

A gating function of the cAMP signalling pathway has been reported for particular cellular processes, such as transformation of fibroblasts (Chen and Iyengar, 1994) and morphogenic patterning of sonic hedgehog (Fan *et al.*, 1995). The results from this study strongly support a PP1-mediated gating mechanism for hippocampal L-LTP and for cAMP-induced synaptic facilitation. The impairment of forskolin-induced facilitation in R(AB) mutant slices, and its rescue by OA, directly show that intact hippocampal PKA is required for this facilitation. It also demonstrates that, like tetraburst L-LTP, cAMP-induced synaptic facilitation in these mutant slices occurs through regulatory interactions between PKA and protein phosphatases. Blitzer at al. (1995) did not observe enhanced

synaptic transmission following postsynaptic somatic injection of a PKA activator (Sp-cAMPS), suggesting that, for early LTP, the cAMP signalling cascade does not actively transmit signals critical for early LTP, but may act as a "gate" instead. It is unclear whether the injected Sp-cAMPS significantly activated PKA at *synaptic* sites, as Blitzer *et al.* (1995) had monitored somal spike afterhyperpolarization to assess the effectiveness of their injected cAMP analog. The results with cAMP-induced facilitation in R(AB) mutant slices suggest that PKA may actually contribute to the transmission of signals *through* the gate to modulate cAMP-induced synaptic facilitation, since genetic reduction of PKA activity attenuated cAMP-induced synaptic facilitation, and this facilitation was rescued by inhibition of PP1/2A. Further work is needed to elucidate the exact signal that triggers cAMP-induced facilitation. Activation of other signalling molecules (*e.g.* MAP kinases) by the effects of cAMP may contribute to this type of facilitation.

It should be noted that inhibition of PP1/2A did not affect E-LTP, induced by a single 100Hz train, in mutant slices. This is consistent with the studies of Blitzer *et al.* (1995) and Huang and Kandel (1994), who showed that pharmacological inhibition of PKA did not affect one-train LTP in area CA1 of rat slices. One-train E-LTP is less dependent on PKA than multi-train LTP (but see Otmakhova *et al.*, 2000), and thus, E-LTP should not be affected by PP1/2A inhibition in mutant slices.

PP1 is a ubiquitous enzyme with a broad spectrum of actions, and it has also been implicated in long-term depression (LTD) (Mulkey *et al.*, 1993; Morishita *et al.*, 2001). The most likely mechanism for cAMP-induced suppression of PP1 activity involves inhibitor-1 (I-1). PKA phosphorylates I-1, which can then inhibit PP1 (Shenolikar, 1994; Blitzer *et al.*, 1998). PKA may influence PP1 activity through anchoring proteins

(Schillace *et al.*, 2001). Recent evidence suggests that anchoring and targeting of PP1 to specific intracellular compartments is required to regulate synaptic strength (Morishita *et al.*, 2001). As PKA is also anchored to particular subcellular compartments such as postsynaptic dendrites, this tethering may enable spatially localized regulation of PP1 activity following synaptic activity.

In another related study, it was demonstrated that upregulation of protein phosphatase activity prior to LTP induction selectively impaired L-LTP (Woo and Nguyen, 2002), supporting a critical role for protein phosphatases in the expression of L-LTP. The specific targets of PP1 that regulate expression of L-LTP are, to date, unidentified. However, some downstream actions of protein phosphatases may involve modulation of gene expression, since it is well established that L-LTP requires gene transcription (Nguyen *et al.*, 1994; Frey *et al.*, 1996) and the synthesis of new proteins (Stanton and Sarvey, 1984; Krug *et al.*, 1984; Frey *et al*, 1988; Scharf *et al*, 2002). Protein phosphatases are known to modulate transcription factors as well as upstream effectors that lead to gene expression. One important transcription factor implicated in longer forms of synaptic plasticity, such as L-LTP, is the cAMP-response element binding protein (CREB) (see review by Lonze and Ginty, 2002). Phosphorylation of CREB can be regulated by numerous kinases such as PKA (Gonzalez and Montminy, 1989; Impey *et al.*, 1998; Matsushita *et al.*, 2001) and CaMKIV (Bito *et al.*, 1996; Ho *et al.*, 2000; Kang *et al.*, 2001). Expression of genes containing the cAMP responsive element promoter (CRE), associated with CREB phosphorylation, is stimulated by synaptic activity that induces L-LTP (Impey *et al.*, 1996). Moreover, CREB phosphorylation is enhanced after inhibition of protein phosphatases (Hagiwara *et al.*, 1992; Bito *et al.*, 1996). This negative regulation of

CREB by phosphatases may control the kinetics and duration of CREB phosphorylation and subsequent gene expression required for L-LTP. Further studies are required to elucidate the molecular targets of protein phosphatases that regulate L-LTP expression (further discussed in Klann, 2002).

In summary, this is the first study to combine genetic and pharmacological approaches to show that reduction of hippocampal PKA activity in neurons impairs L-LTP by allowing PP1/2A to attenuate expression of L-LTP. Pharmacological inhibition of PP1/2A relieved this inhibitory constraint on L-LTP, and it also rescued cAMP-induced synaptic facilitation in PKA mutant slices. PP1/2A may act as a "gate" that can attenuate expression of L-LTP through activity-dependent modulation by PKA. In a broader perspective, the data from the present study show that regulatory interactions between distinct, but functionally interdependent, signalling molecules can importantly modulate specific forms of synaptic plasticity in the mammalian brain.

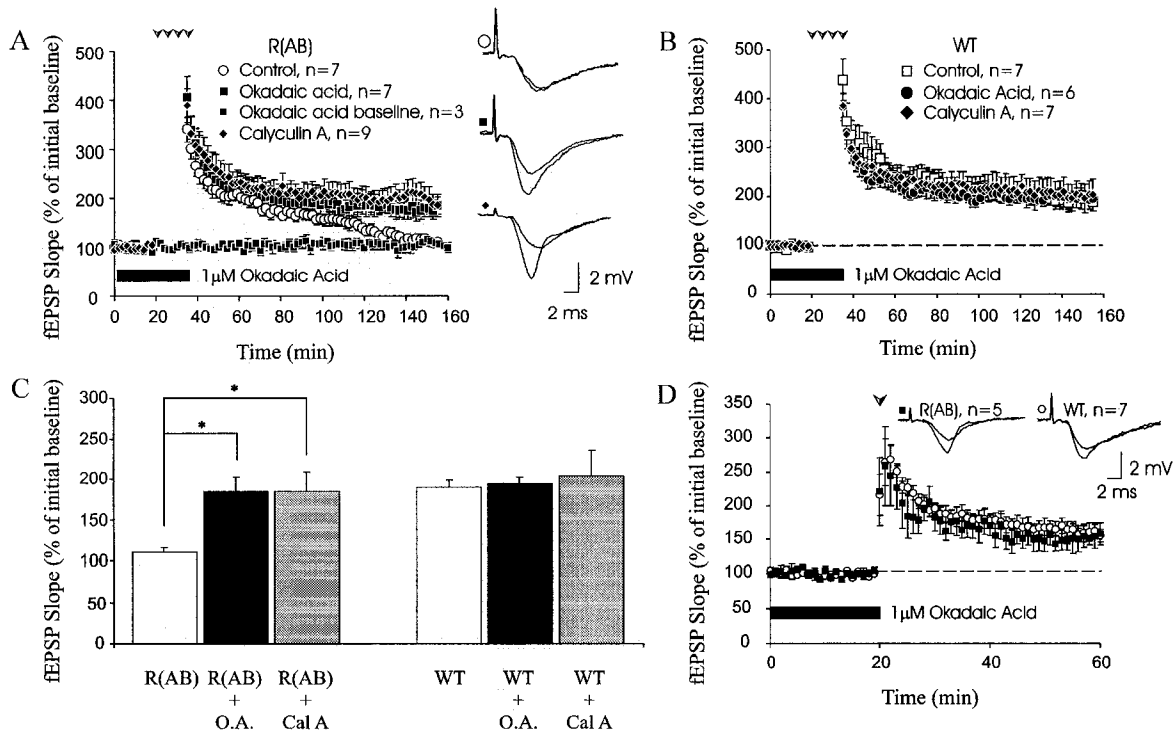

Figure 5.1: Protein phosphatase inhibitors rescue L-LTP in R(AB) mutant slices. **(A)** Bath application of okadaic acid (1μM, horizontal bar, ■), or prior incubation of slices in calyculin A (◆), both inhibitors of PP1/2A, rescues L-LTP that is deficient in R(AB) mutant slices (○). Okadaic acid did not affect transmission in a second, non-tetanized pathway (■). Sample field EPSP traces were recorded 5min before and 2hr after induction of LTP. **(B)** Okadaic acid (●) and calyculin A (◆) did not affect the magnitude or time course of LTP in wildtype slices. **(C)** Summary histogram of mean fEPSP slopes recorded 2hr after LTP induction. Asterisks: $p<0.05$. **(D)** Early-LTP elicited by one train of 100Hz stimulation was unaffected by bath application of okadaic acid (1μM, horizontal bar) in wildtype (○) and in R(AB) mutant slices (■). Field EPSP traces were recorded 5min before and 40min after LTP induction.

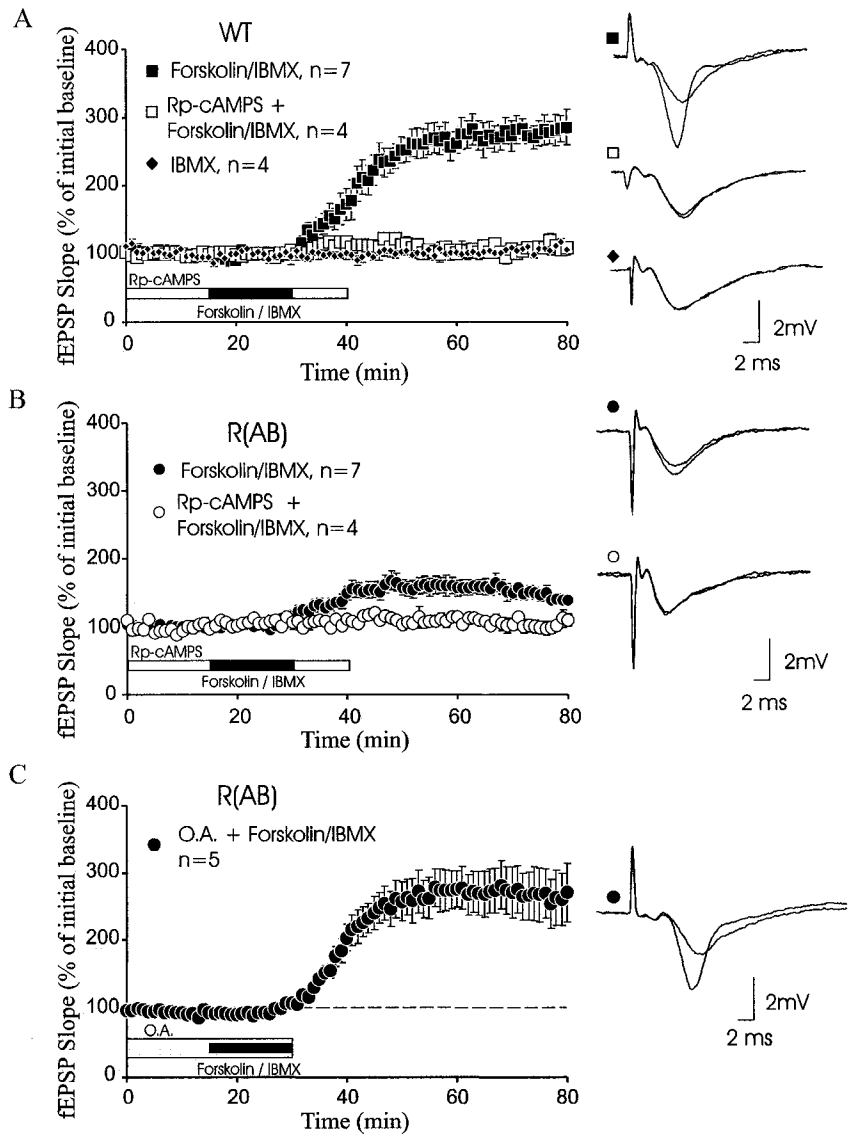

Figure 5.2: Protein phosphatases rescue forskolin/IBMX- induced facilitiation in R(AB) mutant slices. **(A)** Co-application of forskolin and IBMX induces facilitation in wildtype slices (■) that is blocked by a PKA inhibitor, Rp-cAMPS (30μM, □). Acute application of IBMX alone (30μM) does not affect basal transmission (◆). **(B)** Co-application of forskolin (50μM) and IBMX (30μM) to R(AB) mutant slices elicits only modest facilitation (●) that was blocked by Rp-cAMPS (30μM, ○). **(C)** In the presence of okadaic acid, forskolin and IBMX induce substantial facilitation in R(AB) mutant slices (●) that is similar in magnitude to wildtype levels. Field EPSP traces were recorded 5min before and 40min after application of forskolin/IBMX.

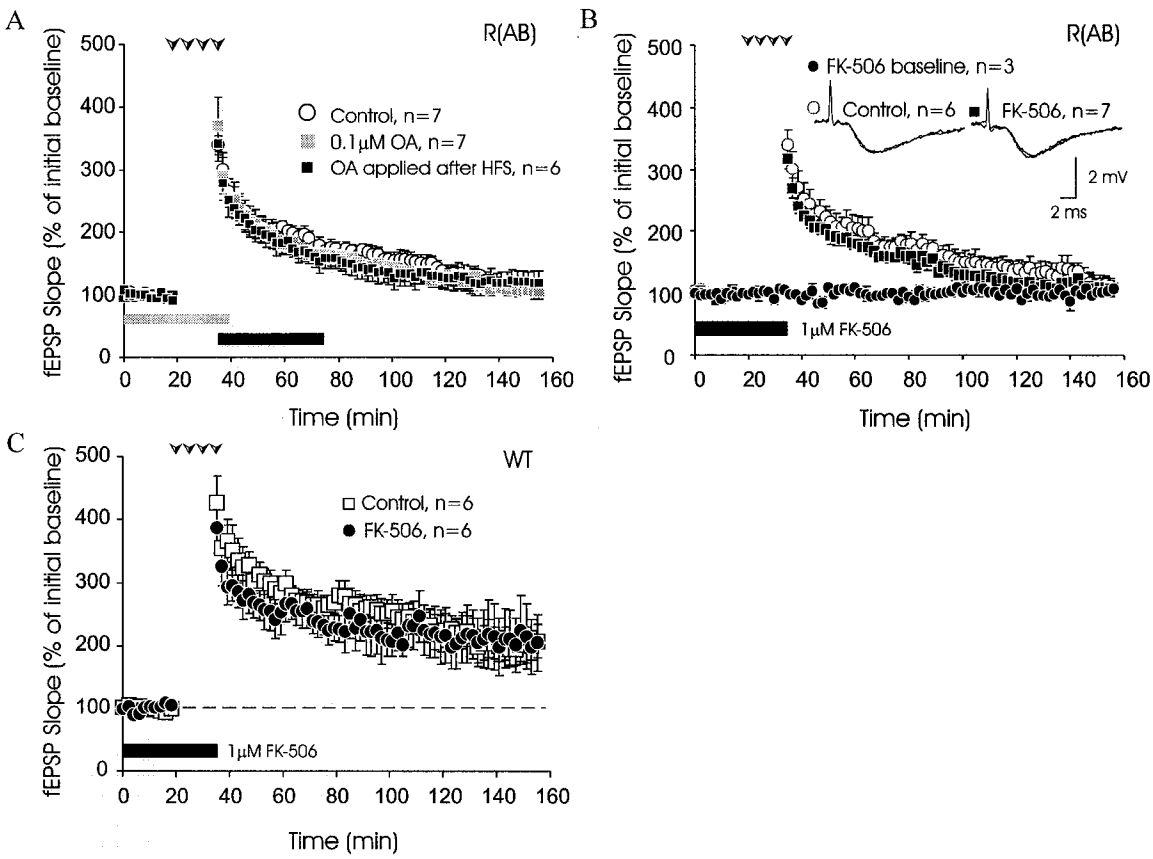

Figure 5.3: Characteristics of protein phosphatase mediated gating of L-LTP expression. **(A)** Application of okadaic acid (1μM, horizontal bar, ■) after LTP induction did not rescue L-LTP in mutant slices (○). Similarly, decreasing the effective concentration of okadaic acid by ten-fold (0.1μM, horizontal bar, *grey squares*) did not rescue L-LTP in mutant slices. **(B)** Bath application of FK-506 (1μM, horizontal bar, ■), a PP2B inhibitor, did not rescue L-LTP in R(AB) mutant slices (○). FK-506 did not affect transmission in a non-tetanized pathway (●). Field EPSP traces were recorded 5min before and 2hr after induction of LTP. (C) FK-506 (●) did not significantly affect the magnitude of L-LTP in wildtype slices (□).

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*CHAPTER VI:

METAPLASTICITY OF THE LATE PHASE OF LONG-TERM POTENTIATION REQUIRES PROTEIN PHOSPHATASES

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Woo, N. H. and Nguyen, P. V. (2002) "Silent" metaplasticity of the late phase of LTP requires protein phosphatases. *Learn. Mem.* **9,** 202-213.

Introduction

Long-term potentiation (LTP) is an enhancement of synaptic strength (Bliss and Lomo, 1973; Schwartzkroin and Wester, 1975; Alger and Tyler, 1976; Andersen *et al.*, 1977) believed to be an important regulator of some types of learning and memory (Bliss and Collingridge, 1993; Moser *et al.*, 1998; Martin *et al.*, 2000; Brun *et al.*, 2001). LTP has at least two temporal phases: an early phase (E-LTP) and a late phase (L-LTP) (Krug *et al.*, 1984; Huang *et al.*, 1994, 1996; see also Winder *et al.*, 1998 for an "intermediate" phase of LTP). In area CA1 of hippocampal slices, E-LTP is induced by a single train of 100Hz stimulation and can last for 1-2h (Huang and Kandel, 1994; see also reviews by Bliss and Collingridge, 1993 and Huang *et al.*, 1996). In contrast, L-LTP is induced by multiple trains of 100Hz stimulation and may last for several hours (Andersen *et al.*, 1977; Huang and Kandel, 1994; Abel *et al.*, 1997). Unlike E-LTP, expression of L-LTP requires activation of cAMP-dependent protein kinase (PKA) (Matthies and Reymann, 1993; Frey *et al.*, 1993; Abel *et al.*, 1997), transcription (Nguyen *et al.*, 1994), and protein synthesis (Stanton and Sarvey, 1984; Frey *et al.*, 1988). Although the biochemical signalling mechanisms underlying L-LTP are well characterized (see reviews by Huang *et al.*, 1996; Kandel, 2001), nothing is known about the mechanisms that underlie the modulation of L-LTP expression by prior synaptic activity.

