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CRITICAL ROLE FOR PKA IN THE INTEGRATION AND REGULATION OF MULTIPLE SYNAPTIC EVENTS IN HIPPOCAMPAL CA1

by

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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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Slartibartfast coughed politely.

'...These creatures you call mice, you see, they are not quite as they appear. They are merely the protrusion into our dimension of vast hyper-intelligent pan-dimensional beings. The whole business with the cheese and the squeaking is just a front.'

The old man paused, and with a sympathetic frown continued.

'They've been experimenting on you, I'm afraid.'

Arthur thought about this for a moment, and then his face cleared.

'Ah no,' he said, 'I see the source of the misunderstanding now. No, look you see, what happened was that we used to do experiments on *them*. They were often used in behavioural research, Pavlov and all that sort of stuff. So what happened was that the mice would be set all sorts of tests, learning to ring bells, run round mazes and things so that the whole nature of the learning process could be examined. From our observations of their behaviour we were able to learn all sorts of things about our own...'

Arthur's voice tailed off.

'Such subtlety...' said Slartibartfast, ' one has to admire it.'

The Hitchhiker's Guide to the Galaxy
Douglas Adams

Dedicated to the memory of Sau-Ling Young

ABSTRACT

Long-term potentiation (LTP) is an enduring activity-dependent enhancement of synaptic transmission that may contribute to information storage in the mammalian brain. In the hippocampus, these processes may play a critical role in certain types of learning and memory. Long-lasting LTP (L-LTP) in mouse hippocampal area CA1 requires cAMP-dependent protein kinase (PKA) activity, transcription and translation. L-LTP is also input specific – a critical aspect that allows the synapse, rather than individual neurons, to be the unit of information storage, thus significantly increasing the potential storage capacity of the brain. The "synaptic tag" model proposes that gene products can only be captured and utilized by synapses that have been "tagged" by previous activity. My research elucidates how the history of synaptic activity can affect synaptic tagging and the integration of different synaptic events over time.

L-LTP expression can be regulated by the history of activity at synapses, a process known as "metaplasticity". The work presented here describes a novel form of metaplasticity whereby low-frequency stimulation (LFS) decreases the stability of L-LTP induced later at the same synapses (homosynaptic inhibition) and at other synapses converging on the same postsynaptic cells (heterosynaptic inhibition). The phosphatase inhibitor okadaic acid blocked homosynaptic and heterosynaptic inhibition of L-LTP by prior application of LFS. Additionally, prior LFS impaired chemical facilitation by forskolin/IMBX in a homosynaptic and heterosynaptic manner, suggesting that a cell-wide dampening of cAMP/PKA signaling occurs concurrent with phosphatase activation. I propose that prior LFS impairs expression of L-LTP by regulating synaptic tagging through its actions on the cAMP/PKA pathway. I show that synaptic capture of L-LTP

gene products is impaired by electrical (i.e., LFS-mediated), pharmacological, or genetic downregulation of PKA signaling. Moreover, pharmacological activation of the cAMP/PKA pathway can produce a synaptic tag that captures L-LTP gene products and results in persistent synaptic facilitation.

Collectively, my results suggest that PKA plays a critical role in synaptic tagging and in synapse-specific long-lasting potentiation in CA1 pyramidal neurons. PKA-mediated signaling can be constrained by prior episodes of synaptic activity to regulate subsequent L-LTP expression and the integration of many synaptic events over time.

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1X	1 x 100-Hz trains
4X@3s	4 x 100-Hz trains with 3 second intertrain interval
4X@5min	4 x 100-Hz trains with 5 minute intertrain interval
5-HT	serotonin
5-Hz	stimulation at 5-Hz for 3 minutes
AC	adenylyl cyclase
ACSF	artificial cerebrospinal fluid
Act D	actinomycin D
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (receptor)
ANOVA	analysis of variance between groups
BCM theory	Bienenstock, Cooper, and Monro theory
BDNF	brain-derived neurotrophic factor
C	catalytic subunit of PKA
CA	cornu ammonis
Ca^{2+}	calcium
CaM	calmodulin
CaMKII	calcium/calmodulin-dependent protein kinase II
CaMKIV	calcium/calmodulin-dependent protein kinase IV
cAMP	adenosine 3',5'-cyclic monophosphate
CCAC	Canadian Council on Animal Care
CRE	cyclic AMP response element
CREB	cyclic AMP response element binding protein
CS	conditioned stimulus
DAG	diacylglycerol
DG	dentate gyrus
DMSO	dimethylsulfoxide
Dpt	depotentiation
E-LTD	early phase of long-term depression
E-LTP	early phase of long-term potentiation
fEPSP	field excitatory postsynaptic potential
FSK	forskolin
fyn	non-receptor tyrosine kinase
glu	glutamate
GluR	glutamate receptor
gly	glycine
G-protein	guanine nucleotide-binding regulatory protein
Het	heterosynaptic
HFS	high-frequency stimulation
Hom	homosynaptic
I-1	inhibitor-1
IACUC	Institutional Animal Care and Use Committee
IBMX	3-isobutyl-1-methylxanthine
iPPF	interpathway paired-pulse facilitation
K^+ channel	potassium channel

K^+	potassium
KT	KT-5720
LA	lateral nucleus of amygdala
LFS	low-frequency stimulation
L-LTD	late phase of long-term depression
L-LTP	late phase of long-term potentiation
LTD	long-term depression
LTF	long-term facilitation
LTM	long-term memory
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
Mg^{2+}	magnesium
mRNA	messenger ribonucleic acid
msec	milliseconds
mV	millivolts
Na^+	sodium
Nif	nifedipine
NMDA	N-methyl-D-aspartate (receptor)
OA	sodium okadaic acid
PKA	cAMP-dependent protein kinase/protein kinase A
PKC	protein kinase C
PKM(ζ)	protein kinase M-zeta
PLC	phospholipase C
PP	protein phosphatases
PP1/PP2A	protein phosphatase 1 / 2A
PP2B	protein phosphatase 2B/calcineurin
PPF	paired-pulse facilitation
PRPs	plasticity-related proteins
PTP	post-tetanic potentiation
R	regulatory subunity of PKA
R(AB)	dominant negative inhibitory of PKA
S	subiculum
S1	stimulating electrode #1
S2	stimulating electrode #2
SEM	standard error of the mean
STF	short-term facilitation
TG	transgenic
US	unconditioned stimulus
VGCC	voltage-gated calcium channel
WT	wildtype

CHAPTER 1:

GENERAL INTRODUCTION

LEARNING AND MEMORY

Localization and Specification of Memory

The study of learning and memory encompasses many different fields of study, and utilizes a range of approaches. Indeed, until the beginning of the nineteenth century, the study of normal mental activity was largely philosophical. In the early 1800's, Franz Joseph Gall attempted to attribute specific aspects of mental function and personality to specialized brain regions that he believed were reflected in the size of the overlying skull area. Gall's attempts to differentiate cognitive functions across brain structures would prove to be prescient, and although flawed, his methodology represented a major departure from philosophy and movement towards biology. In the latter half of the 19th century, more compelling evidence for the compartmentalization of mental functions came with case studies reported by Paul Broca (1861, 1861A) and Carl Wernicke (1894) of patients with selective impairments in speech production and speech comprehension, respectively. In both cases, dysfunction could be ascribed to selective damage to specific cortical areas. However, the search for a specific brain area responsible for learning and memory remained elusive, and as recent as 1929, Karl Lashley's work suggested that memory could not be localized to specific structures (Lashley, 1929).

A breakthrough came in the 1950's through the study of patients who had undergone surgery to alleviate seizures related to epilepsy. Due its epileptogenic qualities, surgery to remove parts of the medial temporal lobe area (temporal lobectomy) was carried out to alleviate stubborn forms of epilepsy that could not be controlled by anticonvulsant drugs. At the Montreal Neurological Institute, unilateral removal of the

temporal lobe was carried out to ameliorate seizures and this was usually accompanied by mild mental deficits. However, in a few cases, notably patient P.B., a severe and persistent memory deficit resulted. P.B.'s case is significant because his surgery occurred in two phases, and it was only after the second surgery, which included removal of the hippocampal formation, that he developed extreme forgetfulness (amnesia). It was proposed that pre-existing damage to the medial temporal lobe in the opposite hemisphere led to a loss of bilateral functionality after surgery (Milner and Penfield, 1955). Post-mortem examination of P.B. confirmed this hypothesis and focused attention on one particular medial temporal lobe structure. In the non-surgical hemisphere, P.B. showed extensive atrophy in the hippocampus. In contrast, other medial temporal lobe structures, such as the amygdala, parahippocampal gyrus, and entorhinal cortex, remained intact (Penfield and Mathieson, 1974).

P.B.'s case study brought attention to the role of the hippocampus and medial temporal lobe structures in memory and led to the discovery of more patients with similar memory impairments. Amnesic patient H.M. has been famously studied for the past five decades following bilateral removal of parts of the medial temporal lobe, including the hippocampus, to relieve epilepsy (Scoville, 1954; Scoville and Milner, 1957; Milner et al., 1968; Corkin, 1984; Corkin et al., 1997; reviewed in Corkin, 2002). Patient R.B. developed similar memory impairments following an ischemic episode and post-mortem histological analysis confirmed bilateral damage restricted to the CA1 subfield of both hippocampi (Zola-Morgan, et al., 1986). Clinical cases like P.B., H.M., and R.B. are all remarkable in the specificity of memory impairment with otherwise spared intellectual

function. Moreover, it became apparent that amnesia arising from medial temporal lobe damage is not global, but is selective to particular domains of memory.

Multiple Memory Systems

Extensive neuropsychological analyses of these clinical cases of temporal lobe amnesia revealed that memory is not unitary, but rather is composed of distinct types mediated by separate brain systems (Cohen and Squire, 1980; Mishkin et al., 1984; Squire and Zola-Morgan, 1988; for review, see Squire LR, 2004; **Figure 1.01**). H.M. and other patients with hippocampal damage show a selective and pervasive memory deficit for declarative (also known as explicit) memory (Corkin, 1965; Penfield and Mathieson, 1974; Zola-Morgan, 1986). This type of memory requires conscious recollection and includes episodic memory (memory for events within a specific spatial and temporal context) and semantic memory (general knowledge of objects, facts, and concepts; Tulving, 1972). In characterizing the extent of H.M.'s amnesia, Milner and her colleagues (1968; reviewed in Corkin, 2002) also discovered that some aspects of memory were spared. It has subsequently been shown that the spared memory capabilities of H.M. generally include what is now known as non-declarative (implicit) memory. Non-declarative memory differs from declarative memories in that they do not require conscious recollection. It includes habits, perceptual learning, classical conditioning, and non-associative learning such as habituation and sensitization (**Figure 1.01**). For example, H.M. can successfully learn new motor skills, such as those required for a mirror-drawing task, and remains proficient over a year while retaining absolutely no recollection that he has ever performed that task (Gabrieli et al., 1993). These intact

forms of learning and memory demonstrated the existence of parallel and independent memory systems.

Animal Models of Hippocampal Function

Soon after publication of case studies like H.M., efforts began to develop animal models in which the amnesic syndrome might be examined. However, declarative memories are those that require *conscious* recollection. Because animals cannot explicitly report their experiences, how can one examine conscious recollection in animals? Early attempts to apply the concept of declarative memory to experimental animals highlighted inadequacies in defining human amnesia. For example, another temporal lobe amnesia patient, E.P., learned over many trials to react correctly to test materials in preparation for a task without instruction and successfully acquired factual information (Stefanacci et al., 2000; Bayley and Squire, 2002). However, closer examination revealed that these cases were not examples of episodic and semantic memory in the strictest sense. Significantly, these changes in behaviour remain distinctive from true declarative memories in that E.P. has no conscious recollection of how he knows what to do with test materials nor confidence that his answers are correct in the semantic task. In addition, changing test conditions or replacing words with a synonym in the sentences E.P. had memorized negated any evidence of memory (Stefanacci et al., 2000; Bayley and Squire, 2002). Thus, E.P.'s memories differ critically from declarative memories in that they are rigidly organized, and unavailable as conscious knowledge. It is believed that repeated exposure to the same material over many trials allowed for gradual acquisition of knowledge outside of awareness in a

process more reminiscent of non-declarative habit learning (Stefanacci et al., 2000; Bayley and Squire, 2002). These show that different strategies can be recruited for the same learning problem, and that remaining memory systems can compensate for declarative memory impairments to some extent. By extending the characteristics of declarative memory to include flexibility in its expression and the ability to use it inferentially in novel situations, a more accurate concept of declarative memory might be modeled in animals (Cohen, 1984; Eichenbaum, 1997).

Primates

The development of the delayed nonmatch to sample (DNMS) and other object discrimination tasks established a way to assess declarative memory in animals (Zola-Morgan et al., 1982; Zola-Morgan and Squire, 1990). Studies of human case studies are often limited by the diffuse nature of lesions that develop as a result of surgery, accident, or disease. The versatility of animal models and application of restricted lesions offered a way to clarify the contribution of various brain structures to explicit memory formation and led to the development of an animal model of amnesia in monkeys (Zola-Morgan and Squire, 1990A).

In combination with clinical case studies of patients such as H.M., studies in non-human primates have revealed that the hippocampus is a critical locus responsible for declarative memory. Selective ablation of various medial temporal lobe structures, such as the amygdala, and surrounding cortical regions, do not result in type of amnesia similar to that seen in human case studies (Zola-Morgan et al., 1982, 1989; Squire and Zola-Morgan, 1991). In addition, these studies suggest that the process of acquiring new

declarative memories can be broken down into four steps. (1) Encoding: newly learned information is attended to and processed (e.g., associated in a meaningful and systematic way with previously acquired knowledge). (2) Consolidation: conversion of newly stored, labile information into a more stable representation for long-term storage. (3) Storage: mechanisms in the sites where memory is retained over time. (4) Retrieval: processes that allow for the recall and use of stored information. Bilateral damage to the hippocampi selectively impairs consolidation of new long-term declarative memories. Encoding episodic information does not require hippocampal function, as both humans and primates with hippocampal damage have intact working memory (Scoville and Milner, 1957; Alvarez et al., 1994). In addition, the hippocampus is not the final repository for memory traces (Mishkin et al., 1982; Squire et al., 1984). Although there may be some retrograde amnesia that extends pre-morbidly, remote memories are generally spared after hippocampal damage (Scoville and Milner, 1957; Zola-Morgan and Squire, 1990; Corkin, 2002; Bayley et al., 2003).

Rodents

Initial studies of hippocampal function in rodents focused on maze learning tasks, and it was proposed that the hippocampus is critically important in spatial learning. The discovery of place cells – neurons in the hippocampus that selectively increase firing when an animal is in a particular location within its environment – supported the hypothesis that the hippocampus is involved in the formation of "cognitive maps" that represent spatial information in the animal's environment (O'Keefe and Dostrovsky, 1971; O'Keefe, 1976). Moreover, hippocampal lesions impair many forms of spatial

learning as assessed by a variety of behavioural tests. In the Morris water maze, a rodent learns the location of a platform hidden in opaque water based on specific spatial cues placed outside the maze (Morris et al., 1982; Morris, 1984). Hippocampal lesions or pharmacological interruption of hippocampal function confirmed a critical role of the hippocampus in consolidating long-term spatial memories (Morris et al., 1982; Davis et al., 1992; Riedel et al., 1999). Other hippocampus-dependent tasks include contextual fear conditioning, in which an animal is trained to associate a noxious stimulus (e.g., foot shock) with the training environment (Kim and Fanselow, 1992; Phillips and Ledoux, 1992). During contextual fear conditioning, the animal acquires knowledge about the spatial context of the task as a whole, instead of individual spatial cues (e.g., Morris water maze).

The identification of place cells in the hippocampus suggested a special role for the hippocampus in spatial mapping. Subsequent studies have shown that the hippocampus is critically involved in encoding both spatial and non-spatial information (Wood et al., 1999, 2000), and a wide range of non-spatial behaviours that are mediated by the hippocampus have also been identified. Social transmission of food preference is a non-spatial, hippocampus-dependent form of social learning in which animals develop a preference for a particular type of food based on their interactive experience with a demonstrator animal that had previously eaten that food (Winocur, 1990; Bunsey and Eichenbaum, 1995). In separate studies taking advantage of the rodent's keen sense of smell, rats learned overlapping sets of associations between odour stimuli. Following training (e.g., odour A is paired with odour B, and odour B is paired with odour C) rats were tested for inferential judgement (e.g., by extension, odour A is related to odour C,

but not to a novel odour X). Although animals with hippocampal damage can successfully acquire the sets of associations with many repeated training sessions, they are significantly impaired in the portion of the task that requires transitive inference (Bunsey and Eichenbaum, 1996; Fortin et al., 2002). These results support the view that rodents have "episodic" memory that guides the expression of behaviour in novel ways that do not merely mirror previously learned behaviour (Eichenbaum, 1997, 2000; Morris, 2001). Indeed, evidence of "explicit" memory in rodents has been reported as early as the 1930s when it was demonstrated that rats could infer detours and shortcuts in a maze-learning paradigm (Tolman, 1932).

The Hippocampus and the Medial Temporal Lobe Memory System

The systematic study of the different components of the medial temporal lobe memory system in animals coupled with comparisons to human clinical case studies and neuroimaging of normal individuals have presented a more complete picture of the role the hippocampus plays in memory (Squire and Zola-Morgan, 1991; Mishkin et al., 1998; Tulving, 1998; Cohen et al., 1999; Eichenbaum, 2004; Squire, 2004). In humans, the role of the hippocampus includes the capacity for conscious recollection of facts (semantic memory) and events (episodic memory). Similarly, in animals, the hippocampus is required for a broad range of spatial and non-spatial tasks. Across species, hippocampus-dependent types of memory have a flexible representation of relationships among multiple items and events. This allows the subject to compare and contrast acquired knowledge, and to express this knowledge in a flexible way to guide their performance (Morris RG, 2001; Clayton et al., 2003; Eichenbaum, 1997, 1999, 2003; Squire, 2004). It

has also become apparent that although the hippocampus is critical in the initial storage of declarative memory, it is also part of a larger neural system working in concert to encode, consolidate, store, and mediate retrieval of memory representations of previous experiences and knowledge (Cohen et al., 1999; Eichenbaum2000; Squire, 2004; **Figure 1.02**). Although selective hippocampal damage produces marked memory impairments, damage to the many medial temporal lobe regions including the hippocampus results in significantly more severe amnesia (Alvarez et al., 1995; Reed and Squire, 1998).

The hippocampus is critically involved in the formation of new long-term declarative memories but many events that the hippocampus encodes into memory cannot be anticipated, and may only occur once. Thus, it has been proposed that the hippocampus engages in moment-to-moment processing of the outside world and rapidly encodes these episodes in real time, as they occur, in a process termed the "automatic recording of attended experience" (Morris and Frey, 1997; Wood et al., 1999). Rapid relational representations are formed among elements of experience in the hippocampus to produce a cohesive percept of ongoing experience. Moreover, these representations are formed in a labile manner, such that episodes can be linked by common features to allow for flexible inferential memory expression (Morris and Frey 1997; Morris, 2001; Eichenbaum et al., 1999; Shimamura, 2002; Schendan et al., 2003; Ergorul and Eichenbaum, 2004). Indeed, the anatomical location of the hippocampus at the apex of a cortical processing hierarchy makes it well suited to this task. The hippocampus receives extensive inputs from the unimodal and polymodal association areas through the entorhinal cortex to integrate cortical processing streams from various sensory modalities (Squire and Zola, 1996; Mishkin et al., 1998; **Figure 1.02**). However, the hippocampus

is not the final storage place for memories (Squire et al., 1984; Bayley et al., 2003). Information flow also occurs in the reverse direction, from the hippocampus back to these same neocortical areas (Squire and Zola, 1996). Continued interactions between the hippocampus and cortical regions lead to more permanent memory traces that are stored in the neocortex (Squire and Alvarez, 1995; Buzsáki, 1996; Bontempi et al., 1999), a hypothesis that is consistent with Karl Lashley's earlier findings that memory impairments are better correlated with the size of cortical lesions, rather than the site of lesioning (Lashley, 1929).