"Metaplasticity" is the modulation of synaptic plasticity by previously imposed activity (Yang and Faber, 1991; Huang *et al.*, 1992; Dudek and Bear, 1993; Wexler and Stanton, 1993; see also reviews by Abraham and Bear, 1996; Abraham and Tate, 1997). An example of metaplasticity is "priming" of synapses using high-frequency stimulation (HFS), which can facilitate subsequent induction of long-term depression (Christie and

Abraham, 1992; Wexler and Stanton, 1993). Conversely, LFS that is subthreshold for inducing synaptic plasticity ("innocuous" LFS) can impair subsequent LTP induction (Huang *et al.*, 1992; Christie and Abraham, 1992; Fujii *et al.*, 1996), or it can erase previously established LTP ("depotentiation" or DPT: Barrioneuvo *et al.*, 1980; Staubli and Lynch, 1990; Fujii *et al.*, 1991). LFS-induced LTD and DPT both require phosphatases for their induction (Mulkey *et al.*, 1993, 1994; O'Dell and Kandel, 1994), but the roles of phosphatases in metaplasticity of LTP, elicited by prior innocuous patterns of LFS, are undefined.

Studies of hippocampal metaplasticity have examined the roles of activity in regulating the subsequent induction and expression of LTP without making a clear distinction between the early and late phases of LTP. Differential, anterograde regulation of expression of E-LTP or L-LTP by prior synaptic activity, and the possible involvement of phosphatases in such regulation, have not been explored. Mammalian neurons receive thousands of synaptic inputs, many of which may be subthreshold for altering synaptic strength. These inputs may, nonetheless, alter the capability of neurons to undergo lasting changes in synaptic strength, such as L-LTP, in response to *future* activity. As L-LTP is linked to some forms of long-term memory in mice (Abel *et al.*, 1997), identifying the mechanisms by which innocuous synaptic activity elicits anterograde regulation of L-LTP may shed light on the question of how long-term memory is influenced by previous neural experience.

In the present study, the following central question was posed: can innocuous synaptic activity, imposed *prior* to HFS, critically modulate the subsequent expression of L-LTP? The present study shows that LFS prior to HFS does not modify E-LTP, but it

does selectively inhibit the expression of L-LTP. When applied by itself, LFS had no lasting effects on synaptic strength. This anterograde inhibition of L-LTP is persistent, and it is dependent on the amount and frequency of prior LFS. Anterograde inhibition of L-LTP by LFS was blocked by inhibitors of PP1/2A, and it required activation of NMDA receptors during LFS. Because this inhibition of L-LTP is caused by prior synaptic activity that alone produced no net effect on synaptic efficacy, this is a "silent" form of metaplasticity that may influence long-term information storage by modulating the capacity of synapses to express L-LTP following repeated bouts of activity.

Materials and Methods

Animals

Female C57BL/6 mice, aged 9-12 weeks (Charles River, Canada) were used for all experiments. At these ages, hippocampal slices did not show LTD when LFS at 1Hz, 15min was applied to the Schaffer collateral pathway in area CA1 (data not shown).

Electrophysiology

Transverse hippocampal slices (400μm thickness) were prepared as described in *Chapter II*. Slices were maintained in an interface chamber at 28°C and were perfused (1 mL/min) with artificial cerebrospinal fluid (ACSF; composition as described in *Chapter II*) aerated with 95% O_2 and 5% CO_2. Extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded with a glass microelectrode positioned in stratum radiatum of area CA1. A bipolar nickel-chromium stimulating electrode was used to elicit fEPSPs by

stimulation of the Schaffer collateral fibres. Evoked fEPSPs were elicited once per minute at this test stimulation intensity.

LTP was induced by applying either a single one-second train (100Hz, at test strength) to induce E-LTP, or four one-second trains spaced 5min apart to elicit L-LTP (Table 2.1). Low-frequency stimulation (LFS) was applied by giving one of the following protocols: 5Hz for 3min, 5Hz for 30s, 1Hz for 15min or 1Hz for 3min, *prior* to LTP induction. For some experiments, a second independent pathway was monitored to gauge the effects of applied drugs on basal synaptic strength.

2-amino-5-phosphonopentanoic acid (APV) was prepared as a concentrated stock solution in distilled water, and it was bath applied (25μM) to slices in some experiments. The protein phosphatase-1/2A inhibitors, sodium okadaic acid (OA) and calyculin A (cal A), were prepared as concentrated stock solutions in distilled water and in DMSO, respectively (Table 2.2). Each drug was diluted in ACSF to the desired concentration. Sodium okadaic acid was bath-applied for 20min prior to start of baseline acquisition and it was washed out starting immediately after LFS (**Figure 6.4B**) or, in some experiments, after HFS (**Figure 6.4D**). All experiments using sodium okadaic acid were performed under dimmed-light conditions. For cal A experiments, slices were incubated in drug for 40min prior to start of baseline recordings. Drug experiments were interleaved with drug-free controls.

Results

LFS reverses E-LTP when given after, but not before, its induction

Low-frequency stimulation (LFS) elicits LTD in hippocampal slices (Dudek and Bear, 1992; Bear and Abraham, 1996; Morishita *et al.*, 2001), and expression of LTD is age-dependent: hippocampal slices from very young animals show robust LTD, whereas slices from adult animals lack, or show attenuated, LTD following LFS (Dudek and Bear, 1992; Mulkey and Malenka, 1992; Wagner and Alger, 1995; Mayford *et al.*, 1995). LFS can also induce depotentiation (DPT), which is a rapid reversal of LTP previously elicited by 100Hz stimulation (Barrioneuvo *et al.*, 1980; Staubli and Lynch, 1990; Fujii *et al.*, 1991). As an initial step towards characterizing the effects of LFS on subsequent expression of L-LTP, a LFS protocol that would not induce persistent or significant changes in fEPSP slopes was tested. A previous study has shown that 5Hz LFS for 3min does not persistently alter fEPSPs in area CA1 of mouse slices (Thomas *et al.*, 1996). As a result, synaptic efficacy following LFS was examined in area CA1 of hippocampal slices cut from mice that were 9-12 weeks of age. LFS at 5Hz for 3min elicited only a transient synaptic depression (**Figure 6.1A,** ○), consistent with the findings of Thomas *et al.* (1996). Mean fEPSP slopes were initially depressed to 67±5 % of pre-LFS values, but they recovered to pre-LFS fEPSP slope values within 7min after LFS (**Figure 6.1A**). This protocol is referred to as "innocuous LFS," because it elicited no persistent changes in synaptic efficacy under these conditions (see also Winder *et al.*, 1999, Thomas *et al.*, 1996, 1998). However, this innocuous LFS protocol induced rapid DPT when given 5min *after* two trains of 100Hz (20s apart) (**Figure 6.1B, C**), thereby confirming the results of other studies performed on rat and guinea pig slices (Barrioneuvo *et al.*, 1980; Staubli and

Lynch, 1990; Fujii *et al.*, 1991, O'Dell and Kandel, 1994). This rapid, retrograde influence of LFS on previously induced LTP represents a form of metaplasticity in which erasure of LTP is caused by LFS applied *after* LTP induction. Can the same LFS protocol affect LTP induction or expression when applied *before* its induction?

To address this question, a 7min interval between the end of LFS and the start of high-frequency stimulation (HFS) for LTP induction was imposed. This time period was sufficient to allow fEPSPs to fully recover to pre-LFS fEPSP slope values following LFS (**Figure 6.1A**). An HFS protocol that elicits E-LTP, consisting of a single train of 100Hz (1s duration) was first tested (Huang and Kandel, 1994). E-LTP gradually decayed to 130±7% at 1h after induction (**Figure 6.1D**, □). Slices receiving innocuous LFS *prior* to induction of E-LTP (**Figure 6.1D**, ●) showed potentiation that was similar in time course and magnitude to E-LTP seen in slices receiving only HFS: the mean fEPSP slope measured in slices receiving LFS before HFS was 123±5% of pre-LFS values (1h after E-LTP induction; **Figure 6.1D**, $p>0.1$ for comparison with experiments using HFS alone, at all time points tested). Thus, innocuous LFS at 5Hz had no significant effect on the induction or expression of E-LTP when it was applied *prior* to E-LTP induction. These data underscore the idea that the time of application of LFS is critical in determining whether LTP can be modulated by LFS (Abraham and Bear, 1996).

Anterograde modulation of L-LTP by prior innocuous low-frequency stimulation

Does innocuous LFS affect subsequent induction or expression of L-LTP? To address this question, a tetraburst protocol was used to elicit L-LTP in mouse hippocampal slices (Abel *et al.*, 1997; Nguyen *et al.*, 2000; Woo *et al.*, 2000); this protocol consisted of

four trains of 100Hz, each 1s duration, spaced 5min apart (**Figure 6.2A**). This regimen produced robust L-LTP in area CA1 of hippocampal slices: fEPSP slopes remained potentiated for at least 2h (**Figure 6.2B**, □). However, slices receiving innocuous LFS at 5Hz (for 3min, equivalent to 900 pulses) prior to tetraburst stimulation showed a significant reduction in the magnitude of L-LTP beginning at 20min post-induction. L-LTP decayed back to near pre-LFS fEPSP slope values within 100min (**Figure 6.2B**, ●). Mean fEPSP slopes measured 5min and 2h after L-LTP induction in control slices receiving no LFS were 332±45% and 300±27%, respectively (**Figure 6.2B**). In slices receiving innocuous LFS *prior* to induction of L-LTP, the corresponding slope values were 283±32% and 105±6%, respectively (**Figure 6.2B**, ●, $p<0.01$ for 2h value).

The effects of varying the duration of LFS on subsequent L-LTP were explored next. When the duration of LFS was decreased to 30s (and keeping the frequency constant at 5Hz), L-LTP elicited by subsequent HFS was intact (**Figure 6.2B**, ▲). Mean fEPSP slopes, measured 5min and 2h post-HFS, were 337±32% and 248±20%, respectively, in slices receiving prior LFS at 5Hz, 30s ($p>0.1$, for comparison with control slices at all time points tested). Thus, a critical number of pulses during LFS is required for LFS-induced suppression of L-LTP.

The effects of giving 900 pulses at a lower frequency, 1Hz (*i.e.* 1Hz for 15min) was subsequently investigated. Like 5Hz LFS, this 1Hz protocol blocked expression of L-LTP. However, it also significantly attenuated induction of E-LTP (**Figure 6.2C**, ◆). The initial fEPSP slope value immediately after HFS with prior 1Hz LFS was 252±22% and was significantly smaller than the corresponding initial potentiation of 451±61% seen after HFS alone (compare □ of **Figure 6.2B** with ◆ of **Figure 6.2C**, $p<0.05$). Since the early

phase was attenuated with 900 pulses at 1Hz (**Figure 6.2C**), the total number of pulses applied at 1Hz was reduced. A 1Hz, 3min regimen produced a smaller initial depression of fEPSPs (mean fEPSP slopes were 89±5% of pre-LFS values) than 5Hz stimulation (74±5% of pre-LFS values), but this depression recovered fully to pre-LFS fEPSP slope values prior to HFS (**Figure 6.2C**, ○). When HFS was applied 7min after 1Hz, 3min LFS, L-LTP similar to that seen in control slices receiving only HFS was observed (compare **Figure 6.2C**, ○, and **Figure 6.2B**, □). Mean values for fEPSP slopes measured 5min and 2h post-HFS in slices receiving 1Hz 3min stimulation were 315±17% and 251±19%, respectively. These values were not significantly different from corresponding values measured from slices receiving only HFS ($p>0.2$). These data are summarized in the histograms of **Figure 6.2D, E**.

These findings establish, for the first time in the literature, that innocuous LFS at 5Hz for 3min selectively inhibits expression of multi-train L-LTP, without affecting single-train E-LTP, when given before L-LTP induction. This anterograde metaplasticity of L-LTP is dependent on both the amount and the frequency of LFS applied prior to L-LTP induction.

LFS cannot reverse L-LTP

Because innocuous LFS prior to tetraburst stimulation inhibits the expression of L-LTP, the study addressed the question whether LFS applied shortly after L-LTP induction could reverse, or depotentiate, L-LTP. To date, depotentiation of tetraburst L-LTP by 5Hz stimulation has not been examined. When 5Hz was applied for 3 min shortly (5min) after

tetraburst stimulation, this LFS protocol did not depotentiate L-LTP (**Figure 6.3A**, ▲). Thus, innocuous LFS at 5Hz, 3min cannot reverse L-LTP in a retrograde manner.

The inhibitory effect of innocuous LFS is limited in its duration of action

Previous studies have shown that there is a narrow critical time window within which LFS effectively erases previously induced LTP (O'Dell and Kandel, 1994; see also Chen *et al.*, 2001 for mossy fibre data). LFS effectively erases LTP when given within the first 5-10min after LTP induction (O'Dell and Kandel, 1994). By contrast, LFS is less effective at persistently reversing LTP when applied 20min after LTP induction (O'Dell and Kandel, 1994). It is unclear whether a time window of effectiveness also exists for the inhibitory influence of LFS applied *prior* to L-LTP. Can innocuous LFS exert *anterograde* inhibition of L-LTP over an extended period of time before L-LTP induction?

The effect of innocuous LFS on L-LTP with a 20- or 40min delay period imposed between the end of LFS and the start of HFS was investigated (**Figure 6.3B, C**). A 20min delay still permitted innocuous LFS (5Hz, 3min) to significantly attenuate L-LTP (**Figure 6.3B**, ●). Mean fEPSP slopes measured 5min and 2h after L-LTP induction in LFS slices were 278±25% and 106±8%, respectively. The mean fEPSP slope value measured 2h post-HFS using the 20min LFS-HFS delay period was significantly lower than the corresponding value measured from slices receiving only HFS ($p<0.01$, at all time points), but it was not significantly different from the mean slope value measured from slices receiving LFS with a 7min delay period (**Figure 6.3C**). However, when the LFS-HFS interval was increased to 40min, innocuous LFS did not attenuate L-LTP (**Figure 6.3B,** ◆). Robust potentiation was observed with mean fEPSP slopes of 331±33% and

245±22%, measured 5min and 2h after HFS, respectively. These were not statistically different from values of slices that received only HFS ($p>0.1$, at all time points). In **Figure 6.3C**, the data are presented so that fEPSP slopes measured at various times after induction of L-LTP can be compared. With the 20min LFS-HFS interval, the decay of L-LTP back to pre-HFS baseline slope values (**Figure 6.3B**, ●) was similar to the decay seen with the shorter 7min LFS-HFS interval (**Figure 6.3A**, ●). Levels of potentiation in slices receiving HFS alone, in slices receiving LFS 40min before HFS, and in slices receiving LFS 5min after the end of HFS, were not statistically different from each other ($p>0.1$).

These results show that innocuous LFS (5Hz, 3min) maintains its inhibitory effect on L-LTP over a substantial period of time before induction of L-LTP. Innocuous LFS inhibited expression of L-LTP when HFS was applied up to 20min *after* LFS. The inhibitory constraint of LFS on L-LTP had a limited duration, as L-LTP was intact when induced 40min after LFS. This type of synaptic plasticity should be regarded as a "silent" anterograde modification of L-LTP that is caused by prior synaptic activity, which, by itself, did not produce persistent changes in synaptic strength.

Inhibition of L-LTP by prior LFS requires activation of NMDAR and PP1/2A

The LFS protocols that were used to inhibit L-LTP resemble those known to induce hippocampal LTD and DPT (Barrioneuvo *et al.*, 1980; Staubli and Lynch, 1990; Fujii *et al.*, 1991; Dudek and Bear, 1992). Both LTD and DPT require activation of protein phosphatases subsequent to NMDA receptor activation (Mulkey *et al.*, 1993, 1994; O'Dell and Kandel, 1994; see also Lisman, 1989 for modelling data). To address whether L-LTP suppression by prior LFS requires NMDA receptor activation, an NMDA receptor

antagonist (APV) was applied to hippocampal slices prior to 5Hz, 3min LFS. After LFS, application of APV was stopped before subsequent HFS. In contrast to the decay of L-LTP observed when LFS was applied 20min prior to induction (**Figure 6.3B**, ●), slices treated with APV during LFS (**Figure 6.4A**, ■) showed robust L-LTP that was not significantly different from L-LTP in slices receiving APV that was allowed to washout for a period of 20min before receiving HFS (*no LFS*) (**Figure 6.4A**, □; $p>0.1$ at all time points). Mean fEPSP slopes for APV-treated, LFS+HFS slices and APV-treated HFS slices were 252±32% and 271±28% at 2h post induction, respectively ($p>0.5$). Thus, activation of NMDA receptors is required for LFS at 5Hz to inhibit subsequent L-LTP (see also Fujii *et al.*, 1996 for E-LTP data).

Activation of PP1 and PP2A is critical for LFS-induced LTD and DPT (Mulkey *et al.*, 1993, 1994; Wang and Kelly, 1996, 1997; O'Dell and Kandel, 1994; Huang *et al.*, 2001; Morishita *et al.*, 2001). PP1/2A can also modulate the induction of LTP, as evidenced by block or enhancement of LTP by PP1/2A inhibitors applied *during HFS* (Blitzer *et al.*, 1995, 1998; Figurov *et al.*, 1993). However, it is unclear whether PP1/2A can modulate L-LTP by being activated well *before* its induction. Does anterograde inhibition of L-LTP by prior LFS require activation of PP1/2A?