CELLULAR AND MOLECULAR BASIS OF LEARNING AND MEMORY

The late 1800's saw the development of the "neuron doctrine" and recognition that neurons are the primary signaling unit of the nervous system. In parallel with these anatomical discoveries came the proposal that the neural substrate of information storage in the brain, including learning and memory, may reside in the ability of neurons to undergo long-term changes in strength and number (Ramón y Cajal, 1893; Tanzi, 1893). In 1949, Donald Hebb put forth his famous and much quoted postulate:

> "When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased." pg. 62

Today, it is generally accepted that information storage and processing in neurons occur through long-term changes in the strength (i.e., *synaptic plasticity*) and number (i.e., *structural plasticity*) of their connections. Activity-dependent long-term synaptic plasticity has been most extensively studied in the marine snail *Aplysia* and in the mammalian hippocampus.

EXPERIENCE-DEPENDENT PLASTICITY IN AN INVERTEBRATE SYSTEM

Non-Declarative Learning and Memory in *Aplysia*

Use-dependent changes in synaptic strength were first proposed as a cellular mechanism for learning and memory over a hundred years ago (Ramón y Cajal, 1893; Tansy, 1893). However, the large number of neurons and complex interconnections in the mammalian nervous system made detailed cellular analysis very difficult. Invertebrate systems offer major technical advantages over more complex mammalian systems due to (1) smaller number of cells; (2) larger cells than those in mammalian systems; and (3) easily identifiable and invariantly located cell types.

Eric Kandel and his colleagues successfully used this reductionist approach in their pioneering behavioural, physiological, and molecular studies of the gill-withdrawal reflex in the marine snail, *Aplysia* (Kandel and Tauc, 1965; Pinsker et al., 1973; **Figure 1.03**). The gill-withdrawal reflex is part of the normal behavioural repertoire of the animal that allows it to rapidly withdraw its delicate gills and siphon under a protective mantle shelf in reaction to potentially harmful stimuli in the environment. In the laboratory, this defensive reaction can be triggered by lightly stroking the siphon with a

paintbrush. The gill-withdrawal reflex can be modified by experience to produce a smaller (habituation) or larger (sensitization) behavioural response (Carew et al., 1972; Pinsker et al., 1973). With repeated benign stimulation of the siphon, the withdrawal reflex decreases over time as the animal learns to ignore the harmless stimuli. On the other hand, noxious stimulation (e.g. electric shock to the tail) results in a period of increased sensitivity to previously neutral stimulation such that benign siphon stimulation produces a more vigorous (e.g., increased number and duration) gill-withdrawal response. With repeated stimulation, the memory for short-term habituation and sensitization of the withdrawal reflex can be extended into long-term memory that lasts for days and even weeks (Carew et al., 1972; Pinsker et al., 1973).

At the cellular level, these two types of non-associative learning are mediated by an increase (sensitization) or decrease (habituation) in the strength of synaptic transmission at the nerve cells that mediate this reflex (Castellucci et al., 1976, 1978). The basic circuitry includes sensory neurons that receive input from the siphon skin and synapse onto motor neurons that innervate the gill and siphon musculature. Sensory neurons also form synapses with various inhibitory and excitatory interneurons that make up a polysynaptic component of the circuit between sensory input and motor output (Kupfermann and Kandel, 1969; **Figure 1.03B**).

Synaptic Plasticity in *Aplysia*

During short-term sensitization, noxious stimulation to the tail results in activation of serotonergic facilitating interneurons. These interneurons form axo-axonic synapses with siphon sensory neurons to enhance transmitter release from presynaptic

sensory neurons (Mackey et al., 1989; Glanzman et al., 1989). As a result of this heterosynaptic facilitation, subsequent siphon stimulation elicits a larger postsynaptic potential in the motor neuron and produces an enhanced withdrawal response (Kandel and Tauc, 1965). Due to the relatively large size of *Aplysia* neurons, pharmacological agents can be directly injected into the presynaptic sensory neuron. These studies provided the first clues as to how molecular signals can lead to synaptic modifications and underlie changes in behaviour (Brunelli et al., 1976). Studies of the sensorimotor synapse in vivo and in semi-intact preparations have also been complemented by the development of cell culture techniques to reconstitute in vitro the three-cell (one sensory neuron, one motor neuron, one interneuron) elementary gill-withdrawal circuit (Montarolo et al., 1986; Rayport and Schacher, 1986).

Heterosynaptic facilitation of synaptic transmission at the sensorimotor synapse is initiated with the release of serotonin (5-HT) by facilitating (heterosynaptic) interneurons (Mackey et al., 1989; Glanzman et al., 1989). In the cell culture model, application of serotonin to the sensory neuron mimics the response of facilitating interneurons to noxious stimulation (Montarorlo et al., 1986; Rayport and Schacher, 1986). Serotonin receptors on the presynaptic sensory neuron are coupled to G-proteins and second messenger signaling cascades, including cAMP and phospholipase C (PLC). Activation leads to the recruitment of cAMP-dependent protein kinase (PKA) and protein kinase C (PKC), respectively (Brunelli et al., 1976; Castellucci et al., 1980; Castellucci et al., 1982; Braha et al., 1990; Sacktor and Schwartz, 1990). PKA and PKC act in concert to: (1) mobilize neurotransmitter vesicles to the active zone for release; (2) decrease potassium conductance to prolong action potentials; and (3) enhance Ca^{2+} conductance

through voltage-gated calcium channels to facilitate Ca^{2+}-dependent transmitter release (Castellucci et al., 1980; Klein and Kandel, 1980; **Figure 1.03C, Arabic numerals**). Together, activation of these biochemical pathways enhances the effects of presynaptic action potential firing and facilitates neurotransmitter release by the sensory neuron.

Long-term sensitization of the gill-withdrawal reflex occurs after multiple exposures of the animal to noxious stimuli (Pinsker et al., 1973). Similarly, repeated application of 5-HT to the sensory neuron in the reduced preparation results in long-term facilitation of the sensorimotor synapse (Montarolo et al., 1986). The loci for short-term and long-term changes at the sensorimotor synapse involve similar biophysical changes that result in enhanced transmitter release from the sensory neuron (Frost et al., 1985; Scholz and Byrne, 1987). Unlike short-term changes, long-term behavioural and synaptic plasticity (1) is supported by persistent protein kinase activity (Greenberg et al., 1987; Sweatt et al., 1989; Sossin et al., 1994); (2) requires de novo protein synthesis and changes in gene expression (Schwartz et al., 1971; Montarolo et al., 1986; Castellucci et al., 1986, 1989); and (3) results in structural changes to the sensorimotor synapse (**Figure 1.03C, Roman numerals**; Bailey and Chen, 1983, 1988, 1988A).

With repeated activation of the 5-HT signalling cascade, such as during long-term sensitization, mitogen-activated protein kinases (MAPK) are recruited by PKA activity and work in concert to regulate gene expression from cAMP-response element binding protein (CRE-binding protein; CREB) transcription factors (Martin et al., 1997; Michael et al., 1998; Müller and Carew, 1998). MAPK removes inhibitory constraints on transcription by phosphorylating CREB-2, while PKA-dependent phosphorylation of CREB-1 allows for CRE-driven gene expression (Schacher et al., 1988; Kaang et al.,

1993; Martin et al., 1997; see Abel et al., 1998 for review). Long-term facilitation-associated gene products include ubiquitin hydrolase whose activation results in the breakdown of PKA regulatory subunits to produce persistent PKA activity at basal cAMP levels (Hegde et al., 1993, 1997; Chain et al., 1995, 1999). Activity-dependent regulation of transcription and translation also results in lasting structural changes to the sensorimotor synapse that parallel the time course of behavioural changes (Bailey and Chen, 1989, 1992; Kim et al., 2003; reviewed by Bailey et al., 2004). Changes in the surface expression of cell adhesion molecules (CAMs) and reorganization of cytoskeletal elements, such as actin, have been implicated in the structural remodelling and growth of new synaptic connections (Mayford et al., 1992; Michael et al., 1998; Hatada et al., 2000).

Aplysia has been a good system for the study of learning and memory because the neuronal circuitry underlying behaviour has been well characterized with a relatively straightforward mapping of learned behaviour onto synaptic change. The integration of molecular, cellular and systems physiology in studying the neurobiology of learning and memory in *Aplysia* paved the way for multidisciplinary approaches to studying memory in more complex mammalian systems. Significantly, many emerging principles of experience-dependent synaptic plasticity in *Aplysia* would prove to be conserved in the mammalian hippocampus.

HIPPOCAMPAL SYNAPTIC PLASTICITY

Long-term Potentiation

In the mid-1960's and early 1970's, long-lasting activity-dependent changes in synaptic efficacy in a mammalian system were finally discovered. Brief tetanic (high frequency) stimulation produced a long-lasting potentiation of synaptic efficacy. Moreover, this persistent enhancement of synaptic transmission, or long-term potentiation (LTP), was discovered in the hippocampus (Lømo, 1966; Bliss and Lømo, 1973) – the brain structure that only ten years prior had been shown to be critical in declarative learning and memory (Scoville and Milner, 1957). The hippocampus was chosen for study due to the laminar organization of neurons and inputs that allowed for in vivo extracellular recordings of synaptic responses (Lømo T, 1966; for review, see Johnston and Wu, 1995). This layered arrangement of the hippocampus would also facilitate the development of the in vitro slice preparation that is commonly used today (Schwartzkroin and Wester, 1975; Andersen et al., 1977; **Figures 1.04 and 1.05**).

A defining property of LTP is that it is rapidly induced by brief episodes of activity, and produces lasting effects for much longer than the initial synaptic activity. Indeed, this significance was not lost on the authors of the original LTP paper (Bliss and Lømo, 1973) as they stated at the end of the discussion:

> "The interest of these results derives both from the prolonged duration of the effect...a time scale long enough to be potentially useful for information storage" and "...from the fact that an identifiable cortical

pathway is involved... [in] a region of the brain which has been much discussed in connexion with learning and memory." pg. 355

Other forms of short-term synaptic plasticity, such as post-tetanic potentiation (PTP), had previously been described, but PTP decays within minutes of induction (Lloyd, 1949). In contrast, LTP can last for several hours in vitro (Andersen et al., 1977; Reymann et al., 1985; Huang and Kandel, 1994), and LTP induced in vivo can last for many weeks (Bliss and Gardner-Medwin, 1973; Douglas and Goddard, 1975), and even up to a year (Abraham et al., 2002). This key characteristic makes LTP a potentially useful mechanism for information storage at the cellular level and in accord, the persistence of LTP has been statistically correlated with the degree of retention of spatial memory over time (Barnes, 1979; Barnes and McNaughton, 1985).

Further investigation found that LTP fits well with many aspects of Hebb's "neurophysiological hypothesis" regarding the cellular basis of learning and memory (**Figure 1.06**). For example, LTP induction requires cooperativity –there is a threshold of stimulus strength or frequency of stimulation that must be reached for LTP induction to occur. Cooperativity reflects a need for the synchronous activation of a crucial number of presynaptic fibres to sufficiently depolarize the postsynaptic neuron and elicit LTP (Bliss and Lømo, 1973; Bliss and Gardner-Medwin, 1973; McNaughton et al., 1978; Malenka, 1991). This co-activation of presynaptic and postsynaptic activity is an integral part of Hebb's postulate (1949) and also predicts that LTP can be generated in an associative manner (i.e., presynaptic activity "enough to excite a cell...or persistently takes part in firing it" should exhibit synaptic plasticity). Indeed, LTP can be elicited with sub-

threshold stimulation at one set of inputs if it is temporally concurrent with LTP-inducing stimuli at another set of synapses on the same cell (Levy and Steward, 1979; Barrionuevo and Brown, 1983). This property of LTP makes it an attractive mechanism for associating separate pieces of information in one postsynaptic cell, in effect a cellular analogue for classical conditioning.

Lastly, LTP is also characterized by its input specificity - LTP is only expressed at stimulated synapses, and not at convergent inputs that were not active during LTP induction (Bliss and Lømo, 1973; Lynch et al., 1977; Andersen et al., 1977; **Figure 1.06C**). CA3 neurons form multiple *en passant* synapses with CA1 dendrites and each CA1 neuron is innervated by multiple CA3 afferents (Ishizuka et al., 1990; Sorra and Harris, 1993). Input specificity maintains the fidelity of the information that is being encoded by limiting plasticity to activated synapses. In addition, this critical aspect of LTP allows the synapse, rather than individual neurons, to be the unit of information storage, thus significantly increasing the potential storage capacity of the brain (Bliss and Collingridge, 1993).

Long-term Depression

If synapse strength can only increase, all synapses will eventually saturate and no information will be stored. In 1973, Gunther Stent proposed the following complementary postulate to Hebb's (1949):

> "When the presynaptic axon of a cell A repeatedly and persistently fails to excite the postsynaptic cell B while cell B is firing under the

> influence of other presynaptic axons, metabolic change takes place in one of both cells such that A's efficiency, as one of the cells firing B, is decreased." pg. 997

In other words, when activity in one set of inputs occurs, inactivity at other convergent inputs will result in synaptic depression at those connections. Indeed, this form of "heterosynaptic" depression has been commonly reported at synapses "silent" during strong postsynaptic depolarization, such as that associated with LTP induction (Lynch et al., 1977; Alger et al., 1978). However, like post-tetanic potentiation, this type of heterosynaptic depression recovers to baseline levels within minutes, making it an unlikely cellular mechanism for long-term information storage.

In 1979, Leon Cooper, a theoretical physicist, and his colleagues developed a model to explain receptive field plasticity in the visual cortex during development. They proposed a "modification threshold" (θ_m) of synaptic activity such that active synapses grow stronger when activity reaches this threshold. Conversely, consistent synaptic activity that fails to activate the postsynaptic neuron beyond this threshold would result in synaptic depression (Cooper et al., 1979).

As long-term potentiation is generally elicited with high-frequency stimulation, it would be reasonable to assume that low-frequency stimulation would produce synaptic depression. Indeed, an early study by Dunwiddie and Lynch (1978) showed that stimulation at low frequencies (100 pulses at 1-Hz) produces a consistent and stable synaptic depression. Although responses were only followed for 5 min "since the return to control levels occurred gradually if at all" (Dunwiddie and Lynch, 1978), the use of

low-frequency stimulation to elicit synaptic depression would prove to be quite effective in inducing long-lasting depression (Dudek and Bear, 1992). Consistent with earlier models (Stent, 1973; Cooper, 1979), hippocampal LTD results from prolonged stimulation that is subthreshold for inducing LTP (e.g., 900 pulses at 1-Hz; Dudek and Bear, 1992). Unlike heterosynaptic depression, prolonged LFS induces an input specific and long-lasting synaptic depression in vitro and in vivo (Dudek and Bear, 1992; Heynen et al., 1996).

Metaplasticity

Hebbian plasticity can be viewed as a positive-feedback process - if some inputs onto a post-synaptic neuron undergo LTP, this will increase the probability that any given input will make the neuron fire. This will in turn make it easier for those inputs to undergo LTP, and synapses will eventually be saturated. On the flip side, if ineffective synapses are continually weakened, postsynaptic firing rates will eventually be reduced to zero. Thus, the proposal by Cooper (1979) was amended (now called the "BCM" theory, after its authors) so that "whether synaptic strength increases or decreases depends upon the magnitude of the postsynaptic response as compared with a variable modification threshold" (Bienenstock et al., 1982). In other words, synaptic plasticity is in itself plastic (a phenomenon termed "metaplasticity") - changes in synaptic efficacy can be affected by the prior history of synaptic activity (Abraham and Bear, 1996). For example, high frequency stimulation to one set of inputs can "prime," or facilitate, subsequent induction of LTD at separate converging inputs (Christie and Abraham, 1992; Wexler and Stanton, 1993). Alternately, low frequency stimulation can impair

subsequently induced LTP in the same pathway (homosynaptic inhibition; Huang et al., 1992; Christie and Abraham, 1992; Fujii et al., 1996; Woo and Nguyen, 2002), and can also erase previously established LTP in that pathway (depotentiation; Barrionuevo et al., 1980; Stäubli and Lynch, 1990; Fujii et al., 1991). Metaplasticity may represent homeostatic processes that maintain the maximum flexibility and learning capacity of a neural network. For example, inverse heterosynaptic plasticity (priming of heterosynaptic depression following LTP induction) allows distributed synaptic strengths to change without destabilizing total synaptic weights (Royer and Paré, 2003) and homosynaptic inhibition may be one way of limiting LTP expression to prevent repetitive synaptic facilitation from saturating synaptic strength. Metaplasticity is also a mechanism to regulate the integration of multiple synaptic events over tens of minutes (cf. temporal summation of synaptic potentials over tens of milliseconds during induction of associative LTP). For example, depotentiation represents a mechanism to erase prior LTP that may have been incidentally induced or extraneous to information being encoded (Zhou and Poo, 2004). Metaplasticity adds to the behavioural repertoire of synapses by allowing them to take the past history of synaptic activity into account. Significantly, although the BCM theory predicts that metaplastic effects should be expressed cell-wide to regulate synaptic plasticity effectively (Bienenstock et al., 1982), examples of heterosynaptic metaplasticity remain scarce.

LTP AND MEMORY

Synaptic strengthening and weakening, and metaplasticity are believed to underlie and regulate information storage in the brain. In the mammalian hippocampus, these processes have been proposed to play a vital role in certain types of learning and memory (Bliss and Collingridge, 1993). Four general criteria have been proposed to establish whether changes in synaptic efficacy (i.e., LTP) mediate memory (Martin et al., 2000; Martin and Morris 2002). (1) Detectability: changes in synaptic efficacy should be detectable with the formation of memory; (2) Mimicry: artificial induction of synaptic plasticity should result in "apparent" memory for past experiences which did not occur; (3) Anterograde alteration: interventions that prevent synaptic plasticity should impair memory formation; and (4) Retrograde alteration: interventions that reverse recent synaptic changes should impair recent memory.

Learning induced changes in the hippocampus have been hard to detect, and this may be due to the storage of information over a spatially distributed set of synapses (Hosokawa et al., 2003). The criterion of mimicry in the hippocampus has been difficult to achieve for the same reasons that detectability of synaptic changes in following learning has been elusive and a lack of evidence for sufficiency (i.e., mimicry) remains a major shortfall in confirming the synaptic plasticity and memory hypothesis (Stevens, 1998; Martin and Morris, 2002; Morris et al., 2003). However, considerable success in detecting experience-dependent changes in synaptic efficacy has been achieved in the amygdala, a medial temporal lobe structure involved in emotional memory (for review, see LeDoux, 2003). In particular, the lateral nucleus of the amygdala (LA) is a crucial

site of experience-dependent plasticity during cued fear conditioning (reviewed in Blair et al., 2001; Rodrigues et al., 2004). Cued fear conditioning employs a behavioural paradigm similar to contextual fear conditioning, in which animals learn a new relationship between two stimuli. In cued fear conditioning, animals learn to associate a neutral stimulus (i.e., a sound) with an unconditioned stimulus (e.g., an electric shock; Phillips and LeDoux, 1992). The neural circuitry underlying conditioned fear is well characterized. Projections from the medial geniculate nucleus in the auditory thalamus enter the amygdala via the lateral nucleus. LA neurons are necessary for the acquisition and expression of cued fear conditioning (LeDoux et al., 1990; Phillips and LeDoux, 1992; Fanselow and LeDoux, 1999). LA neurons can undergo LTP (Chapman et al., 1990; Clugnet and Ledoux, 1990) and LTP-like changes can be detected in the thalamo-amygdala pathway following fear conditioning (Rogan et al., 1997; McKernan and Shinnick-Gallagher, 1997; Tsvetkov et al., 2002). Experience-dependent enhancement of synaptic transmission in vivo with LTP-like properties has been observed in the area for whisker representations (barrel cortex) in the rat primary somatosensory cortex (Glazewski et al., 2000; Takahashi et al., 2003; Hardingham et al., 2003) and in the primary motor cortex following motor skill learning (Rioult-Pedotti et al., 1998, 2000).