Application of okadaic acid (OA), or preincubation of slices in calyculin A (Cal A), both inhibitors of PP1/2A, blocked anterograde inhibition of L-LTP by prior LFS at 5Hz, 3min (**Figure 6.4B,C**). Although transient synaptic depression was observed after LFS, L-LTP was still expressed after recovery from this depression in slices pretreated with OA or Cal A (**Figure 6.4B**). Mean fEPSP slope at 2h post-HFS was 113±10% in drug-free control slices receiving LFS 20min before HFS (**Figure 4B**, ●). By contrast, the corresponding

values in OA- and Cal A-treated slices (**Figure 6.4B**, ◆ and △ open triangles) receiving prior LFS were 215±17% and 232±33%, respectively, and they were significantly higher than control values ($p<0.05$). Preincubation of slices in Cal A, or application of OA *just before and during* tetraburst HFS, *without prior LFS*, did not affect L-LTP expression under the experimental conditions used here (**Figure 6.4D**, $p>0.1$, at all time points). When a second independent pathway was monitored in some experiments, no effects of OA on fEPSPs elicited at 0.017 Hz (once/min) were observed (**Figure 6.4B**, ▲). These data show that LFS-induced anterograde inhibition of L-LTP requires activation of PP1/2A (data summarized in **Figure 6.4E**).

In summary, the data show that innocuous LFS at 5Hz significantly inhibits the expression of the L-LTP without affecting E-LTP or persistently altering basal synaptic strength. This selective anterograde inhibition of L-LTP by *prior* innocuous synaptic activity is sensitive to the frequency, as well as the total amount, of stimulation. The inhibitory effect of innocuous LFS has a persistent duration that can last up to 20min after LFS, but it is absent 40min after LFS. Activation of PP1/2A, and NMDA receptor activation, are required for anterograde inhibition of L-LTP by innocuous LFS.

Discussion

Anterograde metaplasticity involves modulation, by electrical activity, of the capability for expression of *future* synaptic plasticity (Abraham and Bear, 1996; Abraham and Tate, 1997). The present work has revealed a "silent" form of synaptic metaplasticity that specifically modulates L-LTP by prior synaptic stimulation that is subthreshold for eliciting persistent changes in synaptic strength. The selectivity of the suppressive effects

of prior innocuous LFS on L-LTP has a number of interesting implications. By inhibiting L-LTP, innocuous LFS may allow patterns of synaptic activity (*e.g.* multiple spaced trains) that would otherwise elicit persistent potentiation to produce shorter-lasting facilitation. This inhibition would prevent persistent saturation of postsynaptic responses over a time scale of 1-2 hours. The lack of effect on single-train E-LTP suggests that innocuous LFS may modulate mechanisms that are specifically needed for expression of L-LTP (see below). Thus, innocuous LFS prior to LTP induction may represent a "low-pass filter" that allows short-lasting facilitation (like single-train E-LTP) to occur while reducing future expression of L-LTP following repeated trains of synaptic stimuli.

Metaplasticity of LTP by stimulation prior to LTP induction

LTP can be modulated by previous bouts of synaptic activity (Abraham and Bear, 1996; Abraham and Tate, 1997). For example, LFS at 1Hz (1000 stimulation pulses), given prior to single-train LTP induction, substantially impaired E-LTP (Fujii *et al*, 1996). However, when LFS was increased to 5Hz with the same number of stimulation pulses, no change in E-LTP was observed (Fujii *et al*, 1996; L-LTP was not examined in this study). The finding that 5Hz LFS prior to tetraburst stimulation impaired L-LTP is consistent with the findings of Fujii *et al.* (1996) in that it supports a selective role for 5Hz LFS in modulating L-LTP, but not E-LTP.

Because stimulation-induced changes in basal synaptic efficacy can influence subsequent expression of LTP (see Huang *et al.*, 1992, and Abraham and Huggettt, 1997), hippocampal slices from adult mice that did not exhibit LTD following 5Hz LFS were used. This pattern of LFS did not persistently alter fEPSPs, but it still inhibited subsequent

L-LTP without affecting single-train E-LTP. By comparison, prior 5Hz LFS of guinea pig slices impairs LTP induction when given just 5 seconds before LTP induction (O'Dell and Kandel, 1994). This inhibition of LTP induction may be dependent on protocol conditions (stimulation by O'Dell and Kandel was applied at a weaker intensity than that used here) and on animal species, but it too required phosphatase activity (O'Dell and Kandel, 1994). In other studies, Abraham and Huggett (1997) and Huang *et al.* (1992) showed that repeated HFS that did not substantially alter synaptic strength blocked subsequent LTP induction. However, these studies did not test for selectivity of the effects of prior stimulation on early and late phases of LTP elicited by distinct amounts of stimulation. They also did not explore the roles of phosphatases in anterograde suppression of LTP by prior stimulation. Nonetheless, it is now evident that anterograde metaplastic regulation of LTP induction (Abraham and Huggett, 1997; O'Dell and Kandel, 1994; Fujii *et al.*, 1996) and L-LTP expression (present study) may be achieved by imposing various patterns of stimulation prior to LTP induction. The data from the present study reveal a previously unidentified, selective inhibition of the late phase of LTP by innocuous patterns of LFS that requires PP1/2A and NMDA receptor activation.

Protein phosphatases and synaptic plasticity

Protein kinases have long been regarded as key players in synaptic plasticity (see reviews by Micheau and Riedel, 1999 and Martin *et al.*, 2000), but a plethora of studies has highlighted the importance of protein phosphatases in regulating bidirectional changes in synaptic strength (Mulkey *et al.*, 1992, 1993, 1994; Wang and Kelly, 1996, 1997; Blitzer *et al.*, 1995, 1998; O'Dell and Kandel, 1994; Winder *et al.*, 1998; Yan *et al.*, 1999;

Allen *et al.*, 2000; Winder and Sweatt, 2001). In calcineurin knockout mice, bidirectional plasticity was modified with a change in the LTP/LTD threshold (Zeng *et al.*, 2001). It has also been suggested that protein phosphatases may function as a "gate" that exerts a modifiable, PKA-dependent inhibitory constraint on expression of LTP (Blitzer *et al.*, 1995, 1998; Brown *et al.*, 2000). Evidence that calcineurin acts as an inhibitory constraint on LTP is found in studies showing that genetic inhibition and enhancement of calcineurin facilitated and impaired LTP, respectively (Winder *et al.*, 1998; Malleret *et al.*, 2001). In support of the notion that phosphatases exert an inhibitory constraint on LTP, the data implicate PP1/2A in blocking the expression of L-LTP. Application of PP1/2A inhibitors during LFS prior to L-LTP induction abolished anterograde inhibition of L-LTP. This finding supports the prediction that prior activation of PP1/2A, in this case by LFS, should impair the expression of LTP (Blitzer *et al.*, 1995).

How might phosphatases mediate anterograde suppression of L-LTP by prior LFS?

Recently, Huang *et al.* (2001) demonstrated that an LFS paradigm, similar to one employed in this study, significantly increased protein phosphatase activity in area CA1 of hippocampal slices. Also, LFS *in vivo* increased protein phosphatase activity (Thiels *et al.*, 1998). Although hippocampal levels of PP1/2A activity were not measured, the blockade of anterograde suppression of L-LTP by two different inhibitors of PP1/2A, and the findings of these earlier studies, suggest that LFS can engage phosphatases critical for anterograde suppression of L-LTP.

LFS-induced suppression of L-LTP may be caused by PP1/2A acting on gene transcription. L-LTP requires transcription that is linked to the cAMP signalling pathway

(Nguyen *et al.*, 1994), and L-LTP may be caused by CREB-mediated transcription. Phosphatases may move to the nucleus and dephosphorylate key transcription factors, such as CREB (Hagiwara *et al.*, 1992; Bito *et al.*, 1996). L-LTP may arise from augmented CREB-mediated transcriptional activity following reduced CREB dephosphorylation by PP1. This notion is consistent with previous work showing that CRE-mediated gene expression is stimulated by activity that induces L-LTP (Impey *et al.*, 1996), and that CREB phosphorylation is enhanced after inhibition of phosphatases (Hagiwara *et al.*, 1992; Bito *et al.*, 1996). In the present study, LFS-induced engagement of PP1/2A may have decreased CREB phosphorylation, reduced CRE-mediated transcription, and thereby impaired L-LTP. Further work is needed to test whether the metaplastic suppression of L-LTP reported here involves increased dephosphorylation of CREB.

Another potential mechanism for the phosphatase-mediated anterograde suppression of L-LTP may involve ERK/mitogen-activated protein kinases (ERK/ MAPKs). A link between phosphatases and the MAPK pathway was shown when an LTD-inducing protocol decreased ERK immunoreactivity (Norman *et al.*, 2000). This was simulated by incubation of hippocampal homogenates with purified PP1/2A (Norman *et al.*, 2000). MAPK inhibitors block the expression of L-LTP (English and Sweatt, 1997; see also reviews by Impey *et al.*, 1999, and Sweatt, 2001) and inhibit activity-dependent gene transcription (Impey *et al.*, 1998). Furthermore, the MAPK cascade is activated by synaptic stimulation (English and Sweatt, 1996) and by increases in cAMP, implicating cross-talk between the PKA and ERK/MAPK pathways (Impey *et al.*, 1998). Thus, activation of phosphatases by innocuous LFS might inhibit subsequent L-LTP by suppressing activation of the MAPK and PKA pathways.

Behavioral implications

The demonstration that L-LTP may be linked to specific types of long-term memory in mice (Abel *et al.*, 1997) prompts one to hypothesize that anterograde metaplasticity of L-LTP in the hippocampus might translate into altered expression of some types of hippocampus-dependent long-term, but not short-term, memory. Experience can modify memory processing and consolidation, and it is interesting that specific types of behavioral experiences, such as chronic drug abuse and addiction, can distort long-term memory (reviewed in Schacter, 1995). For example, suppression of traumatic memories in humans may involve neuromodulatory actions of endogenous substances, such as release of opiates during a painful experience (Gallagher *et al.*, 1985). However, linking certain behavioral experiences to synaptic activity and metaplasticity of L-LTP in brain structures important for long-term memory remains a stimulating challenge.

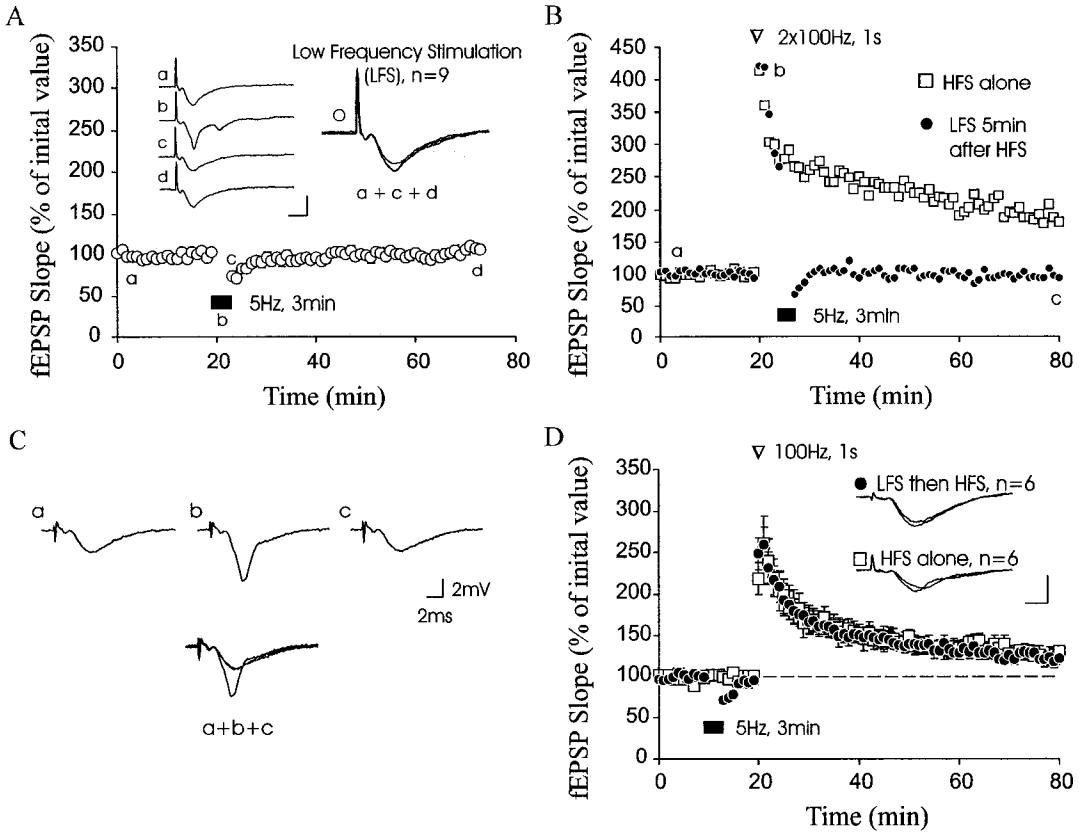

Figure 6.1: Low-frequency stimulation (LFS) does not affect either basal synaptic transmission or the early phase of LTP (E-LTP). **(A)** Transient depression was observed after 5Hz stimulation (for 3min) was applied to area CA1 of hippocampal slices. However, fEPSP slopes recovered back to pre-LFS slope values within 7min after the end of LFS (○). Sample fEPSP traces from one experiment are shown; these were recorded 15 min. before (a), during (b), immediately after (c), and 20min after (d), LFS. Calibration bars: 2mV, 4ms. **(B)** LFS at 5Hz, applied after two trains of 100Hz, (1s duration, spaced 20s apart), erases previously established LTP (●, "depotentiation"). **(C)** Sample fEPSP traces recorded from one experiment at time points labeled in part B. **(D)** Prior LFS at 5Hz does not affect E-LTP induced by a single 100Hz train. Control slices (□) generated E-LTP that was similar in magnitude and time course to E-LTP induced in slices that received LFS prior to tetanization (●). Inset: Sample fEPSP traces from one experiment, measured 10min before and 60min after E-LTP induction. Calibration bars: 2mV, 2ms.

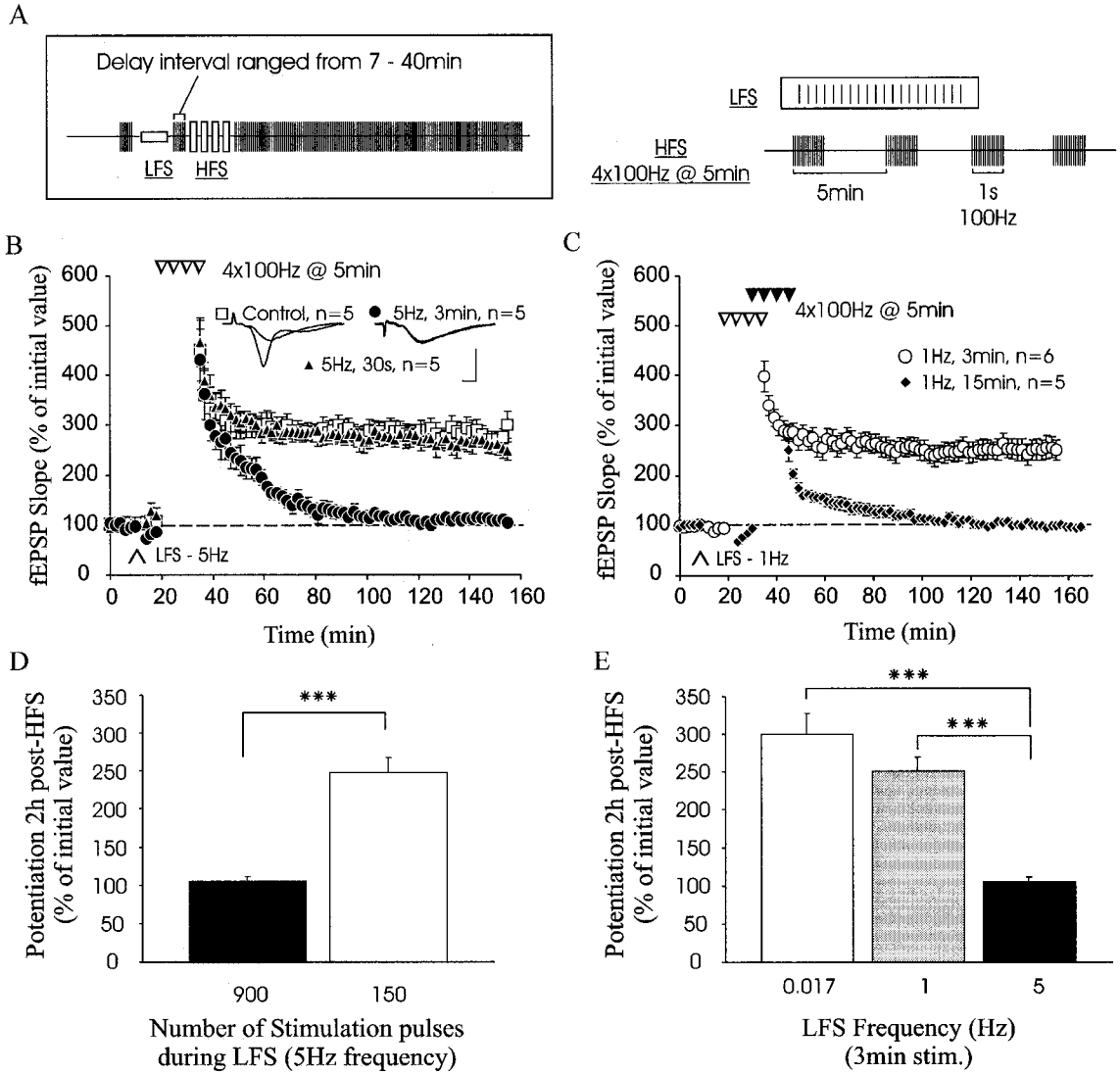

Figure 6.2: Activity-dependent anterograde metaplasticity of L-LTP. **(A)** Experimental protocols used for synaptic stimulation of hippocampal slices. LFS was applied by giving 5Hz or 1Hz stimulation for various durations (see Materials and Methods). Subsequent LTP was then induced by applying four 100Hz trains spaced 5min apart (tetraburst stimulation). fEPSPs were monitored for 2 hours post-induction. The interval between LFS and subsequent induction of LTP ranged from 7 - 40min. **(B)** Tetraburst stimulation reveals a deficit in L-LTP when LFS (5Hz, 3min) was given prior to induction of L-LTP. Four successive trains of 100Hz, spaced 5min apart, induced robust L-LTP in control slices (□) and in slices that received a brief prior episode of LFS (5Hz, 30s; ▲). No L-LTP was seen in slices that received more prolonged LFS (5Hz, 3min; ●) prior to HFS. Inset: Sample traces from an experiment, measured 10min before and 2h after L-LTP induction. Calibration bars: 5mV, 2ms **(C)** Anterograde metaplastic inhibition of L-LTP expression is frequency-dependent. Decreasing the frequency of LFS to 1Hz, without altering the duration of stimulation (1Hz, 3min; ○), had no effect on subsequent L-LTP. However, when 1Hz LFS was increased to 15min in duration, L-LTP was blocked and E-LTP was attenuated (1Hz, 15min; ◆). **(D)** Summary histogram showing mean levels of potentiation seen when the number of pulses during prior LFS was varied at a constant frequency of 5Hz (* denotes $p<0.001$). **(E)** Summary histogram depicting the level of potentiation observed when only the frequency of prior LFS was varied (duration of stimulation was constant at 3min).