Much of the evidence linking LTP and memory has been obtained using pharmacological and genetic anterograde alterations. For example, numerous early studies demonstrated the importance of protein synthesis in learning and memory (Flexner and Flexner, 1966; Agranoff BW, 1967; Barondes and Cohen, 1967). More recent studies on hippocampus- and amygdala-mediated types of memory reveal a selective effect of protein synthesis inhibitors on long-term memory, and not short-term

memory (Bourtchouladze et al., 1998; Schafe et al., 2000). LTP also consists of different phases, resembling the phase model of memory formation. Significantly, long-lasting LTP, and not transient LTP, in both the hippocampus and amygdala also requires protein synthesis and gene transcription (Krug et al., 1984; Stanton and Sarvey, 1984; Frey et al., 1988; Otani et al.., 1989; Nguyen et al., 1994; Huang et al., 2000). Protein kinase A (PKA) plays a critical role in long-lasting LTP (Matthies and Reymann, 1993; Frey et al., 1993). Transgenic animals with impaired PKA signaling in the hippocampus are also selectively impaired in long-lasting LTP, but not transient LTP, and perform significantly worse on tests of long-term memory for contextual fear conditioning, but have intact short-term memory (Abel et al., 1997).

Pharmacological or genetic manipulations that impair hippocampal LTP induction also impair memory acquisition in hipoocampus-dependent tasks (Morris et al., 1982, 1986; Tsien et al., 1996). NMDA receptor blockade with pharmacological inhibitors does not affect normal synaptic transmission, but selectively prevents LTP induction and performance on a hippocampus-dependent learning task (Collingridge et al., 1983; Harris et al., 1984; Morris et al., 1986). Transgenic mice with NMDA receptors "knocked out" display only a transient form of LTP that lasts for minutes (post-tetanic potentiation) in response to LTP-inducing stimulation (Tsien et al., 1996). When tested in the Morris water maze, a hippocampus-dependent learning task (Morris et al., 1982), mutant mice were impaired in remembering a hidden platform location. Although mutant mice showed an improvement in finding the hidden platform during individual training sessions, they consistently performed at baseline levels over several training blocks, demonstrating an inability to remember the platform location. These mice were

proficient at learning a version of the task that does not require the hippocampus, demonstrating that memory impairments were not due to sensorimotor or motivational deficencies (Tsien et al., 1996). The mice in this study were also unique due to the restricted deletion of NMDA receptors. Previous attempts to knock out NMDA receptors generated mutant mice that died neonatally (Forrest et al., 1994). The novel knockouts generated by Tsien et al., selectively targeted NMDA receptors in hippocampal CA1 pyramidal neurons (Tsien et al., 1996A). Their results provide strong support for a link between LTP and memory, and point to a pivotal role for CA1-LTP in learning and memory. Neuropsychological and neuropathological analyses of a human case study support this finding. Following an ischemic episode, patient R.B., like H.M., presented with profound anterograde amnesia for declarative memories, mild retrograde amnesia, and spared general intellectual functions. Post-mortem neuropathological analysis revealed complete and bilateral damage restricted to the CA1 field, suggesting that area CA1 is critically involved in the formation of long-term declarative memory (Zola-Morgan et al., 1986).

Other anterograde manipulations include attempts to occlude experience-dependent synaptic plasticity and prevent new memory encoding by saturating LTP prior to learning. Widespread induction of LTP with high-frequency stimulation (HFS) to hippocampal afferents impairs acquisition of spatial memories, suggesting that LTP and experience-dependent synaptic plasticity share common mechanisms of expression (McNaughton et al., 1986; Moser et al., 1998).

The strategy of saturating LTP has also been applied to manipulate synaptic plasticity and affect memory in a retrograde manner – if learning induces changes in

synaptic efficacy in a specific pattern, then inducing LTP at a large proportion of synapses may add "noise" and disrupt recently acquired information. Indeed, LTP-inducing LFS applied to hippocampal inputs impairs retention of recently acquired spatial memory. Performance in a spatial working memory problem (hippocampus-independent), and established spatial reference memories were unaffected, consistent with a selective role of the hippocampus in the consolidation of new long-term memories (McNaughton et al., 1986). A more recent study using more efficient stimulation methods confirmed these results and showed that LTP induction in hippocampal afferents, without reaching saturation, is sufficient to impair retention of recently learned spatial information (Brun et al., 2001). Consistent with the synaptic plasticity and memory hypothesis, memory impairments by HFS are NMDA receptor-dependent – drugs that block NMDA receptor activation during HFS block both in vivo LTP and retrograde disruption of memory by HFS (Brun et al., 2001).

The concluding sentence in Bliss and Lømo's seminal LTP paper (1973) reads:

> "Our experiments show that there exists at least one group of synapses in the hippocampus whose efficiency is influenced by activity.... Whether or not the intact animal makes use in real life of a property which has been revealed by synchronous, repetitive volleys to a population of fibres the normal rate and pattern of activity along which are unknown, is another matter."

Over the last 30 years, several lines of research provide strong support that experience-dependent plasticity is not confined to the laboratory (Boroojerdi et al., 2001; Rodrigues et al., 2004; Foeller and Feldman, 2004; Wilson et al., 2004) and much evidence has been gathered supporting the necessity of synaptic plasticity in learning and memory (Martin et al., 2000; Martin and Morris, 2002; Morris et al., 2003). Although LTP, LTD, and metaplasticity remain experimental phenomena, they effectively demonstrate the range of long-lasting modifications of which synapses are capable.

HIPPOCAMPAL LONG-TERM POTENTIATION (LTP)

LTP Induction

At the CA3-CA1 synapses, LTP induction involves a critical rise in postsynaptic calcium (Ca^{2+}) levels (Lynch et al., 1983; Malenka et al., 1988; Malenka et al., 1992). This is chiefly accomplished through activation of N-methyl-D-aspartate (NMDA) receptors (Collingridge et al., 1983; Harris et al., 1984). Because NMDA receptors are blocked by extracellular magnesium ions (Mg^{2+}) at resting membrane potentials, they do not usually contribute to basal synaptic transmission (Mayer et al., 1984; Nowak et al., 1984). However, with repeated synaptic activation (such as that during tetanic stimulation), co-localized α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) glutamate receptors provide sufficient depolarization to relieve the Mg^{2+} block from NMDA receptors, thus allowing Ca^{2+} ions to flow into the postsynaptic neuron (Collingridge et al., 1983, 1988; Kauer and Malenka, 1988; **Figure 1.07**).

Due to its combined voltage- and ligand-gated properties, NMDA receptors are an ideal "coincidence detector" for conjoint presynaptic (transmitter release) and postsynaptic (depolarization) activity, analogous to Hebb's "neurophysiological hypothesis" (Hebb, 1949; Collingridge, 1985; Wigström and Gustafsson, 1986). The critical involvement of NMDA receptors during LTP induction also provides a molecular basis for the key characteristics of LTP that makes it such an appealing cellular model for learning and memory. Input specificity can be explained by the requirement of NMDA receptors for presynaptic transmitter release in addition to postsynaptic depolarization, thus limiting LTP expression to synapses with conjoint pre- and post-synaptic activity. The voltage-dependent Mg^{2+} block of NMDA receptors also explains the requirement for coordinated activation of sets of fibres (cooperativity) and appropriately timed activity from separate pathways (associativity) in LTP induction (Collingridge GL, 1985; Wigström and Gustafsson, 1986). In addition to NMDA receptors, extrasynaptic L-type voltage-gated calcium channels (VGCCs) and intracellular Ca^{2+} stores are additional sources of postsynaptic calcium that may play a role in different types of synaptic plasticity (Grover and Telyer, 1990; Westenbroek et al., 1990; Harvey and Collingridge, 1992; **Figure 1.07**).

Prior to the discovery of LTP, short-term plasticity, such as post-tetanic potentiation (PTP), which decays within minutes of induction, was the best example of synaptic plasticity in the mammalian system (Lloyd, 1949). The molecular basis of conversion from short-term plasticity to long-term plasticity depends on the postsynaptic Ca^{2+} signal generated during LTP induction. Manipulations that enhance NMDA receptor activation or increase the influx of Ca^{2+} into the postsynaptic cell during

induction can convert PTP to LTP (Malenka, 1991). Downstream of Ca^{2+} entry into the postsynaptic cell, numerous signaling cascades have been implicated in hippocampal synaptic plasticity (**Figure 1.08**). Different patterns of synaptic activity recruit several signaling pathways to elicit mechanistically distinct forms of LTP (Grover and Teyler, 1990; Çavus and Teyler, 1996; Woo et al., 2000; for modeling data, see also Bhalla, 2002) and pharmacological, biochemical, and genetic studies have implicated a vast number of molecules in hippocampal LTP (for reviews, see Sanes and Lichtman, 1999; Soderling and Derkach, 2000). Ca^{2+}/diacylglycerol-dependent protein kinase C (PKC; Hu et al., 1987; Malinow et al., 1989; reviewed in Angenstein and Staak, 1997) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII; Malenka et al., 1989; Malinow et al., 1989; Silva et al., 1992; reviewed in Lisman et al., 2002; Colbran and Brown, 2004) are two serine/threonine kinases that have been implicated in LTP induction. In particular, CaMKII appears to be critically involved across a wide variety of induction protocols and is viewed by many as a "mediator" or requisite component of LTP (Sanes and Lichtman, 1999). Ca^{2+} entry through NMDA receptor activation has also been linked to activation of the cAMP signaling cascade through Ca^{2+}/calmodulin-dependent adenylyl cyclases (Chetkovich et al., 1991; Chetkovich and Sweatt, 1993). Downstream effectors of cAMP include protein kinase A (PKA; Frey et al., 1993; Matthies and Reymann, 1993; reviewed in Nguyen and Woo, 2003) and cross-talk between signaling pathways can also lead to mitogen-activated protein kinase (MAPK) activation (English and Sweatt, 1996, 1997; reviewed in Thomas et al., 2004). In addition, receptor tyrosine kinases, such as those activated by brain-derived neurotrophic factor (BDNF), and non-receptor tyrosine

kinases, such as fyn, have also been implicated in synaptic plasticity (Grant et al., 1992; Korte et al., 1995; Çavus and Teyler, 1996).

LTP Expression (Early-Phase LTP)

Many insights from studies of synaptic plasticity in *Aplysia* have been extended to the mammalian hippocampus. In the reduced *Aplysia* preparation or in cell culture, one application of serotonin elicits short-term facilitation (STF) of the sensorimotor synapse (Montarolo et al., 1986). Analogously, transient LTP (early phase LTP; E-LTP) in hippocampal slices is commonly induced with one brief train of high-frequency stimulation (HFS). E-LTP lasts for less than a few hours and like STF in *Aplysia*, it involves the covalent modification of pre-existing proteins and does not require translation or transcription (Montarolo et al., 1986; Reymann et al., 1985; Huang and Kandel, 1994).

Activity-dependent enhancement of sensorimotor transmission in *Aplysia* is mediated by pre-synaptic facilitation of transmitter release from sensory neurons (Kandel and Tauc, 1965, 1965A) and there is evidence that up-regulation of transmitter release from the presynaptic neuron also contributes to the early stages of LTP (Bolshakov and Siegelbaum, 1995; Zakharenko et al., 2001). Because LTP induction is postsynaptic (e.g. rise in postsynaptic intracellular Ca^{2+}), retrograde messengers, such as arachidonic acid and nitric oxide, have been proposed to communicate postsynaptic activation back to presynaptic neurons (Williams et al., 1989; Schuman and Madison, 1991). In addition, unlike facilitation of sensorimotor transmission in *Aplysia*, the initial expression of LTP also involves significant postsynaptic mechanisms (Nicoll, 2003). The AMPA receptor-

mediated component of synaptic transmission can be enhanced through changes to the biophysical properties of AMPA receptors (Benke et al., 1998; Derkach et al., 1999) and trafficking of additional AMPA receptors to the synaptic membrane (Kauer et al., 1988; Shi et al., 1999; Lee et al., 2003; reviewed by Malinow, 2003; **Figure 1.09**).

Both CaMKII and PKC are critical for the initial expression of LTP - pharmacological inhibition of CaMKII, PKC, or a constitutively active form of PKC, protein kinase M zeta (PKMζ), during LTP induction causes LTP to decay to baseline within minutes (Hu et al., 1987; Malinow et al., 1989; Ling et al., 2002). LTP is also dramatically impaired in CaMKII knock out mice and decays to baseline levels within minutes of induction, similar to that seen with PKC/ PKMζ inhibition (Silva et al., 1992). Consistent with a critical role for CaMKII and AMPA receptor trafficking in early LTP, enhancement of CaMKII activity in organotypic slices is sufficient to drive AMPA receptor trafficking (Hayashi et al., 2000).

In contrast, PKA appears to play a more modulatory role in LTP expression. Mutations that prevent PKA phosphorylation of AMPA receptors show that it plays a critical role in stable LTP expression and is necessary, but not sufficient, to drive synaptic delivery of AMPA receptors (Esteban et al., 2003). Significantly, AMPA receptor delivery to the synaptic membrane is also blocked by mutations at the PKA phosphorylation site that mimic phospho-serine (Esteban et al., 2003), suggesting that both phosphorylated and un-phosphorylated states of the AMPA receptor are involved in the trafficking process, and that regulated PKA activity is required for LTP expression. Biochemical studies show that PKA is transiently activated for 2-10 min after induction of LTP (Roberson and Sweatt, 1996). However, while CaMKII or PKC inhibitors blocks

LTP induction completely (Malinow et al., 1989), genetic or pharmacological inhibition of PKA signaling does not affect transient E-LTP elicited with a single tetanic train (Huang et al., 1994; Abel et al., 1997; Duffy and Nguyen, 2003).

Instead, PKA has been proposed to "gate" the expression of LTP, in part, by regulating opposing protein phosphatase activity (Blitzer et al., 1995, 1998; **Figure 1.10**). The relative balance of signaling through protein phosphatases and kinases regulates trafficking of AMPA receptors in and out of the synaptic membrane, and determines the direction of changes in synaptic efficacy (Lisman, 1989; Coussens and Teyler, 1996; Wang and Kelly, 1996; Malinow and Malenka 2002). Phosphorylation by PKA enables inhibitor-1 (I-1) to bind to and inactivate protein phosphatase 1 (PP1; Hemmings et al., 1984). PP1 has been implicated in synaptic depression, and reciprocally, suppression of PP1 activity promotes synaptic potentiation (Mulkey at al., 1993; Blitzer et al., 1998). During E-LTP, PKA-mediated suppression of PP1 may gate autophosphorylation of CaMKII to generate persistent kinase activity (Blitzer et al., 1995, 1998).

LTP Maintenance (Late-phase LTP)

Like long-term facilitation of the sensorimotor synapse in Aplysia, long-lasting LTP (late phase LTP; L-LTP) can be induced by repeated bouts of synaptic activation. A single application of serotonin to the sensory neurons in cell culture produces short-term facilitation, and repeated synaptic activation with multiple applications of 5-HT leads to long-term facilitation that is dependent on de novo protein synthesis and gene expression (Montarolo et al., 1986; Castellucci et al., 1986, 1989). Analogously, whereas a single high frequency train elicits E-LTP, multiple trains of HFS (generally 3 or more) induce

long-lasting potentiation that can last for several hours in vitro (Andersen et al., 1977; Reymann et al., 1985; Huang and Kandel, 1994), and for many months in vivo (Abraham et al., 2002). Like long-term facilitation in *Aplysia*, hippocampal L-LTP is dependent on translational and transcriptional processes (Krug et al., 1984; Deadwyler et al., 1987; Frey et al., 1988; Nguyen et al., 1994; Bourtchouladze et al., 1994, 1998; **Figure 1.11**).

In *Aplysia*, long-term facilitation involves protein kinase A (PKA) and gene expression mediated by cAMP responsive element binding protein (CREB), a transcription factor that regulates expression of genes with cAMP responsive element (CRE) promoter sequences (Martin et al., 1997; Michael et al., 1998; Müller and Carew, 1998). PKA and CREB also play critical roles in the cellular consolidation of L-LTP in the mammalian hippocampus (Matthies and Reymann, 1993; Bourtchouladze et al., 1994; Impey et al., 1996, 1998; Matsushita et al., 2001; Barco et al., 2002). Interfering with PKA signaling selectively impairs L-LTP elicited with multiple tetanic trains, but does not affect E-LTP elicited with a single train (Huang and Kandel, 1994; Abel et al., 1997; Duffy et al., 2003). Strong tetanic stimulation that elicits L-LTP, but not weaker E-LTP-inducing stimuli, also triggers CRE-mediated gene expression that can be attenuated by PKA inhibitors (Impey et al., 1996). Specific blockade of nuclear PKA activity prevents CREB phosphorylation and impairs L-LTP, but not E-LTP (Matsushita et al., 2001). Furthermore, chemical activation of cAMP/PKA signaling elicits long-lasting facilitation of synaptic transmission (chemLTP) that is sensitive to protein synthesis inhibitors and can occlude electrically induced L-LTP (Frey et al., 1993; Huang and Kandel, 1994). cAMP/PKA activators are also sufficient to induce CRE-mediated transcription, an effect that can be occluded by prior application of L-LTP-inducing tetanic stimulation (Impey et

al., 1996). Together, these results suggest that PKA is a critical trigger for consolidating E-LTP into L-LTP by activating CREB-dependent transcription that stabilizes the expression of long-lasting LTP.

Other second messenger systems, such as MAPK and Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV), can also regulate CREB phosphorylation (Bito et al., 1996; Impey et al., 1998; Roberson et al., 1999; Kang et al., 2001; for review, see Lonze and Ginty, 2002). Other regulators of CREB include protein phosphatases that antagonize the actions of protein kinases. In particular, protein phosphatases 1 (PP1) and 2A (PP2A) have been implicated in dephosphorylating CREB at the PKA phosphorylation site (Hagiwara et al., 1992; Wadzinski et al., 1993). PP1-mediated dephosphorylation of Ca2+-activated CREB has been reported in vitro (Bito et al., 1996) and in vivo (Genoux et al., 2002). These reports demonstrate that CREB phosphorylation is tightly regulated through activity-dependent recruitment of protein kinases and opposing phosphatases (Bito et al., 1996; West et al., 2002).

Regulation of L-LTP expression through a PKA/PP1 gating mechanism (Blitzer et al., 1995, 1998) is supported by recent studies. R(AB) transgenic mice overexpressing PKA regulatory subunits (RIalpha) have reduced hippocampal cAMP/PKA signaling and selective impairments in L-LTP, with intact E-LTP (Abel et al., 1997). Deficient L-LTP in mutants can be rescued by applying protein phosphatase inhibitors, suggesting that PP1-mediated inhibitory constraints on L-LTP expression can be overcome by PKA activity (Woo et al., 2002). One possibility is that activation of CREB by kinases and inhibition by phosphatases compete to regulate expression of long-lasting plasticity, and long-term memory. Consistent with this hypothesis, genetic inhibition of PP1 activity by

expressing a constitutively active form of inhibitor 1 (I-1) enhances both the acquisition and retention of hippocampus-dependent memories (Genoux et al., 2002).

Many genes have been shown to be up-regulated with L-LTP, including immediate early genes (IEGs) that encode regulatory transcription factors for other down-stream effector genes (Cole et al., 1989). Other identified genes support a role for reported synaptogenesis and morphological changes (Yuste and Bonhoeffer, 2001) in stabilizing long-lasting hippocampal LTP. L-LTP is associated with the synthesis of new AMPA receptors (Nayak et al., 1998; Heynen et al., 2000) and recruitment of new sites of synaptic transmission (Bolshakev et al., 1997), including the conversion of "silent synapses" (synapses that exhibit only NMDA receptors) into functional synapses through the addition of AMPA receptors (Isaac et al., 1995; Liao et al., 1995). Components of NMDA receptors, associated receptor anchoring proteins, and cytoskeletal elements have also been identified with LTP-associated gene expression (Steward et al., 1998; Williams et al., 2003; for review, see Abraham and Williams, 2003).