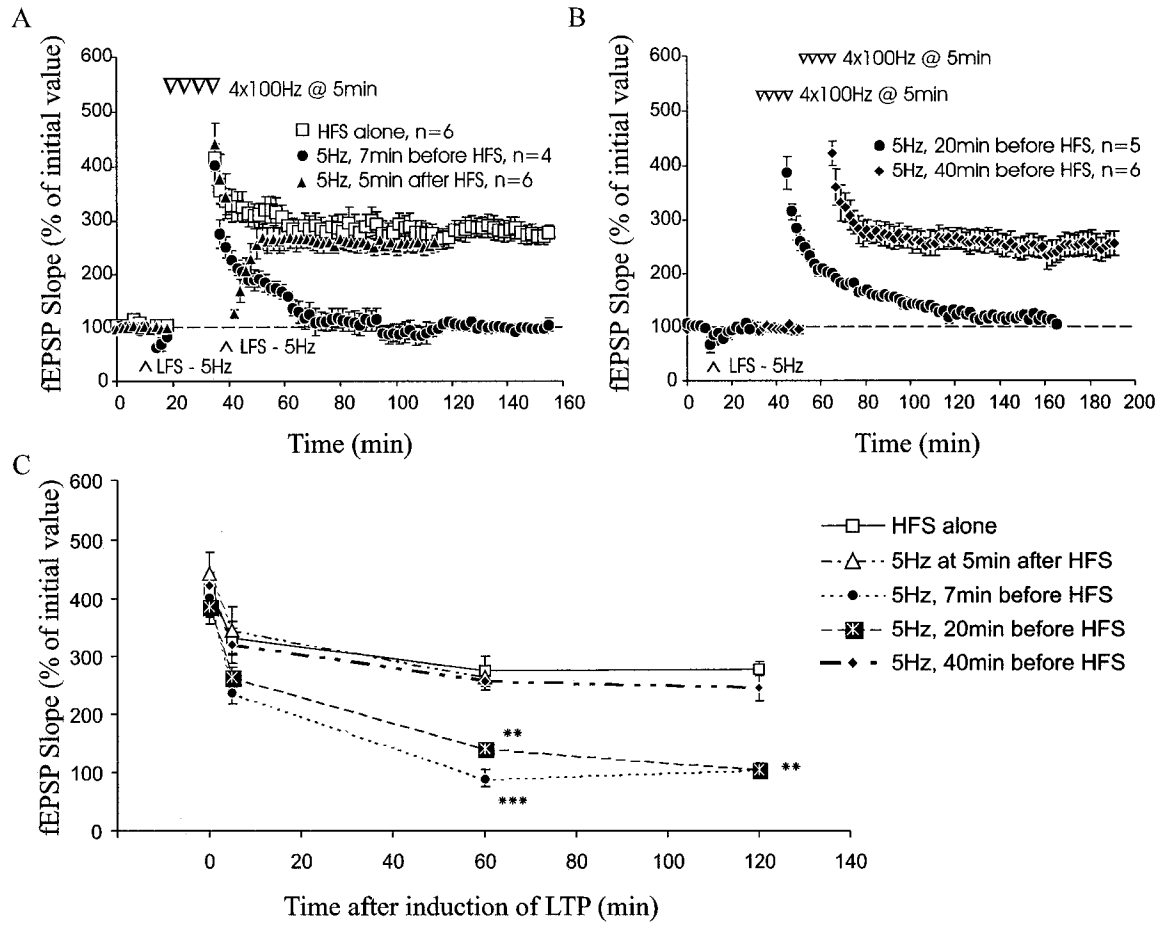

Figure 6.3: Innocuous LFS elicits anterograde metaplasticity of L-LTP only when given within a critical time window before L-LTP induction. **(A)** LFS induces anterograde, but not retrograde, suppression of L-LTP. When LFS (5Hz, 3min) was applied 7min prior to tetraburst stimulation, L-LTP was blocked (●). However, when the same LFS was applied 5min after tetraburst stimulation (▲), transient depotentiation occurred, and the level of potentiation recovered to levels not significantly different from slices receiving tetraburst stimulation alone (□). **(B)** Increasing the time interval between LFS and L-LTP induction abolishes LFS-induced anterograde metaplasticity of L-LTP. Defective L-LTP was observed when LFS was applied 20min before L-LTP induction (●). By contrast, normal L-LTP was seen when the time interval between LFS and tetraburst stimulation was extended to 40min (◆). **(C)** Summary plot showing levels of potentiation measured 5min, 1h, 2h, and immediately after tetraburst stimulation when the time between LFS and HFS was varied. (** denotes $p<0.01$, *** denotes $p<0.001$).

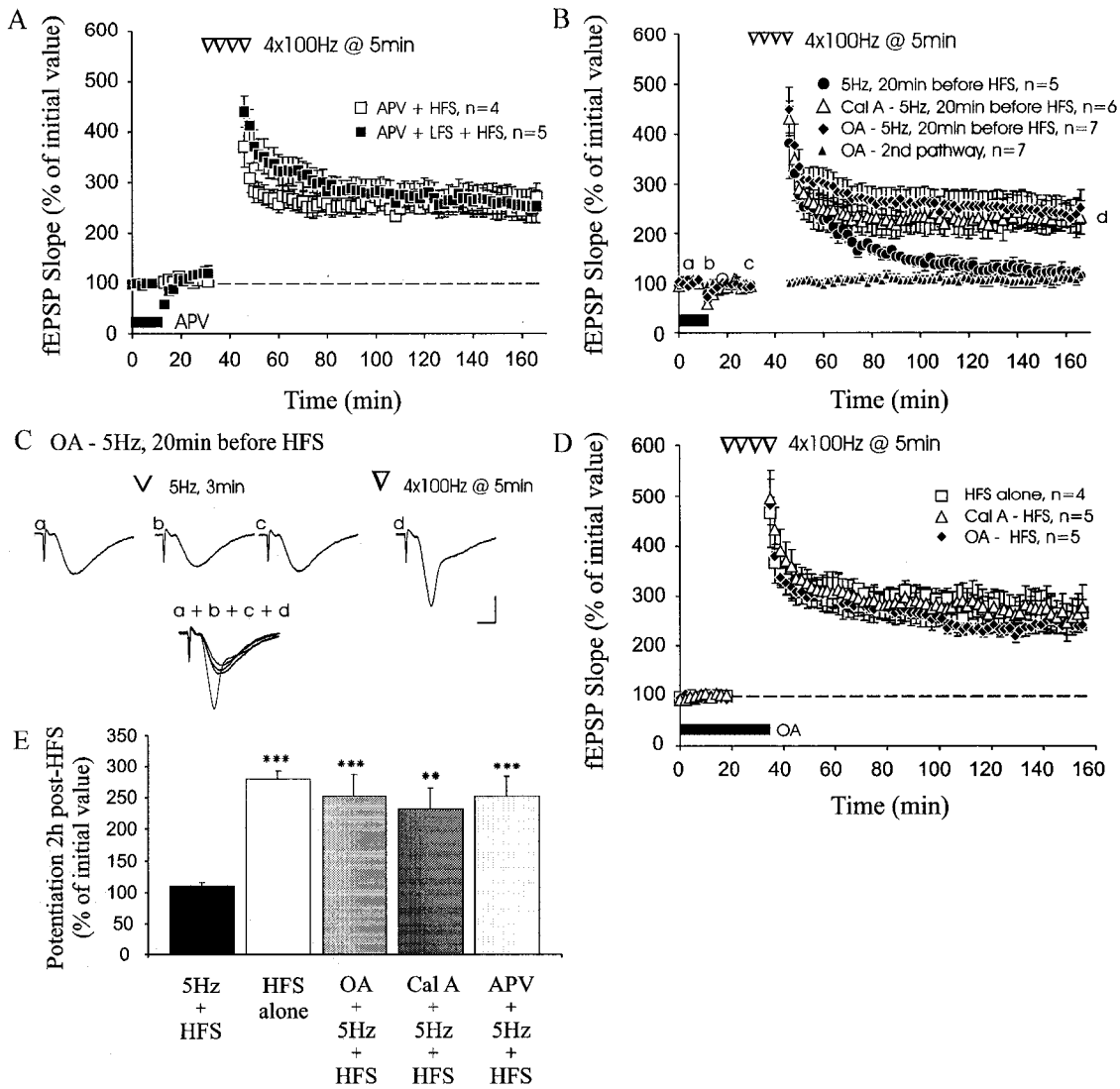

Figure 6.4: Inhibition of protein phosphatases or NMDA receptors blocks LFS-induced metaplasticity of L-LTP. **(A)** Application of APV, an NMDA receptor antagonist, blocks LFS-induced suppression of L-LTP. When APV was applied to slices and allowed to washout for 20min (no LFS), robust L-LTP was elicited by HFS (□). Similarly, when APV was applied to slices before and during LFS, robust L-LTP was induced by subsequent HFS (■). **(B)** Robust L-LTP was observed in slices that received 5Hz LFS in the presence of okadaic acid (1μM, horizontal bar; ◆) or calyculin A (△). By contrast, slices receiving LFS without drug (●) showed deficient L-LTP. Application of okadaic acid did not alter basal synaptic transmission in a second independent pathway which did not receive either LFS or tetraburst stimulation (▲). **(C)** Sample fEPSP traces from one experiment, showing okadaic acid blocking LFS-induced inhibition of L-LTP. Sample traces were recorded at times marked on the graph (a-d). Note that fEPSPs have recovered to pre-LFS amplitudes and slope values before initiation of tetraburst stimulation. Calibration bars: 2mV, 2ms. **(D)** Protein phosphatase inhibitors do not affect L-LTP. LTP in slices pretreated with either okadaic acid (◆) or calyculin A (△) was similar to LTP in drug-free slices receiving HFS (□). **(E)** Summary bar graph depicting levels of potentiation seen 2h post-HFS in the presence of various inhibitors. ** denotes $p<0.01$, *** denotes $p<0.001$ compared to 5Hz, HFS.

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*CHAPTER VII:

PROTEIN SYNTHESIS CONFERS SYNAPTIC IMMUNITY TO DEPOTENTIATION

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Introduction

Synaptic efficacy in the hippocampus can be rapidly modified by imposed synaptic activity (see review by Madison *et al.*, 1991, Hawkins *et al.*, 1993, and Bear and Abraham, 1996). Hippocampal synapses can express either a persistent enhancement ("long-term potentiation," or LTP) or reduction ("long-term depression," or LTD) of synaptic transmission (Bliss and Lomo, 1973; Dudek and Bear, 1992). LTP and LTD may critically regulate long-term information storage in the mammalian brain (Bliss and Collingridge, 1993; Moser *et al*, 1998; Martin *et al*, 2000; Brun *et al*, 2001).

Hippocampal LTP, which can be elicited by high-frequency stimulation (HFS), is sensitive to activity-induced reversal, or depotentiation (DPT), during a short time interval immediately after LTP induction (Barrioneuvo *et al.*, 1980; Staubli and Lynch, 1990; Fujii *et al.*, 1991). Low-frequency stimulation (LFS) induces DPT at previously potentiated synapses *in vitro* (Fujii *et al*, 1991; Bashir and Collingridge, 1994; Huang *et al*, 1999) and *in vivo* (Barrionuevo *et al*, 1980; Stäubli and Lynch, 1990). DPT may keep synaptic strength within a dynamic operating range such that synaptic efficacy can remain modifiable in response to electrical activity. Although several studies have extensively characterized DPT, the factors that protect potentiated synapses from DPT have not been previously examined.

Some evidence that susceptibility to DPT can be altered comes from a study that showed that multiple trains of high-frequency stimulation induce a form of LTP that is resistant to depotentiation (Woo and Nguyen, 2002). In addition, genetic overexpression of *one* specific transcription factor (cyclic-AMP response element binding protein, CREB) in transgenic mice can convert decremental synaptic potentiation into a more enduring form

of LTP that is protein synthesis-dependent and resistant to DPT (Barco *et al.*, 2002). In the present study, the molecular mechanisms that critically regulate the susceptibility of synapses to DPT were examined. By using standard electrophysiological techniques to examine LTP and DPT in wildtype mouse hippocampal slices, the findings demonstrate that synaptic immunity to DPT depends critically on the amount of imposed synaptic stimulation. Furthermore, synaptic immunity to DPT is rapidly induced and requires protein synthesis and transcription, and that the signals that are critical for establishing this synaptic immunity may originate locally at synapses, or at the soma. It is proposed that the cellular site of macromolecular synthesis determines whether synaptic immunity to DPT is input-specific or cell-wide.

Materials and Methods

Animals

Female C57BL/6 mice, aged 9-12 weeks (Charles River, Canada) were used for all experiments.

Electrophysiology

Transverse hippocampal slices (400μm thickness) taken from C57BL/6 mice were prepared as described in *Chapter II*. Slices were maintained in an interface chamber at 28°C and were perfused (1mL/min) with artificial cerebrospinal fluid (ACSF; composition described in *Chapter II*) aerated with 95% O_2 and 5% CO_2. Extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded with a glass microelectrode filled with ACSF and positioned in stratum radiatum of area CA1. A bipolar nickel-chromium

electrode was used to elicit fEPSPs by stimulating the Schaffer collateral-commissural fibres. fEPSPs were elicited at the rate of once per min at this "test" stimulation intensity.

For two-pathway experiments, a second independent pathway within stratum radiatum was monitored by placing a second bipolar stimulating electrode in this region. Placement of S2 was adjusted so that paired-pulse facilitation (PPF) was not observed when stimulating electrodes S1 and S2 were sequentially activated by paired S1-S2 stimulus pulses spaced 75ms apart.

For experiments on isolated CA1 dendrites, hippocampal slices were cut in ice-cold ACSF (NaCl was replaced by equimolar sucrose, and $CaCl_2$ was replaced by $MgCl_2$), after which two incisions were made under a dissecting microscope. One cut was applied just below the cell body layer of area CA1, and another incision was applied in area CA3 (**Figure 7.3A**). Slices were then perfused with normal ACSF and allowed to recover in an interface chamber for at least 2hr before recordings commenced. Slices were used if the following criteria were met: **a)** absence of a population spike when the recording electrode was placed at the cell body layer and strong stimulation was applied in stratum radiatum below the cut; **b)** absence of a fEPSP in stratum radiatum below the incision when stimulation was applied at the basilar dendrites in stratum oriens. Brightfield digital images (10x magnification) were captured using a SPOT Digital Microscope (Carsen Group, Ont., Canada) mounted on an Olympus BX40 microscope.

LTP was induced by applying either two trains of HFS (100Hz, 1s duration at test strength), or four trains of HFS (100Hz, 1s duration at test strength) spaced 20s apart. DPT was induced by applying LFS consisting of 5Hz for 3min (Table 2.1)

Two different translational inhibitors, anisomycin (25μM) and emetine (20μM) were prepared as concentrated stock solutions in DMSO and in distilled water, respectively. The transcriptional inhibitor, actinomycin D (25μM), was prepared in DMSO (Table 2.1). Each drug was then diluted in ACSF to the desired final concentration and was bath-applied, during baseline fEPSP acquisition and LFS, for a total period of 30mins. Drug experiments were interleaved with drug-free controls.

Results

Tetraburst stimulation induces LTP that is resistant to depotentiation

Several distinct forms of LTP, elicited by various stimulation protocols, require protein synthesis for their expression (Stanton and Sarvey, 1984; Krug *et al.*, 1984; Frey *et al.*, 1988; Nguyen *et al.*, 1994; Scharf *et al.*, 2002). As an initial step towards defining the roles of activity, transcription, and translation in mediating synaptic immunity to DPT, the protein synthesis-dependence of LTP elicited by two trains versus four trains of 100Hz (HFS) tetanus paradigm was tested. Anisomycin, an inhibitor of translation, was bath applied to mouse hippocampal slices at a concentration that inhibits >85% of protein synthesis (Frey *et al.*, 1988). LTP elicited by two trains of HFS (20s interburst spacing) was unaffected by anisomycin (**Figure 7.1A**): the mean fEPSP slopes measured 5min, 1h and 2h after LTP induction were 263±19%, 193±10% and 159±10% respectively (**Figure 7.1A**, ■). Corresponding slope values in drug-free slices were 229±14%, 181±10% and 158±17% (**Figure 7.1A**, ○), and these were not significantly different ($p>0.05$ at all time points) from anisomycin-treated values. In contrast, when two additional trains of HFS (*i.e.* 4x100Hz) were added to the stimulation regimen (with the interburst interval kept the

same, at 20s duration), slices that received anisomycin showed decaying LTP (**Figure 7.1B**, ■). This stronger tetanus protocol elicited stable LTP in control drug-free slices, with mean fEPSP slope values of 307±25%, 216±21%, and 222±16%, measured 5min, 1h, and 2h post-HFS, respectively. Corresponding slope values in anisomycin-treated slices were 238±27%, 153±27%, and 101±17%, with a significant difference seen beginning at 30min post-HFS (**Figure 7.1B**, ■, $p<0.05$). When a second independent pathway in slices was monitored in the presence of anisomycin, no effect was observed on baseline (**Figure 7.1B**, ▲). It should be noted that the two train tetanus protocol elicited significantly less LTP than the four trains protocol at 5min and 2h after stimulation (**Figure 7.1A, B**, $p<0.05$). These findings show that, in the transition from two to four trains, the addition of two trains of HFS can induce different forms of LTP that differentially engage protein synthesis, consistent with previous studies (Huang and Kandel, 1994; Winder *et al.*, 1998).

The next issue addressed was whether this same increase in the amount of stimulation could critically change the susceptibility of potentiated synapses to DPT. To induce DPT, a LFS protocol consisting of 5Hz stimulation for 3min was used. LFS alone produced only a transient depression of fEPSP slopes that subsequently recovered to pre-LFS values within 10min (**Figure 7.1F**). However, LFS following induction of two train LTP reversed potentiated fEPSP slopes back to pre-LTP baseline values (**Figure 7.1C**, ■), which is consistent with earlier studies (Barrioneuvo *et al.*, 1980; Fujii *et al.*, 1991). Mean fEPSP slopes measured immediately after HFS, and 50min after LFS, were 343±25% and 102±18%, respectively. The corresponding mean fEPSP slopes in control slices receiving only HFS were 354±33% and 166±12% (**Figure 7.1C**, □), with the latter value being

significantly higher than the mean value measured from slices that received HFS followed by LFS ($p<0.01$; **Figure 7.1C**, ■).

In contrast, adding two additional trains of HFS blocked the expression of DPT induced by LFS (**Figure 7.1D**, ●). Four trains of 100Hz stimulation, spaced 20s apart, induced stable potentiation with mean fEPSP slope values of 326±21%, 267±18% and 176±19%, measured immediately, 5min, and 50min, after HFS, respectively (**Figure 7.1D**, □). Application of 5Hz LFS, 5min after LTP induction, initially depressed fEPSP slope values that subsequently recovered to previously potentiated values (**Figure 7.1D**, ●): corresponding mean values were 380±43%, 298±33%, and 167±17%, respectively.

Because the number of stimulation pulses was doubled during four trains of HFS (see **Figure 7.1B**), a stronger LFS protocol may be required to elicit subsequent DPT. Thus, an extended LFS protocol consisting of 5Hz stimulation for a period of 6min was applied 2min after four train LTP. Similar to the 3min LFS protocol, an initial depression followed by a recovery to potentiated values was observed (**Figure 7.1D**, ■). Mean fEPSP slope values for the extended LFS group were 327±41% and 182±31%, measured immediately and 50min after HFS, respectively. An ANOVA did not reveal a difference between the two LFS groups and the control group, which only received HFS ($p>0.5$).

Because induction of DPT is time-dependent (O'Dell and Kandel, 1994; Chen *et al*, 2001), the interval between LTP induction and LFS application was also reduced. This was accomplished by decreasing the time interval between successive trains during HFS. Four trains of 100Hz stimulation using *3s interburst intervals* elicited robust potentiation with mean fEPSP slope values of 354±42%, 307±25%, and 224±18%, measured immediately, 5min, and 50min, after HFS, respectively (**Figure 7.1E**, □). When subsequent LFS was

applied (5 min after this compressed HFS), an initial depression followed by a recovery of fEPSPs to potentiated values was observed (**Figure 7.1E**, ●). The corresponding values in slices that received LFS were 393±53%, 288±58%, and 187±20%, respectively ($p>0.1$, at all time points indicated).

In summary, these data demonstrate that there exists a close correspondence between the amount of stimulation used to induce LTP and the susceptibility of LTP to DPT. Two trains of HFS elicit protein synthesis-independent LTP that is reversed or depotentiated by subsequent LFS. In contrast, four trains of HFS induce protein synthesis-dependent LTP that is immune to DPT.

Local protein synthesis confers homosynaptic immunity to DPT

Because no difference was detected between anisomycin-treated and control slices within 30min after tetraburst stimulation (**Figure 7.1B**), protein synthesis may not play a role in the early stages. However, some studies have reported that protein synthesis can be engaged very early after tetanization (Otani *et al.*, 1989; Ouyang *et al.*, 1999). Thus, early protein synthesis, which may not contribute to the early potentiation observed, may nonetheless be involved in regulating the susceptibility of synapses to DPT.

To identify a critical requirement for early protein synthesis in mediating synaptic immunity to DPT, a translational inhibitor, anisomycin or emetine, was bath-applied to hippocampal slices. Both anisomycin and emetine, at lower concentrations than those used here, blocked protein synthesis >80% in hippocampal slices (for anisomycin data see Frey *et al.*, 1988; for emetine data see Stanton and Sarvey, 1984). Either inhibitor permitted DPT, which was not normally seen following four trains of HFS (**Figure 7.2A, B**). In

slices which did not receive LFS, mean fEPSP slope values 40min after HFS were 165±17% and 155±5% in the presence of anisomycin and emetine, respectively. In contrast, when LFS was applied 5min after LTP induction in the presence of these inhibitors, initially depressed fEPSP slopes recovered only to *pre-HFS baseline values* (**Figure 7.2A, B**, ●). Mean fEPSP slope values seen after LFS in anisomycin- and emetine- treated slices were 113±10% and 99±6%, respectively ($p<0.02$ when compared to controls without LFS).

In contrast to the persistent DPT observed with these translational inhibitors, application of a transcriptional inhibitor, actinomycin-D failed to facilitate DPT. A previous study reported that >70% of transcription was inhibited by actinomycin-D in hippocampal slices (Nguyen *et al.*, 1994). After LFS, initially depressed fEPSP slopes gradually increased to potentiated levels similar to control slices receiving only HFS (**Figure 7.2C**, ○). The mean fEPSP slope value measured 40min after HFS was 148±17% ($p>0.2$ for comparison to a mean value of 168±21% in slices that received LFS after HFS). Thus, homosynaptic immunity of four train LTP to DPT was dependent on protein synthesis, but it did not require transcription (**Figure 7.2D** summarizes these data).

Because four trains of HFS confers immediate synaptic immunity, local protein synthesis may be critical for this process. To test this idea, fEPSPs were recorded from slices containing isolated CA1 pyramidal cell dendrites that were created by applying two small cuts just under the cell body layers of areas CA1 and CA3 (**Figure 7.3A**, *two bold lines*). A brightfield digital image of a representative cut hippocampal slice is shown in **Figure 7.3B**. These slices exhibited smaller maximal fEPSP amplitudes, ranging from 1-3 mV. When four trains of HFS were applied to these slices, robust potentiation was elicited:

a mean fEPSP slope of 164±19% was measured 40min post-HFS (**Figure 7.3C**, □). This potentiation lasted >40 min, but it slowly decayed to pre-HFS baseline slope values within 2h (data not shown). When subsequent LFS was applied to cut slices, a transient depression was observed, but fEPSPs recovered to potentiated values with a mean value of 165±19% (**Figure 7.3C**, ●, $p>0.2$). Application of either anisomycin or emetine to these slices permitted subsequent DPT by LFS after four train LTP (**Figure 7.3D**). The mean fEPSP slope was 175±17% in cut slices, measured 40min post-HFS (**Figure 7.3D**, ○). In contrast, mean corresponding slope values for anisomycin- (**Figure 7.3D**, ●) and emetine- (**Figure 7.3D**, ■) treated cut slices after LFS were 89±13% and 103±6%, respectively ($p<0.01$, compared to corresponding control value). Both inhibitors of translation did not affect four train LTP in these cut slices (data not shown). This finding, when considered alongside the translational inhibitor data in intact cells (**Figure 7.3A, B**), suggests that local protein synthesis initiated immediately after tetanus, without prior transcription, can block DPT.

Input-specific vs. cell-wide synaptic immunity to DPT

Because DPT of four train LTP occurred only when protein synthesis was blocked, inhibition of protein synthesis by anisomycin may have altered synaptic properties. Thus, the input specificity of DPT of four train LTP in the presence of anisomycin was assessed. A previous study characterizing DPT in hippocampal slices reported that reversal of LTP was input-specific (Huang *et al.*, 2001).

Two independent pathways in stratum radiatum of area CA1 were stimulated, in the presence of anisomycin: one pathway (S1) received HFS followed 5min later by LFS,

whereas a second pathway (S2) received only HFS. **Figure 7.4A** shows that application of LFS (5min after HFS) to S1 elicited persistent DPT only in S1. Pathway S2, which was transiently depressed, remained potentiated, with a mean fEPSP slope value of 175±23% measured at 60min post-HFS. This was significantly higher than the corresponding value of 90±10% ($p<0.01$), measured in S1. Interestingly, fEPSP slopes in S2 were transiently reduced immediately after LFS in S1, thereby revealing a short-lasting heterosynaptic effect of 5Hz stimulation. This effect was unlikely the result of non-specific effects of anisomycin or partial overlap of activated synapses in the two pathways, because no heterosynaptic paired pulse facilitation (PPF) was observed between S1 and S2, whereas strong homosynaptic PPF was seen in these pathways (data not shown). In addition, induction of LTP in one pathway produced input-specific changes without modifying transmission in an independent pathway (see **Figure 7.4B, C**). It was previously demonstrated that synaptic activity affected a second independent pathway only if that pathway experienced prior plasticity (Muller *et al.*, 1995); this may explain the present short-lasting reduction in fEPSP slopes seen in S2. These data show that DPT of four train LTP, which occurs only when protein synthesis is inhibited, is input-specific.

The next question addressed was whether synaptic immunity can be transferred between independent pathways (*i.e.* whether it is heterosynaptic) by examining whether stimulation of one pathway can block DPT in an adjacent pathway by synaptic "capture" of proteins (**Figure 7.4B**). Four trains of HFS were applied to one pathway to induce protein synthesis-dependent LTP and 45min was allowed to elapse before two trains of HFS were applied to an adjacent pathway. This delay allowed macromolecular synthesis to occur, and there is evidence that such synthesis is critical for converting short- to long-lasting types of

synaptic plasticity by synaptic capture (Frey and Morris, 1997; Barco *et al.*, 2002). DPT was blocked in the S2 pathway (2x100Hz) pathway if it followed induction of four train LTP in an adjacent (S1) pathway (**Figure 7.4B**). When LFS was applied to S2 after two train LTP induction in S2 (45min after four trains of HFS was applied to S1), fEPSPs were initially depressed, but they recovered to previously potentiated levels, with a mean value of 196±10% measured at 40min post-LFS (**Figure 7.4B** bottom, S2, ○). However, when four trains of HFS was applied to S1 *in the presence of bath-applied anisomycin* (**Figure 7.4B** top, S1, ■) and the S2 pathway was subsequently given *two trains of* HFS, LTP induced in the S2 pathway was persistently reversed by 5Hz LFS (**Figure 7.4B** bottom, S2, ●; **Figure 7.4D**, black bar). These results suggest that activity-induced synaptic capture of newly synthesized proteins can confer immunity to DPT. This heterosynaptic process may involve the transfer, or capture, by synaptic sites, of proteins whose synthesis is triggered by activity at other synaptic sites.

To assess whether products of *local* protein synthesis are critical for heterosynaptic immunity to DPT, a transcriptional inhibitor, actinomycin-D, was applied to intact slices while monitoring two independent pathways. By inhibiting somal transcription, the potential necessity for *somatic* products in mediating the transfer of immunity to DPT from one pathway (or synaptic site) to another could be assessed. When transcription was inhibited by bath-applied actinomycin-D, and cell-wide translation was allowed to occur, four trains of HFS in one pathway (S1 of **Figure 7.4C**) did not elicit immunity to DPT at an adjacent pathway that received two trains of HFS (S2 of **Figure 7.4C**). Two train LTP in this second pathway (S2) was persistently erased after prior four train LTP induction in S1 (**Figure 7.4D**, grey bar). The mean fEPSP slope measured in S2 30min post-LFS was

108±9%, and the corresponding value in S1 was 141±11% ($p<0.05$). These findings mirror the results with *bath-applied anisomycin*: two train LTP in one pathway (S2) was persistently erased by LFS applied 45min after four trains HFS in an adjacent pathway (S1) (**Figure 7.4B**, ■, ●; **Figure 7.4D**). Taken together, these results suggest that protein synthesis initiated at the soma, and not transfer of locally-synthesized proteins between distinct synaptic sites, is critical for heterosynaptic transfer of immunity to DPT.

Discussion

Synaptic immunity to DPT: roles of activity

Synapses in invertebrates and vertebrates can experience long-lasting alterations in their physiological strength (Hawkins *et al.*, 1993; Huang *et al.*, 1996; Martin *et al.*, 2000). Certain forms of synaptic plasticity require protein synthesis and transcription (see reviews by Hawkins *et al.*, 1993, Martin *et al.*, 2000, and Steward and Schuman, 2001), and particular proteins may be synthesized at synaptic sites following changes in the electrical activity of these synapses (reviewed by Steward and Schuman, 2001). A particularly intriguing observation made in the present study is the rapid and critical modulation by activity of the reversibility of LTP. In changing from a two train to a four train tetanus protocol, the addition of just two trains of HFS (representing only 2s of additional imposed activity) was sufficient to convert LTP from a form that was susceptible to one that was immune to DPT.

The mechanisms underlying DPT have been partially elucidated (reviewed by Huang and Hsu, 2001). However, the factors that critically regulate the susceptibility of different forms of LTP to DPT have not been previously identified. Like memory,

hippocampal LTP has several distinct temporal phases (reviewed by Huang *et al.*, 1996). A "late" phase of LTP (L-LTP) is defined by its requirement for protein synthesis and transcription. Inhibitors of either translation (Stanton and Sarvey, 1984; Krug *et al.*, 1984; Frey *et al*, 1988; Scharf *et al*, 2002) or transcription (Nguyen *et al*, 1994, Frey *et al.*, 1996) block expression of L-LTP induced by multiple 100Hz trains, without affecting a more modest form of LTP elicited by weaker stimulation (Huang and Kandel, 1994). These studies demonstrate that *de novo* protein synthesis is engaged at a later stage to mediate the expression of L-LTP, which is in agreement with the results in this study, but they have not identified an early role for these processes. The results from the present study demonstrate an early role for protein synthesis during LTP that is critical for "consolidating" synaptic potentiation into a resilient state that is immune to DPT.

Local dendritic protein synthesis and synaptic immunity to DPT

Application of anisomycin or emetine, both inhibitors of translation, enabled depotentiation of four train LTP without affecting hippocampal slice viability. Many previous studies have demonstrated that protein synthesis inhibition is reversible and does not affect neuronal properties such as excitability (Stanton and Sarvey, 1984). In addition, this study shows that inhibition of protein synthesis does not affect basal transmission in hippocampal slices (**Figure 7.1B**), which confirms many previous findings (Krug *et al.*, 1984; Frey *et al.*, 1988, 1996; Nguyen *et al.*, 1994; Scharf *et al.*, 2002). Moreover, it did not affect the initial magnitude of LTP or the input specificity of DPT.

In contrast to inhibitors of translation, actinomycin-D, an inhibitor of transcription, did not enable DPT following induction of four train LTP. Furthermore, when recordings

were taken from slices containing isolated CA1 neuropil, blockade of DPT was still evident, indicating that the soma is not required for homosynaptic immunity to DPT. Thus, an essential step for initiating immunity may be the translation of pre-existing mRNA, as suggested by the block of synaptic immunity to DPT by translational inhibitors applied to slices containing isolated neuropil. Although, the possibility that the site of local protein synthesis occurs in cells other than pyramidal cells such as glial cells or interneurons cannot be excluded, the most likely source of translation is the dendrites of CA1 pyramidal neurons. Polyribosomes have been observed in dendritic shafts (Steward and Levy, 1982; Steward and Falk, 1986, 1991) that can mediate local protein synthesis in isolated dendrites of hippocampal neurons (Aakalu *et al.*, 2001). In addition, a study reported that a tetanus protocol (4x100Hz, 30s intervals) similar to the one employed in this study can rapidly initiate dendritic protein synthesis in hippocampal slices (Ouyang *et al*, 1999). The axons of presynaptic CA3 neurons as the site of local protein synthesis can be excluded because functional axonal protein synthesis has not yet been identified in the hippocampus (Torre and Steward, 1992).

Several studies suggest that dendritic protein synthesis can mediate persistent changes in synaptic efficacy (Kang and Schuman, 1996; Huber *et al*, 2000). Some of the dendritic molecules needed for initiating translation at local dendritic sites have been identified (Tang *et al.*, 2002), but the activity-dependence of their recruitment has not yet been clearly defined. In the present study, additional synaptic activity imposed during four trains of HFS may have engaged translational machinery at dendrites (or at other extra-somal sites) to elicit synaptic immunity to DPT. This may occur as a result of different calcium stores being activated by the two different tetanus protocols. In a previous study, it

was demonstrated that calcium stores are also narrowly tuned to different forms of LTP (Raymond and Redmond, 2002). Similarly, initiation of local protein synthesis may have different thresholds to different calcium stores. Further studies are required to determine the properties that govern the initiation of local protein synthesis.

Although the identities of the dendritic proteins required for synaptic immunity to DPT are unknown, the observations from the present study suggest that the candidate proteins should be rapidly upregulated, synthesized locally, and that their synthesis should be activity-dependent. Some candidates may include Arc, an actin-binding cytoskeletal protein (Link *et al.*, 1995; Lyford *et al.*, 1995; Steward *et al.*, 1998; Ying *et al.*, 2002), and αCaMKII (Burgin *et al.*, 1990; Ouyang *et al.*, 1999). Another plausible mechanism by which local protein synthesis may confer synaptic immunity to DPT is through alterations of adenosine metabolism by the newly synthesized product(s). In a previous study it was shown that accumulation of extracellular adenosine is required for DPT (Huang *et al.*, 1999). Thus, stronger stimulation protocols may alter adenosine metabolism such that increases in extracellular adenosine by low frequency stimulation is inhibited. Further work, involving the use of functional knock-outs of candidate proteins, is needed to assess their potential roles in conferring synaptic immunity to DPT.