SYNAPTIC TAGGING AND SYNAPSE SPECIFICITY OF LONG-TERM PLASTICITY

The combined input specificity and transcriptional requirements of L-LTP necessitate a mechanism for selective delivery or localization of proteins required for stabilizing LTP to activated synapses. A leading proposal involves a mechanism to mark, or "tag", active synapses that allows newly synthesized gene products to be captured and utilized at appropriately activated synapses (Sossin, 1996; Frey and Morris, 1997, 1998; Schuman, 1997; **Figure 1.12**). Frey and Morris (1997) first provided evidence for the

synaptic tagging hypothesis in a series of elegant two-pathway experiments (here designated "S1" and "S2" for clarity). First, they demonstrated that protein synthesis-dependent L-LTP could be elicited in the presence of translational inhibitors if L-LTP had been previously induced at converging independent inputs. L-LTP requires de novo protein synthesis, and they proposed that induction of L-LTP at S1 resulted in a cell-wide distribution of plasticity-related proteins (PRPs). Subsequently, L-LTP at S2 could be induced in the presence of translational inhibitors because PRPs associated with S1 L-LTP were still in circulation and could be captured by synaptic activity at S2 to stabilize S2 L-LTP (**Figure 1.12B**). Consistent with this model, L-LTP expression can be also be captured by synaptic activation that normally only produces transient potentiation, as long as it reaches a threshold of synaptic activity, presumably required to generate a synaptic tag (Frey and Morris, 1997, 1998; **Figure 1.12C**).

Significant advances into the mechanisms of synaptic tagging and capture have also been carried out in an *Aplysia* culture system in which a single bifurcated sensory neuron forms synaptic contacts with two spatially separated motor neurons. Repeated applications of 5-HT to the connections onto one of the motor neurons induced branch-specific long-term facilitation (LTF) at that connection without any change at the opposite connection (Martin et al., 1997). In experiments analogous to examination of synaptic tagging in the mammalian hippocampus (Morris and Frey, 1997), pairing established LTF at one connection with a single application of 5-HT (which is subthreshold for LTF) to the other connection induced LTF at both sensorimotor synapses (Martin et al., 1997).

By manipulating the pairing of weak and strong synaptic activation, it has been demonstrated in both *Aplysia* and hippocampal systems that the period of PRP distribution and the synaptic tag persists within a limited time period (Frey and Morris, 1997, 1998; Martin et al., 1997; Casadio et al., 1999; **Figure 1.12D-F**). Because synaptic capture can occur in the presence of translational inhibitors, input specific L-LTP is believed to involve a protein synthesis-independent synaptic tag (Frey and Morris, 1997; Kelleher et al., 2004). More recent work showed that generation of the synaptic tag involves NMDA receptor and PKA activation (Barco et al., 2002). Studies carried out in the *Aplysia* culture system revealed that LTF involves two phases of consolidation: (1) an initial phase of PKA-dependent, protein synthesis-independent synaptic capture; and (2) protein synthesis-dependent synaptic capture detectable only 72 h after induction that acts to stabilize LTF and which involves the growth of new synaptic connections (Martin et al., 1997; Casadio et al., 1999).

The validity of the synaptic tagging hypothesis rests on the identification of the tag. However, the identity of the synaptic tag, in both systems, and the mechanisms through which synaptic tagging and capture can be regulated by previous synaptic activity is completely uncharacterized.

OBJECTIVES OF THE PRESENT THESIS

Long-lasting changes in synaptic efficacy are believed to underlie information storage at a cellular level. In the mammalian hippocampus, long-lasting LTP has been correlated with hippocampal long-term memory and both cellular and behavioural processes require

transcription, translation, and PKA. Understanding the critical factors that regulate long-lasting forms of synaptic plasticity, such as L-LTP, should advance our understanding of cellular and molecular processes that underlie information storage at a cellular level.

The first objective of this thesis was to assess the metaplastic effects of prior low frequency stimulation on different forms of LTP (Chapter 2). Having discovered that prior LFS selectively inhibits L-LTP, but not E-LTP, we sought to determine how LFS regulates the transition from E-LTP to L-LTP (Chapter 3). Finally, the third objective was to explore the role of PKA in the expression of input specific L-LTP, and how the balance of PKA and protein phosphatases can be regulated by prior synaptic activity (Chapter 4). Briefly, I have addressed the following questions in my thesis:

1. Can the prior history of synaptic activity regulate subsequent synaptic plasticity in a non-input specific manner?
2. In what way can prior synaptic activity regulate the expression of different types of LTP?
3. Can the balance of phosphatase and kinase activity be regulated by prior synaptic activity to gate the expression of L-LTP in a non-input specific manner?
4. Is PKA necessary for synaptic tagging and capture of L-LTP expression?
5. Is PKA sufficient for synaptic tagging and capture of L-LTP expression?

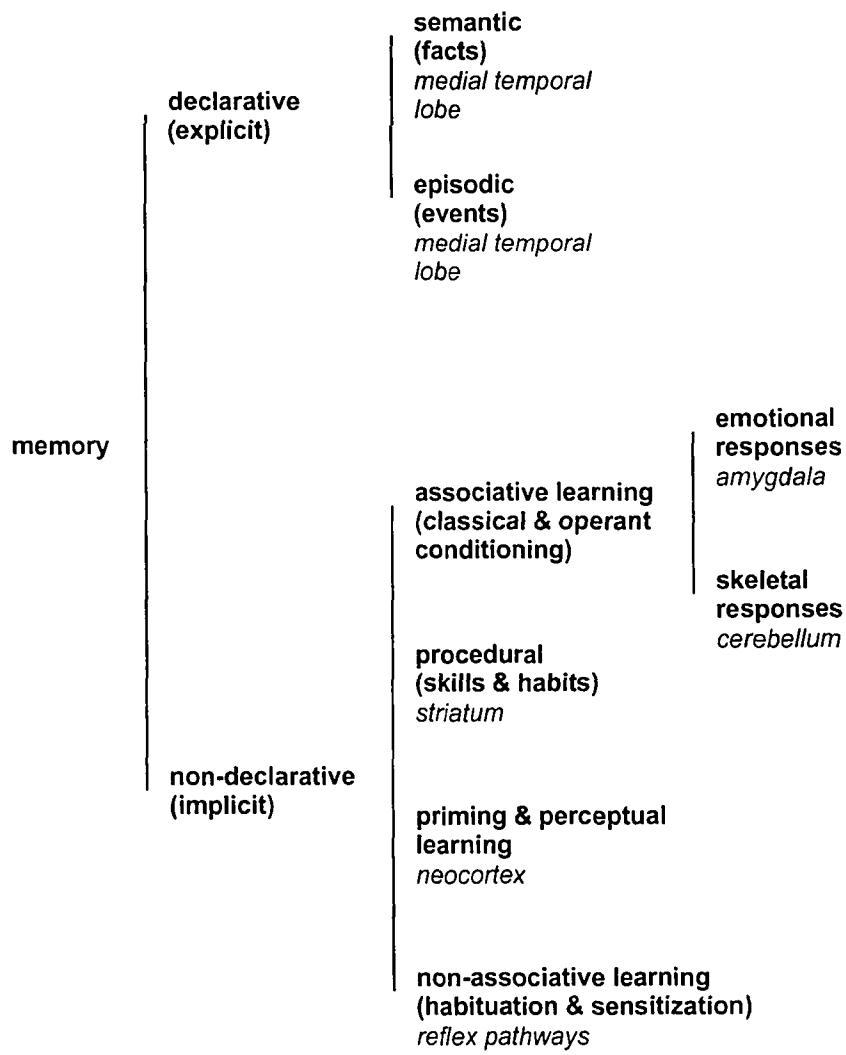

Figure 1.01: Mammalian long-term memory systems. Different types of long-term memory are classified based on declarative or non-declarative characteristics. The brain structures believed to be responsible for that form of memory are italicized. [Adapted from Squire and Zola-Morgan, 1988.]

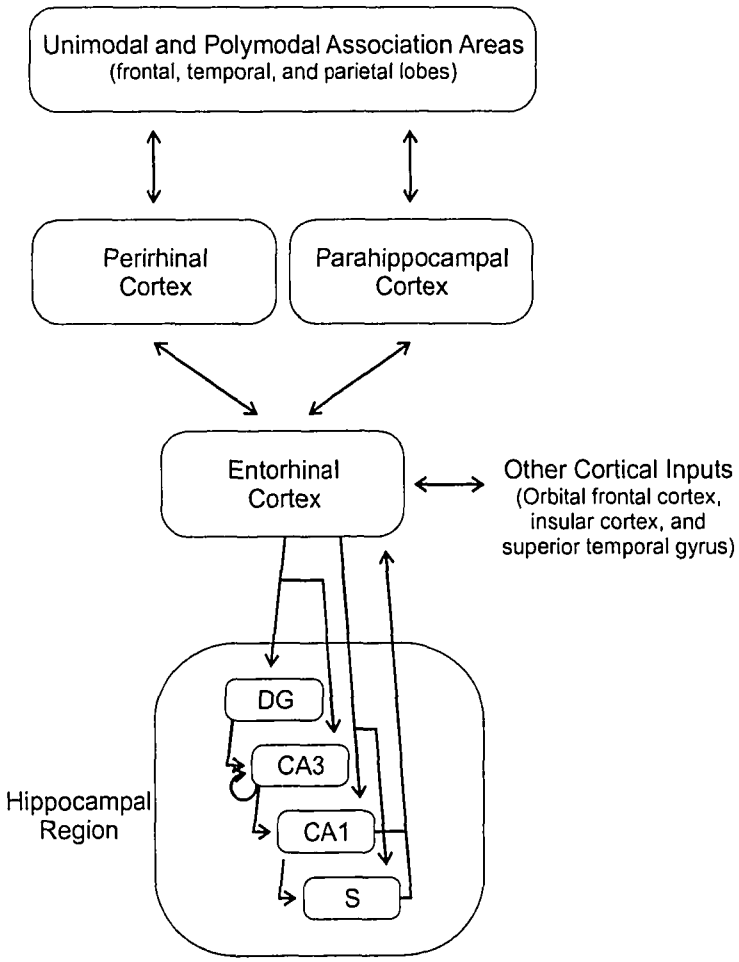

Figure 1.02: Organization of the medial temporal lobe memory system. The entorhinal cortex is a major source of projections from various cortical processing streams to the hippocampal region. Within the hippocampus, the large network of connections, which support plasticity mechanisms, is believed to underlie the rapid encoding of information. All connections are reciprocal and information flows back from the hippocampus to cortical regions where memory traces are believed to be stored. [Adapted from Squire and Zola, 1996.]