Transcription and cell-wide synaptic immunity to DPT

Independent, long-term control of physiological strength at individual synapses has been proposed to involve a synaptic "tagging" process, whereby plasticity-related proteins may be sequestered at tagged synapses that had experienced activity (Martin *et al.*, 1997; Frey and Morris, 1997, 1998). Accumulation of these proteins may convert temporary

synaptic modifications into more persistent alterations. Another function of plasticity proteins that are produced via transcription may be to provide cell-wide synaptic immunity to DPT. Although transcription was not required for homosynaptic immunity to DPT, it was required for heterosynaptic transfer of synaptic immunity to DPT. Earlier studies suggest that the cAMP/PKA signalling pathway can inhibit DPT (Otmakhova and Lisman, 1998; Natsuki *et al.*, 1997). Modulators of this pathway alter cAMP-mediated transcriptional processes in hippocampal neurons (Impey *et al.*, 1996; Matsushita *et al.*, 2001). One important transcription factor involved in cAMP-regulated gene expression is the cAMP response element binding protein (CREB). Signals that induce LTP, such as multiple trains of HFS, can elicit CRE-mediated transcription in hippocampal neurons (Impey *et al.*, 1996; but see also Deisseroth *et al.*, 1996). When a constitutively active isoform of CREB was expressed in mutant mice, unstable LTP was converted into long-lasting LTP that was immune to DPT (Barco *et al.*, 2002). Consistent with this notion, inducing protein synthesis-dependent LTP in one synaptic pathway can elicit cell-wide immunity to DPT, as evident from the inhibition of DPT after two train LTP was elicited at adjacent synaptic inputs (**Figure 7.4B**). In the model presented in **Figure 7.5**, when four trains of HFS was applied to one pathway, the end-products of transcription (*i.e.* somatic proteins and/or mRNA) were sequestered at an independent pathway that became immune to DPT. Two-pathway experiments showed that transcription *and* protein synthesis are required for synaptic immunity. This implies that a product of local protein synthesis, which mediates immunity to DPT at a given synapse, does not pass directly from one synaptic site to another. Instead, a sequential soma to dendrite relay, involving somal transcription, may be needed for cell wide immunity to DPT.

Synaptic immunity: potential functions in the context of LTP, learning, and memory

Increases and decreases in synaptic efficacy may act in tandem to allow for the detection and storage of new information in neural circuits. Synaptic immunity to DPT provides activity-dependent protection of synapses against erasure of LTP. Such immunity may serve as a "high-pass filter" to allow for the expression of resilient, long-lasting types of LTP induced by repeated high-frequency patterns of activity that are also critical for eliciting translation- and transcription-dependent forms of LTP. Because the strong tetanus protocol employed in this study to induce LTP has been correlated with hippocampus-dependent memory in mice (Abel *et al.*, 1997, Miller *et al.*, 2002), activity-dependent synaptic immunity of LTP against DPT may be important for controlling the neural threshold for the consolidation of short-term memories into long-term memories.

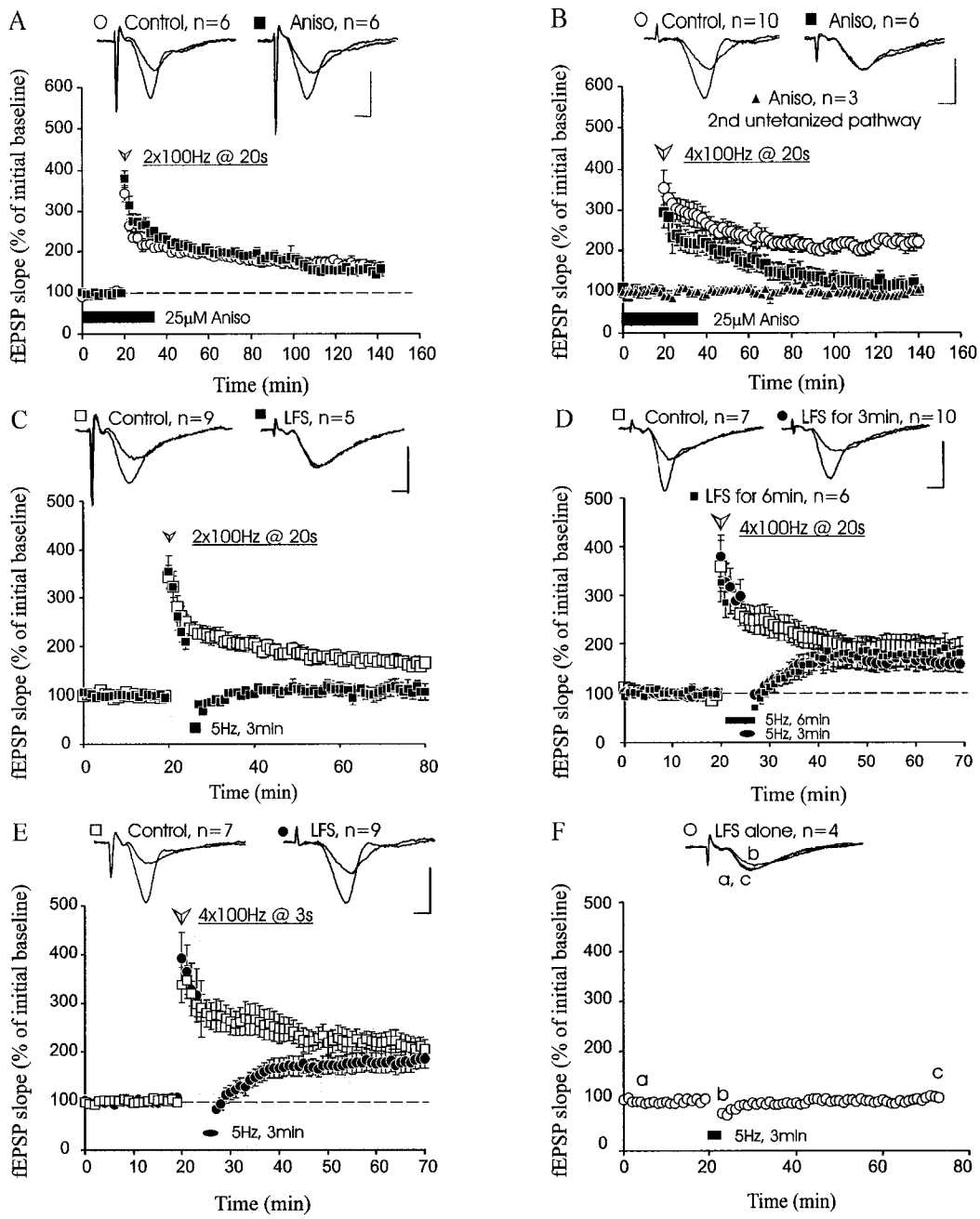

Figure 7.1: Synaptic immunity to DPT is activity-dependent. **(A)** Application of anisomycin did not significantly alter two-train LTP (■). Sample fEPSP traces were recorded 5min pre-HFS and 2h post-HFS. **(B)** Four-train LTP in anisomycin slowly decayed to baseline within 100min (■). Sample traces taken at 5min pre-HFS and 2h post-HFS. **(C)** LFS given 5min after two trains of HFS persistently erased LTP (■), whereas fEPSPs in slices that received HFS without LFS remained potentiated (□). **(D)** In contrast, when LFS was applied after four trains of HFS, spaced 20s apart, persistent DPT was not observed (●). When LFS was applied for 6min, four-train LTP was still not persistently erased (■). **(E)** Similarly, when the spacing between successive trains of HFS was compressed to 3s, no persistent DPT was observed following LFS (●). All sample traces from DPT experiments were taken at 5min pre-HFS and 40min post-LFS. Calibration Bars: 5mV, 2ms. **(F)** LFS alone transiently depressed fEPSPs, which recovered back to pre-LFS slope values.

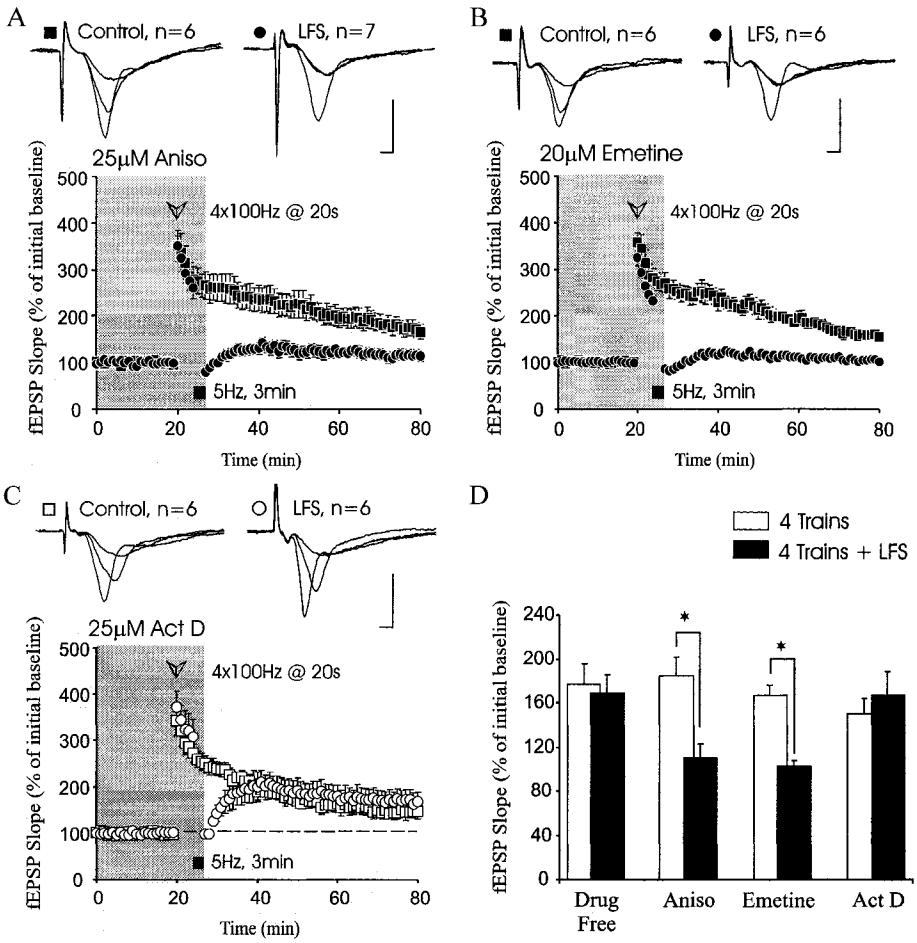

Figure 7.2: Protein synthesis is required to "immunize" LTP against DPT. Application of either **(A)** anisomycin or **(B)** emetine, both inhibitors of translation, permitted persistent DPT of four-train LTP (●). **(C)** Application of a transcriptional inhibitor, actinomycin-D, did not block immunity to DPT, and no persistent DPT was observed (○). Sample sweeps in A-C were taken 5min pre-HFS, immediately after HFS, and 40min post-LFS. Calibration Bars: 5mV, 2ms. **(D)** Summary histogram for these experiments.

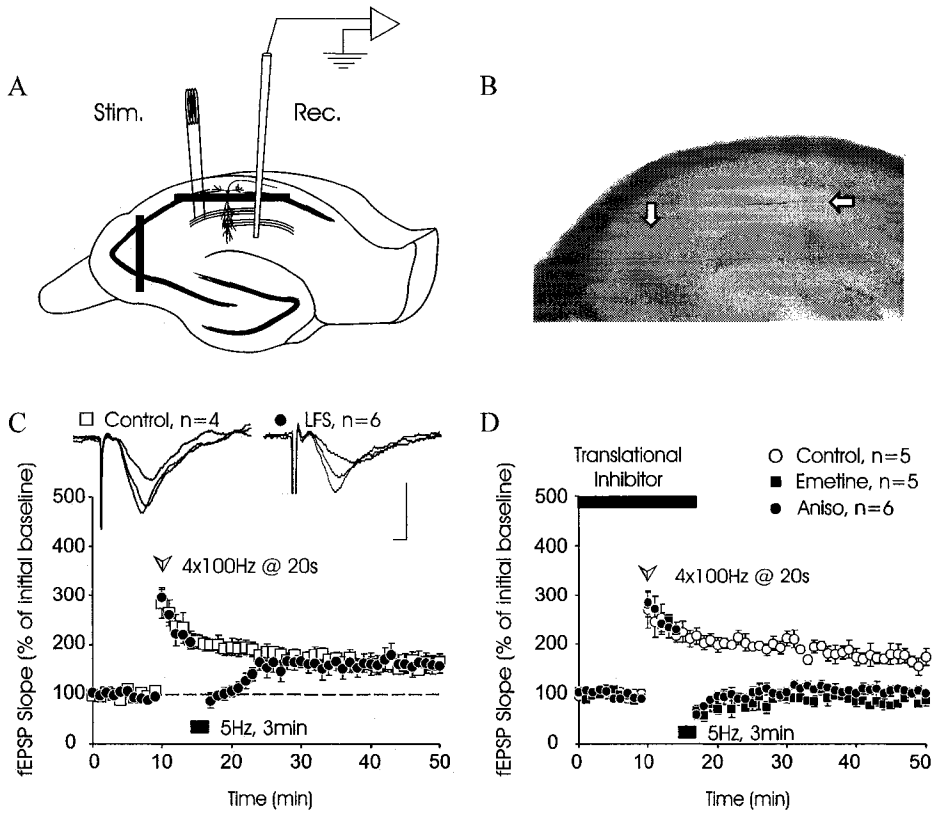

Figure 7.3: Local protein synthesis is required for synaptic immunity to DPT. **(A)** Schematic diagram showing the two incisions (thick black lines) made to isolate CA1 neuropil and the positions of recording and stimulation electrodes. **(B)** Brightfield image of a hippocampal slice after two incisions (arrows) were made to isolate CA1 neuropil (10x magnification). The dentate gyrus is at the bottom right edge of the photograph. **(C)** When LFS was applied shortly after four trains of HFS in isolated CA1 pyramidal cell dendrites, transient synaptic depression was seen (●), but fEPSP slopes recovered to potentiated levels not significantly different from those seen in cut slices that received HFS alone (□). Traces were taken 5min pre-HFS, immediately after HFS, and 30min post-LFS. Calibration Bars: 2mV, 2ms. **(D)** Both anisomycin (●) and emetine (■) permitted the expression of DPT after four trains of HFS in slices containing cut CA1 dendrites.

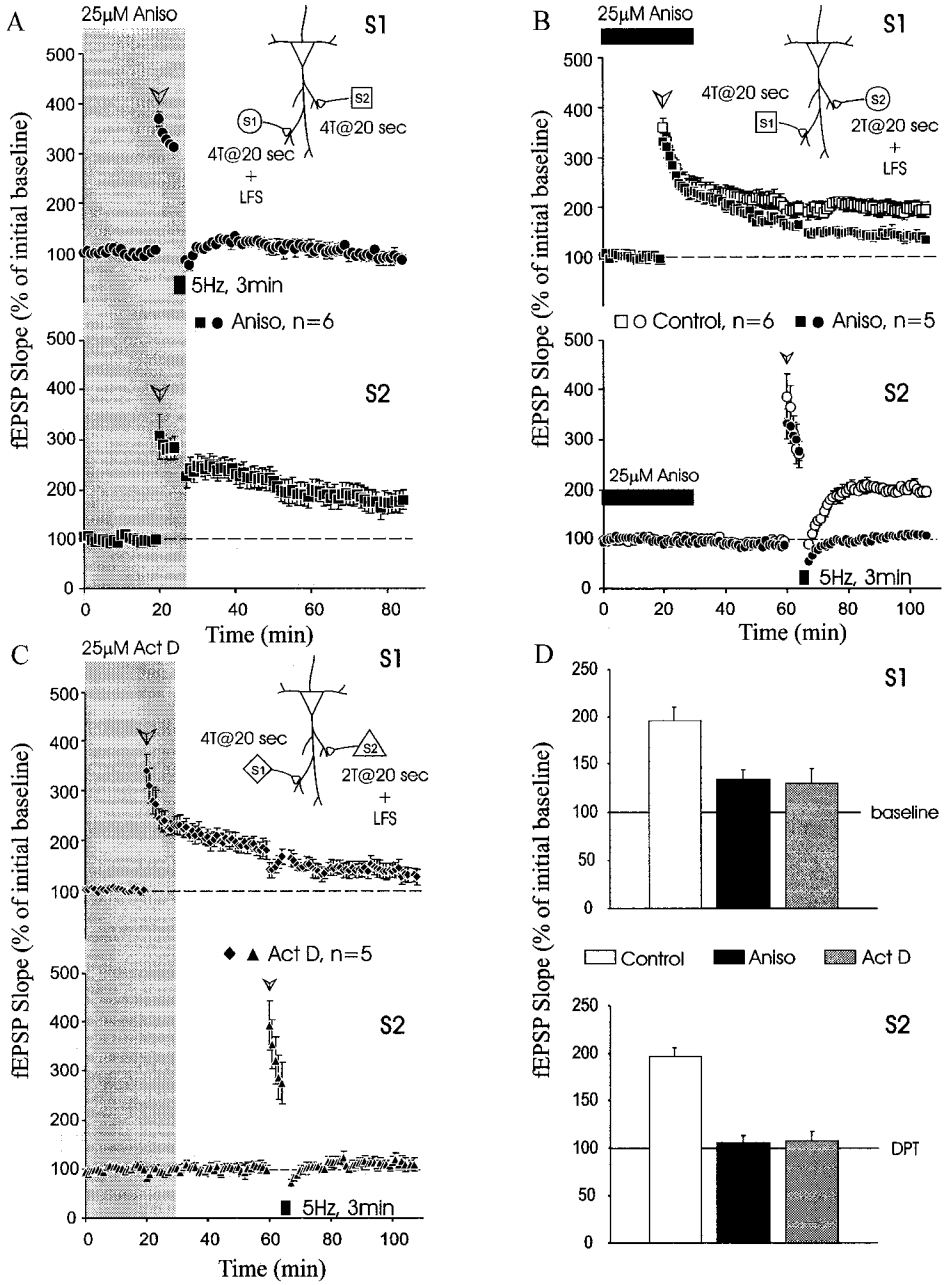

Figure 7.4: Input specificity, homosynaptic, and heterosynaptic properties of synaptic immunity to DPT. **(A)** In the presence of anisomycin, DPT of four-train LTP is input-specific. When two pathways received four trains of HFS in the presence of anisomycin, only the pathway receiving LFS 5min after HFS (S1, ●) showed DPT, whereas the other pathway showed robust LTP (S2, ■). **(B)** Synaptic immunity may be conferred by cell-wide intersynaptic transfer of newly expressed gene products. When four trains of HFS were applied to one pathway (S1, □), two-train LTP in a second adjacent pathway (S2, ○) became resistant to DPT. Inhibition of protein synthesis (●, ■), by bath application of anisomycin to both S1 and S2, blocked heterosynaptic transfer of resistance to DPT at S2. Two-train LTP at S2 could now be erased following prior four trains of HFS priming at S1 (● in bottom half). **(C)** Inhibition of transcription by actinomycin-D blocked transfer of synaptic immunity to pathway S2 following priming by four trains of HFS in pathway S1. **(D)** Summary bar graph depicting the results of the two pathway experiments shown in B & C.

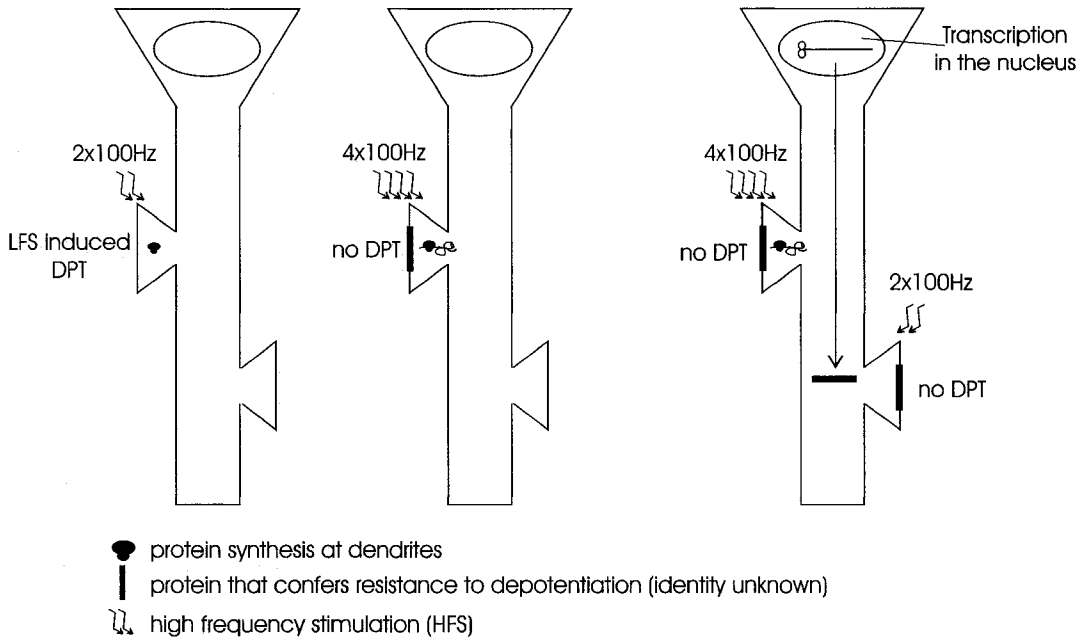

Figure 7.5: Schematic model of protein synthesis-dependent synaptic immunity to DPT. LTP induced by two 100Hz trains can be persistently erased by DPT (*left*). In contrast, LTP elicited by four 100Hz trains cannot be persistently erased, and this homosynaptic resistance to DPT likely requires translation at dendrites (*center*). *Transfer* of the immunity to DPT can occur between distinct synaptic sites on CA1 pyramidal neurons (*right*). Local protein synthesis initiated by four trains of HFS is necessary for homosynaptic immunity to DPT. However, transcription is needed for effective transfer of resistance to DPT from one input pathway to another. Thus, a molecular signalling relay may occur, so that stimulation may trigger production of somatic signals (*e.g.* proteins), which then confer immunity to DPT at remote synaptic sites, via sequestering of these signals at these sites.

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*CHAPTER VIII:

GENERAL DISCUSSION

*Portions of this chapter have been submitted for publication:

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Long-lasting Forms of Hippocampal Synaptic Plasticity: Summary of thesis findings

Cellular and molecular studies of synaptic plasticity and learning in invertebrate models have provided an important conceptual framework for the genesis of research in mammalian models. One idea derived from the work on *Aplysia* is that activity-dependent increases in synaptic strength (facilitation) have distinct temporal phases (Kandel, 2001). Similarly, there exists at least two distinct temporal phases of activity dependent forms of hippocampal LTP, an early phase and a late phase, that are distinguished on the basis of their induction requirements, duration of expression, and molecular mechanisms. The long-lasting form of LTP (L-LTP) has been proposed to be a mechanism for long-term memory (LTM) in the mammalian brain because both share requirements for PKA and protein synthesis. Furthermore, selective disruption of L-LTP is correlated with profound and selective impairment of LTM (Abel *et al.*, 1997; Wong *et al.*, 1999; Guzowski *et al.*, 2000; Jones *et al.*, 2001; Kang *et al.*, 2001; Miller *et al.*, 2002). In light of the importance of L-LTP to LTM, my research findings have identified several critical factors that govern the induction and expression of long-lasting forms of synaptic plasticity in area CA1 of the hippocampus.

Stimulation Parameters

The first objective of my research was to critically assess the importance of stimulation parameters for the induction and expression of L-LTP. As described in *Chapters III & IV*, patterns of stimulation influence the expression of L-LTP in two important ways, by differentially recruiting distinct isoforms of PKA, and by controlling the PKA-dependence of LTP.

Both pharmacological and genetic approaches were used in *Chapter III* to demonstrate that the recruitment of PKA isoforms is differentially regulated by the temporal pattern of stimulation. R(AB) mutant mice were tested because they express substantial reduction of hippocampal PKA activity through transgenic overexpression of an inhibitory isoform of RIα (Abel *et al.*, 1997). L-LTP elicited by spaced stimulation was attenuated in slices taken from mutant mice but not in slices taken from wildtype mice. In contrast, L-LTP elicited by thetaburst stimulation was not impaired in either mutant or wildtype slices. When an isoform-nonspecific PKA inhibitor was applied to mutant slices, thetaburst L-LTP was impaired. Thus, theta-like patterns of stimulation may have recruited endogenous isoforms of hippocampal PKA that do not contain mutant RIα subunits, whereas stronger, multitrain stimulation recruited PKA isoforms that contain the mutant regulatory subunit (**Figure 3.4**).

In *Chapter IV*, genetic and pharmacological techniques were again employed to demonstrate the importance of the intertrain interval during tetanus. By maintaining the number of trains (four) and the number of electrical pulses (400) constant, while varying the intertrain interval, the role of temporal spacing *per se* was specifically addressed. This manipulation significantly altered the PKA-dependence of L-LTP by changing the sensitivity of LTP to PKA inhibitors. A "spaced" protocol (5min intertrain interval) and two "compressed" protocols (intertrain intervals of 20s and 3s) all elicited protein synthesis-dependent forms of LTP (*Appendix A*). However, only the spaced protocol elicited a form of L-LTP that was PKA-dependent. Both compressed protocols elicited LTP that was not attenuated in R(AB) mutant slices. Because the compressed tetraburst protocols may potentially recruit different isoforms of PKA, as was the case of thetaburst

LTP, their PKA-independence was further confirmed by the application of a pharmacological inhibitor, KT5720. This inhibitor did not affect L-LTP elicited by compressed protocols in mutant slices, whereas it attenuated L-LTP elicited by spaced tetraburst stimulation in wildtype slices (positive control).

Protein Phosphatases

Because recent studies have highlighted the importance of protein phosphatases for the expression of different forms of synaptic plasticity (Winder and Sweatt, 2001), the second major objective of my thesis was to determine the significance of protein phosphatases in the expression of L-LTP. As described in *Chapter V*, protein phosphatases, acting downstream of PKA, play an important role in the expression of L-LTP. Previous studies on E-LTP have shown that activation of PKA can inhibit protein phosphatase by phosphorylating I-1, a potent endogenous PP1 inhibitor (Blitzer *et al.*, 1995, 1998). Genetic inhibition of PKA impaired L-LTP in R(AB) mutant slices because protein phosphatases were able to act as an inhibitory constraint of L-LTP expression. When this inhibitory constraint was relieved by pharmacological application of okadaic acid or calyculin A (PP1/2A inhibitors), L-LTP in R(AB) mutant slices was fully rescued. Synaptic facilitation induced by co-application of forskolin and IBMX, an adenylyl cylcase activator and a phosphodiesterase inhibitor respectively, was impaired in R(AB) mutant slices but was fully rescued by okadaic acid. Moreover, the gating mechanism involved in the expression of L-LTP is primarily operated by PP1 because application of FK-506, a calcineurin inhibitor, or a lower concentration of okadaic acid that primarily inhibits PP2A, failed to rescue L-LTP in mutant slices.

The functional importance of protein phosphatases for the expression of L-LTP was further demonstrated in *Chapter VI*. Activation of protein phosphatases by 5Hz stimulation for 3min, a low-frequency stimulation protocol normally used to induce depotentiation, elicited a form of metaplasticity that impaired subsequent expression of L-LTP but not E-LTP. This anterograde inhibition of L-LTP was persistent in duration and it was activity-dependent. This novel form of metaplasticity was blocked by the protein phosphatase inhibitors okadaic acid and calyculin A, and by the NMDA receptor antagonist APV.

Protein Synthesis

Finally, the third and last objective of my research was to examine the role of protein synthesis in long-lasting forms of synaptic plasticity. As described in *Chapter IV*, the temporal pattern of stimulation influences the expression of L-LTP by controlling its PKA-dependence. In a subsequent study in collaboration with Dr. Ted Abel's laboratory at the University of Pennsylvania, the issue of temporal spacing was revisited but with respect to protein synthesis. *Appendix A* demonstrates the importance of intertrial interval between high-frequency trains, and how it can regulate the efficacy of long-term potentiation via protein synthesis. Although both protocols elicited a protein synthesis-dependent form of LTP, spaced tetraburst stimulation elicited a more robust form of L-LTP that was more dependent on protein synthesis than L-LTP induced by compressed protocols. This was clearly evident from the higher levels of potentiation observed after induction and the greater sensitivity of L-LTP to disruption by anisomycin, a protein synthesis inhibitor.

Finally, *Chapter VII* describes a novel function for protein synthesis: protection of potentiated synapses from depotentiation. An intriguing observation in the discovery of protein phosphatase mediated metaplasticity (*Chapter VI*) was that low-frequency stimulation (LFS) caused anterograde, but not retrograde, effects on L-LTP. Previous studies have shown that LTP is sensitive to disruption shortly after induction. However, LFS in this case did not depotentiate LTP elicited by spaced tetraburst stimulation. Because an inverse relationship exists between the onset of LFS application after LTP induction and the magnitude of depotentiation (Fujii *et al.*, 1991; O'Dell and Kandel, 1994; Staubli and Chun, 1996), compressed tetraburst stimulation protocols were utilized to minimize this influence, as the interval between the first and last trains is much less than that in the spaced protocol. Similar to the case of spaced tetraburst stimulation, both compressed protocols elicited a form of LTP that was resistant to depotentiation. This synaptic resistance or "immunity" to depotentiation was activity-dependent and protein synthesis-dependent. Local protein synthesis elicited a spatially input-specific form of immunity that was rapidly induced, whereas transcription mediated a cell-wide immunity that slowly developed because of the lag between the synthesis of immunity-related proteins and their transport to 'tagged' or activated synapses.

Emerging Model of the Late Phase of LTP (L-LTP): An amalgamation of research findings

Advances in molecular biology have significantly strengthened the correlation between L-LTP and LTM. As a result, many studies have focused on the mechanisms underlying L-LTP. The remainder of this section discusses these findings alongside my present findings to construct an emerging model of L-LTP (**Figure 8.1**).

Induction Protocols

One cardinal feature that differentiates L-LTP from E-LTP is the requirement of protein synthesis for L-LTP. All tetanus protocols, except one and two trains of 100Hz stimulation, elicit a form of LTP that requires *protein synthesis* (*Appendix A*; Nguyen and Kandel, 1997). Thus, thetaburst, spaced, and compressed tetraburst stimulation can all elicit L-LTP.

An important property identified in the present thesis is the NMDAR dependence of L-LTP. L-LTP induced by both spaced and compressed tetraburst stimulation was blocked by APV, an NMDA receptor antagonist (*Chapter IV*). Likewise, it was previously shown that thetaburst LTP is also sensitive to APV (Nguyen and Kandel, 1997). Thus, L-LTP, in addition to E-LTP, requires NMDA receptor activation and presumably a concomitant increase in Ca^{+2} influx. A key insight into the downstream signalling events occurring after multiple trains of high-frequency stimulation was the demonstration that NMDA receptor activation is coupled to PKA activation (Chetkovich *et al.*, 1991; Chetkovich and Sweatt, 1993; Roberson and Sweatt, 1996).

As seen in *Chapters III, IV, and V*, L-LTP induced by spaced tetraburst stimulation was selectively impaired by pharmacological inhibition or genetic reduction of PKA. In contrast, thetaburst LTP, although sensitive to a PKA inhibitor, was not attenuated in R(AB) mutant mice (*Chapter III*). Because pharmacological inhibitors of PKA are isoform-nonspecific, these results suggest thetaburst stimulation may recruit specific isoforms of PKA that are not affected by the genetic manipulation in R(AB) mice. In the mammalian brain, several different isoforms of the regulatory and catalytic subunits of PKA have been identified (Scott *et al.*, 1987; Clegg *et al.*, 1987; Cadd and McKnight,

1989; see also: Reimann *et al.*, 1971; Uhler *et al.*, 1986a,b). Different PKA holoenzymes formed by these various isoforms have distinct characteristics and functional properties. For instance, different combinations of catalytic subunits can regulate distinct sets of genes (Morris *et al.*, 2002). The findings in *Chapter III* not only support the notion that different isoforms of subunits may underlie different forms of synaptic plasticity but they also indicate that isoform recruitment can be regulated by synaptic activity. However, to identify which isoforms are preferentially recruited after a particular stimulation protocol, future studies will require molecular probes designed to selectively monitor individual isoforms of PKA (Burns-Hamuro *et al.*, 2003).

Stimulation parameters can also regulate the PKA-dependence of L-LTP (*Chapter IV*). Compressed tetraburst stimulation elicited a form of L-LTP that was not impaired by pharmacological inhibition or genetic reduction of PKA activity. These results indicate that signalling cascades other than cAMP/PKA can initiate protein synthesis to support long-lasting LTP. A candidate signalling cascade that may underlie compressed tetraburst LTP is the ERK/MAPK cascade, because this pathway has been previously shown to regulate CREB-mediated gene transcription and LTP expression (English and Sweatt, 1996, 1997; Impey *et al.*, 1998; Roberson *et al.*, 1999; Davis *et al.*, 2000; Kanterewicz *et al.*, 2000). Many other signalling molecules, such as PKC and CaMKIV, have been implicated in protein synthesis dependent forms of plasticity (Osten *et al.*, 1996; Kang *et al.*, 2001). Thus, further studies are required to identify the underlying signalling cascades involved in controlling protein synthesis-dependent types of LTP. Collectively, these findings indicate that particular signalling transduction pathways are tuned to distinct stimulation parameters.

Expression

Several signalling cascades are implicated in the expression of hippocampal synaptic plasticity (Madison *et al.*, 1991; Micheau and Riedel, 1999; Martin *et al.*, 2000; Soderling and Derkach, 2000). One in particular, the cAMP/PKA cascade, is required for the expression of hippocampal L-LTP. Although the precise underlying molecular mechanisms have not been fully elucidated, many downstream effectors of PKA, such as transcription factors and glutamate receptors, are thought to be important mediators of synaptic plasticity (**Figure 1.8A** in *Chapter I*; for review see Nguyen and Woo, 2003). In addition, PKA interacts with several other important signal transduction enzymes, including protein phosphatases.

Protein phosphatases have an important role in forms of plasticity that involve a persistent decrease in synaptic efficacy (Mulkey *et al.*, 1993, 1994; O'Dell and Kandel, 1994). It is increasingly apparent that protein phosphatases play an extensive role in *bidirectional* plasticity (*i.e.* both LTP and LTD) (Winder *et al.*, 1998; Malleret *et al.*, 2001; Zeng *et al.*, 2001; reviewed by Winder and Sweatt, 2001). Calcineurin (PP2B), a calcium-sensitive phosphatase, has been demonstrated to exert an inhibitory constraint on LTP (Ikegami and Inokuchi, 2000; Malleret *et al.*, 2001). Genetic inhibition of calcineurin enhanced LTP induced by only one 100Hz train, and this form of LTP was attenuated by a PKA inhibitor (Malleret *et al.*, 2001). For this reason, calcineurin is thought to regulate the coupling between LTP induction signals and the activation of cAMP/PKA.

Protein phosphatases can also act downstream of PKA to modulate synaptic plasticity. Early studies demonstrated that activation of the cAMP/PKA pathway through dopamine receptors led to phosphorylation of I-1, a potent endogenous inhibitor of PP1

(Hemmings *et al.*, 1984; Surmeier *et al.*, 1995; reviewed by Greengard *et al.*, 1999). This interaction was later shown to be important in regulating E-LTP, because PP1 is known to inhibit its expression (Blitzer *et al.*, 1995; 1998). In the same way, PP1 was also observed to inhibit the expression of L-LTP. *Chapter V* describes pharmacological evidence that PP1 is disinhibited by genetic suppression of PKA activity and is responsible for impairing L-LTP in R(AB) mutant mice. Application of PP1 inhibitors, but not a calcineurin inhibitor, selectively rescued L-LTP in PKA mutant slices. Moreover, PP1 inhibition neither enhanced LTP nor induced LTP in wildtype slices. Thus, these results suggest that an innate function of PKA activation is to remove the inhibitory gating influence of PP1 to allow for the expression of L-LTP. Furthermore, *Chapter VI* shows that application of LFS prior to tetraburst stimulation selectively impaired the expression of L-LTP. This anterograde inhibition of L-LTP was critically dependent on NMDA receptor activation as well as PP1/2A activation. Thus, these collective data show that PP1 acts as an inhibitory constraint on L-LTP, and that genetic reduction of PKA activity or prior low-frequency stimulation impairs L-LTP by enhancing this inhibitory constraint.

The downstream actions of protein phosphatases in regulating L-LTP were not addressed in the present thesis and are largely unknown. However, interestingly, many of the downstream targets of PKA highlighted in **Figure 1.8A** (*Chapter I*) are also regulated by protein phosphatases. CREB, a transcription factor required for L-LTP (Bourtchouladze *et al.*, 1994; Matsushita *et al.*, 2001), is highly sensitive to protein phosphatase activity (Bito *et al.*, 1996). This stems from the observation that CREB phosphorylation is enhanced after inhibition of phosphatases (Hagiwara *et al.*, 1992; Bito *et al.*, 1996). Thus, a downstream effect of increased phosphatase activity may be to prevent or limit CREB

phosphorylation and impair CREB-mediated transcription. Another potential downstream target of phosphatases is the ERK/MAPK pathway. Incubation of hippocampal homogenates with purified PP1/2A decreased ERK immunoreactivity, which suggests that phosphatases may downregulate or suppress this pathway (Norman *et al.*, 2000). Glutamate receptors are also regulated by phosphatases. Both chemically- and electrically-induced LTD is accompanied by dephosphorylation of Ser845 in GluR1, which can be blocked by okadaic acid (Lee *et al.*, 1998, 2000; Snyder *et al.*, 2000). Further studies are required to elucidate which molecular targets of protein phosphatases are critical for regulating L-LTP expression.

Protein Synthesis

It is widely accepted that appropriate patterns of activity at hippocampal synapses induce the synthesis of particular proteins that are critical for establishing enduring modifications (Stanton and Sarvey, 1984; Deadwyler *et al.*, 1987; Frey *et al.*, 1988; Nguyen *et al.*, 1994). These modifications presumably occur at select populations of synapses (synapse specificity) and during a time window that extends for several hours after the triggering event. In agreement, my data demonstrated that expression of L-LTP is synapse-specific (*Chapter VII*) and is attenuated by a protein synthesis inhibitor (*Appendix A*). The identities and functional roles of the newly synthesized proteins required for the maintenance of L-LTP are largely unknown. But described in *Chapter VII*, a novel role for a newly synthesized product of protein synthesis was identified and was demonstrated to confer synaptic immunity (or resistance) to depotentiation. Interestingly, both transcription and local translation are able to confer this immunity to depotentiation.

What is the mechanism of synapse specificity of L-LTP? In an elegant study, it was demonstrated that plasticity related proteins synthesized in response to strong synaptic activity are distributed cell wide and are captured at tagged synapses (Frey and Morris, 1997, 1998). The work in *Chapter VII* also demonstrates this idea of synaptic tagging, where L-LTP induced in one pathway elicited the synthesis of plasticity related proteins that were captured at previously activated (*i.e.* tagged) synapses and conferred immunity to depotentiation.

Chapter VII also implicates a role for local protein synthesis. Tetraburst stimulation conferred immediate synaptic immunity to depotentiation. Application of anisomycin or emetine, both inhibitors of translation, did not affect the *induction* of L-LTP, but enabled persistent depotentiation of tetraburst LTP. In contrast, actinomycin-D, an inhibitor of transcription, did not enable DPT following induction of tetraburst LTP. Furthermore, when I recorded fEPSPs from isolated dendrites, which were severed from the cell bodies, resistance to DPT was still evident. Thus, an essential step for initiating immunity to depotentiation may be the translation of pre-existing mRNA. Although the identity of the protein that confers immunity is unknown, future studies should examine candidate proteins that are rapidly upregulated in an activity-dependent manner. In addition, the synthesis of this candidate protein at the soma may be CREB-mediated as expression of a constitutively active isoform of CREB converted decremental LTP into long-lasting LTP that was also resistant to depotentiation (Barco *et al.*, 2002).

Theoretical Implications of Research Findings

The frequency and intensity of the stimuli used to induce LTP can determine which signalling pathways contribute to the induction and expression of different forms of LTP (Lisman, 1989; Chapman *et al.*, 1995; Cavus and Teyler, 1996; Micheau and Riedel, 1999). For instance, higher stimulation frequencies, such as a 200Hz tetanus, elicit a form of LTP that requires activation of voltage-dependent Ca^{+2} channels (VDCCs), whereas intermediate frequencies, ranging from 50 – 100Hz, elicit an NMDA receptor-dependent form of LTP (Huber *et al.*, 1995; Izumi and Zorumski, 1998). These studies indicate that receptor activation is tuned to optimal frequencies of stimulation. Similarly, biochemical studies and theoretical modeling have demonstrated that the activities of certain kinases can also be tuned to discrete patterns of electrical impulses (Bhalla, 2002a,b; see DeKoninck and Schulman, 1998, for CaMKII data). For example, the total amount of imposed activity (*i.e.* number of pulses) is a key characteristic of stimulation protocols for eliciting a particular form of LTP that is PKA-dependent. LTP induced by 1 train of 100Hz is relatively insensitive to genetic or pharmacological inhibition of PKA (Matthies and Reymann, 1993; Huang and Kandel, 1994; Blitzer *et al.*, 1995; Qi *et al.*, 1996; Abel *et al.*, 1997; Otmakhova *et al.*, 2000; Duffy *et al.*, 2001; Yasuda *et al.*, 2003; Duffy and Nguyen, 2003). However, LTP induced by stronger stimulation, such as 3-4 trains of 100Hz, is attenuated by PKA inhibition, evident as a gradual decay of potentiated synaptic responses to pretetanic levels of synaptic transmission (Huang and Kandel, 1994; Blitzer *et al.*, 1995; Abel *et al.*, 1997; Matsushita *et al.*, 2001; Duffy and Nguyen, 2003).

In my thesis, I demonstrated that the pattern of stimulation influenced the expression of L-LTP in two important ways, by recruiting distinct isoforms of PKA, and

by controlling the PKA-dependence of LTP. Thus, by altering the frequency and amount of synaptic activity, the mammalian brain may differentially regulate expression of long-lasting forms of synaptic plasticity. However, *in vivo* studies should be performed to further identify the behavioral relevance of these patterns of activity imposed on hippocampal slices.

Two other important findings in my thesis direct attention to the regulation of L-LTP expression. Opposing forms of regulation were observed: protein phosphatases inhibited L-LTP expression (Chapter VI), whereas protein synthesis preserved L-LTP (Chapter VII).

Several studies have shown that prior synaptic activity (*i.e.* "activation history" of the synapse) may influence some forms of synaptic plasticity (Huang *et al.*, 1992; Wexler and Stanton, 1993). This activity-dependent modulation of subsequent synaptic plasticity has been termed "metaplasticity" (Abraham and Bear, 1996; Abraham and Tate, 1997). As described in *Chapter VI*, a novel form of metaplasticity that is protein phosphatase-dependent selectively inhibited the expression of L-LTP. This form of metaplasticity may play an important role in the integration of neuronal signals. Synaptic inputs that may be subthreshold for altering synaptic strength may nonetheless alter the capability of neurons to undergo *future* long-lasting changes in synaptic efficacy. In essence, LFS creates a lowpass synaptic filter that only permits the expression of shorter lasting forms of plasticity.

Depotentiation is another form of metaplasticity. Low frequency stimulation (LFS) that does not affect basal synaptic transmission can reverse potentiation back to near baseline levels after LTP induction *in vitro* (Fujii *et al*, 1991; Bashir and Collingridge,

1994; Huang *et al*, 1999) and *in vivo* (Barrionuevo *et al*, 1980; Stäubli and Lynch, 1990). *Chapter VII* demonstrates that the amount of activity used to induce LTP can subsequently influence the susceptibility of synapses to depotentiation. When tetraburst protocols were used to induce LTP, synaptic immunity was rapidly induced, and subsequent expression of depotentiation was prevented. Such immunity served as a "highpass filter" that preserved the expression of long-lasting types of LTP induced by multiple bursts of high-frequency stimulation.

Collectively, these two forms of synaptic filters alter the threshold for the expression of long-lasting forms of synaptic plasticity. Both types of filter may enable neurons to translate different patterns of synaptic activity into distinct type of synaptic modification that can influence neural information processing over extended periods of time. This idea supports the hypothesis that a synapse's threshold for plasticity is not a static property but is highly dependent on its history of previous activity. Because long-lasting forms of synaptic plasticity have been correlated with hippocampus-dependent memory in mice, these activity-dependent filters that regulate the expression of L-LTP may also control the neural threshold for the consolidation of short-term memories into long-term memories.

Conclusion and Future Directions

In conclusion, my data support several distinct mechanisms that regulate the expression of L-LTP (**Figure 8.1**). First, the temporal pattern of stimulation used for L-LTP induction importantly determines which signalling cascades are recruited for its expression. Second, protein phosphatases regulate L-LTP expression by acting as an

inhibitory constraint. This inhibitory constraint can be enhanced by the genetic suppression of the cAMP/PKA cascade or by prior application of low frequency stimulation. Finally, protein synthesis is important for conferring an immunity factor that protects synapses from depotentiation.

In the preceding paragraphs of this discussion, a number of important issues for future exploration were identified and these include:

1) *The identification of specific isoforms of PKA or other signalling cascades underlying L-LTP induced by different patterns of stimulation.*
2) *The molecular downstream targets of protein phosphatases that mediate the inhibitory constraint in the expression of L-LTP.*
3) *The identification of the immunity factor responsible for conferring the resistance to depotentiation.*

To address these issues, investigators will need to use multiple approaches that integrate electrophysiological and biochemical techniques with cell biological, genetic, and imaging technologies (Zhang *et al.*, 2001; Abraham *et al.*, 2002; Trachtenberg *et al.*, 2002; Zaccolo and Pozzan, 2002; Stosiek *et al.*, 2003; Tonegawa *et al.*, 2003). It will also be critical to examine these issues in the context of animal behavior through *in vivo* experimentation (Abraham *et al.*, 2002; Stosiek *et al.*, 2003). Overall, there is much that remains to be accomplished to extend and further develop the current findings. This daunting task, however, should be accelerated by the notion that the importance of long-lasting forms of synaptic plasticity is not limited to long-term memory but applies to a variety of fundamental brain processes (*e.g.* drug addiction and development).

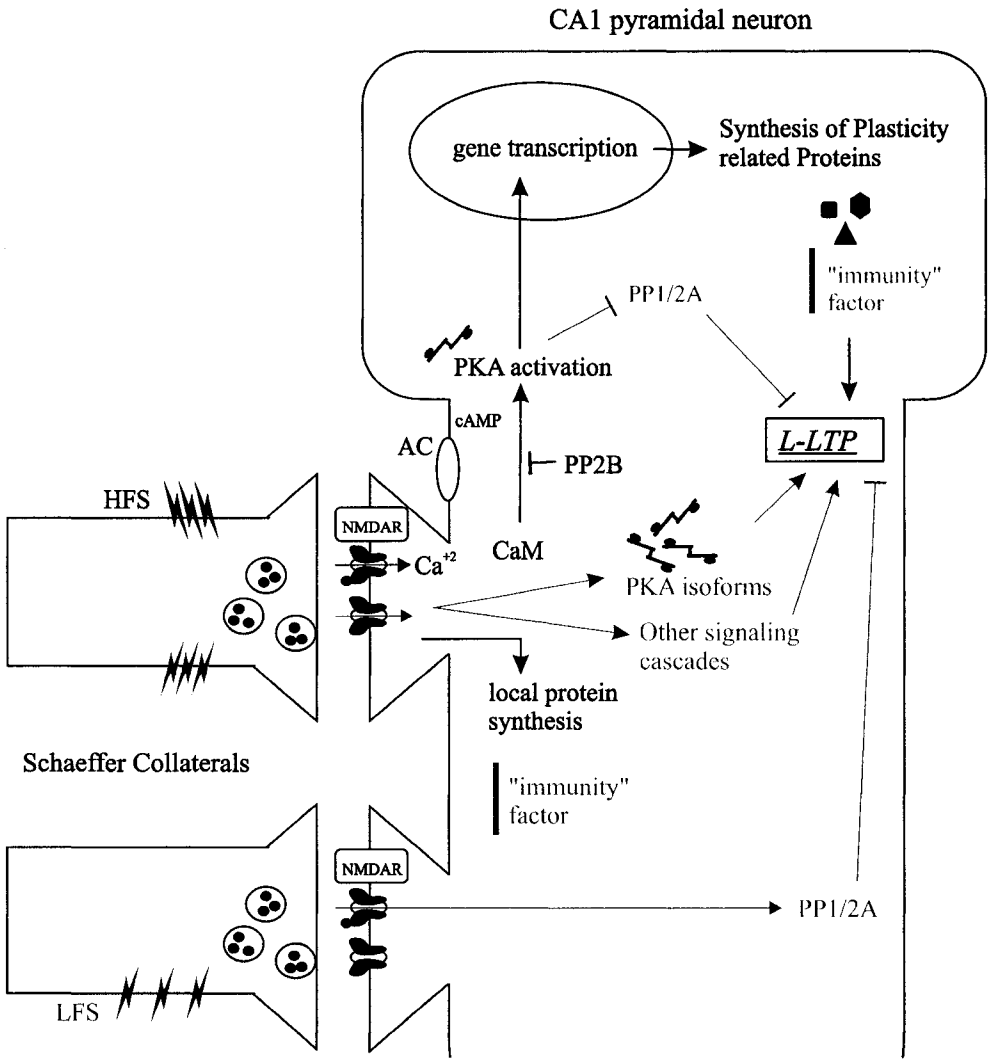

Figure 8.1: Emerging model of L-LTP in the hippocampus: a synthesis of findings. Expression of L-LTP is critically dependent on protein synthesis. One important signalling transduction pathway that initiates gene transcription is the cAMP/PKA pathway. Multiple trains of HFS causes a large influx of Ca^{+2}, which then recruits an adenylyl cyclase (AC). cAMP production allows for the catalytic subunit of PKA to dissociate and become activated. Downstream of PKA, protein phosphatases were identified to be an important regulator of L-LTP expression. These phosphatases can be activated by LFS and act as an inhibitory constraint to the expression of L-LTP. Another finding was that the stimulation parameter of a tetanus protocol, can differentially recruit different isoforms of PKA and/or other signalling cascades to mediate the expression of L-LTP. Many of the protocols used to elicit L-LTP can also mediate synaptic immunity, which serves to preserve L-LTP expression. Amalgamation of previous (shown in black) and present findings (shown in red).

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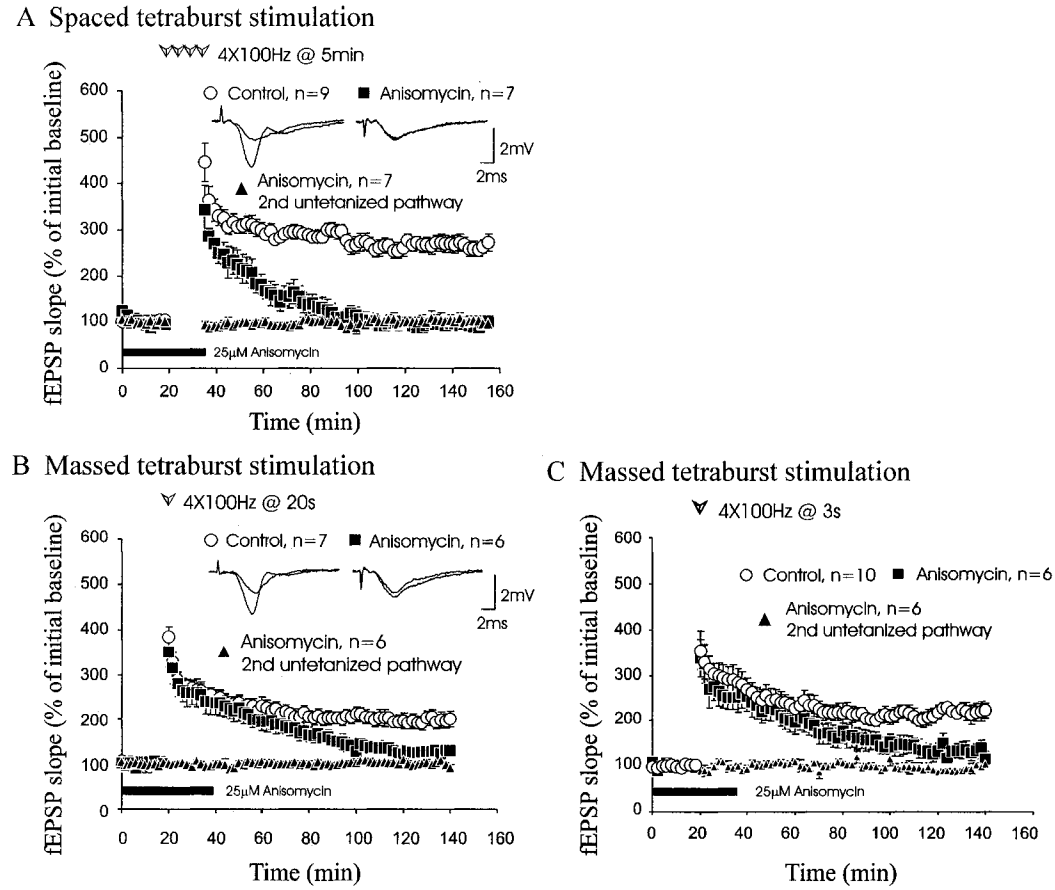
APPENDIX A



Appendix A: Long-term potentiation (LTP) following spaced tetraburst stimulation is more sensitive to anisomycin than LTP following massed tetraburst stimulation. **(A)** Spaced tetanic stimulation induced robust LTP that was attenuated by anisomycin. Slices were stimulated using four trains of 100Hz (1s duration) at 5min intervals. Anisomycin substantially impaired LTP, with mean fEPSPs reaching pre-tetanus baseline values within 1hr after HFS. Anisomycin did not affect a 2nd adjacent pathway that did not experience tetraburst stimulation. **(B & C)** Massed tetanic stimulation induced LTP that was also impaired by anisomycin. Slices were stimulated using four trains of 100Hz at 20s **(B)** or 3s **(C)** interburst intervals. In summary, both spaced stimulation and massed stimulation elicited LTP that was dependent on protein synthesis. However, the time courses of protein synthesis dependence were different between groups, as the decay of LTP in the presence of anisomycin was much slower in the massed group.

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*A version of this Figure (Parts A & B) has been previously published in:

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