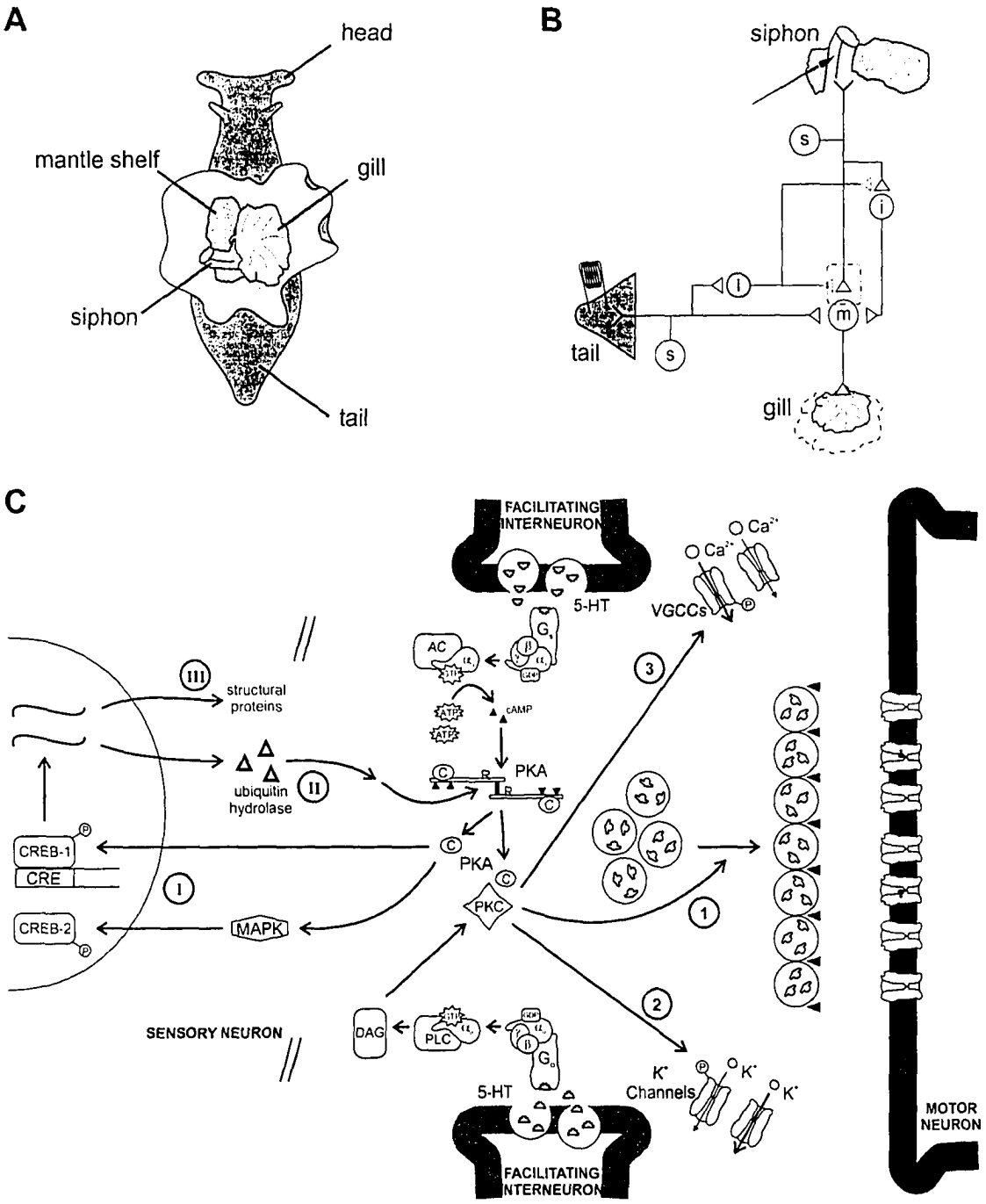

Figure 1.03: Study of non-declarative learning and memory in the marine snail, *Aplysia*. **(A)** Diagram of the marine snail, *Aplysia*, detailing anatomy involved in the gill-withdrawal reflex. **(B)** Simplified diagram of the neural circuit responsible for the *Aplysia* gill-withdrawal reflex. Noxious stimulation to the tail activates facilitating interneurons (i) that form axo-axonic connections with presynaptic sensory neurons (s) and acts to enhance neurotransmitter release onto motor neurons (m) that mediate gill withdrawal. **(C)** Schematic of cellular mechanisms underlying behavioural sensitization. Short-term facilitation involves PKA- and PKC-mediated (1): mobilization of transmitter vesicles to readily-releaseable pool; (2) decrease K+ conductance; and (3) enhanced Ca2+ influx through VGCCs. Long-term sensitization involves: (I) PKA and MAPK translocation to the nucleus to activate transcription; (II) synthesis of ubiquitin hydrolase which leads to the degradation of PKA regulatory subunits to generate persistent PKA activity; (III) translation of proteins involved in mediating structural changes to the sensory neuron.[Adapted from Kandel et al., 2000.]

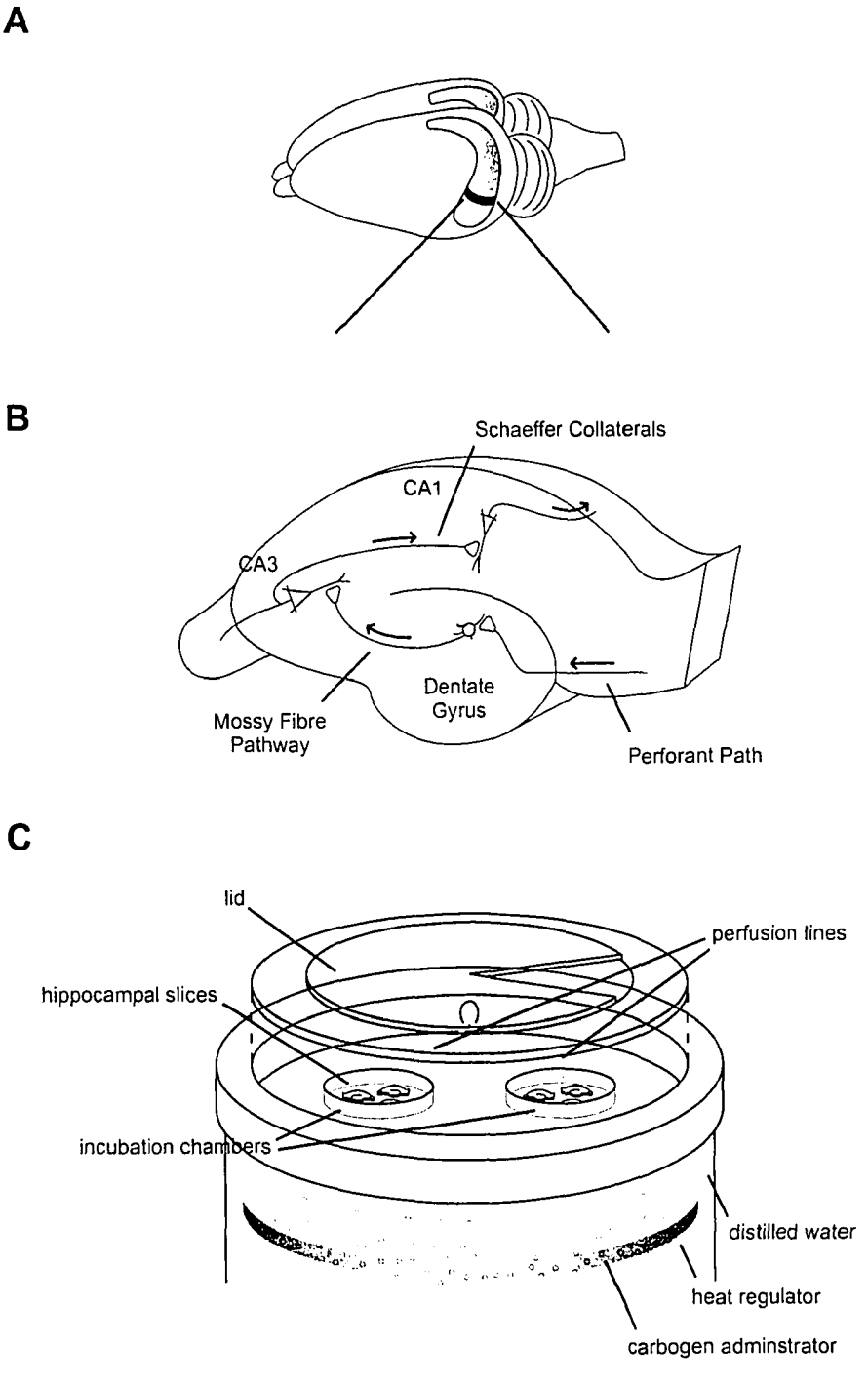

Figure 1.04: The laminar organization of the mammalian hippocampus allows for in vitro electrophysiological examination. (A) Schematic detailing position of the hippocampi in a mouse brain. **(B)** Diagram of a hippocampal slice with major anatomical subfields and principal pathways. **(C)** Schematic of a typical recording interface chamber used in in vitro study of hippocampal slices.

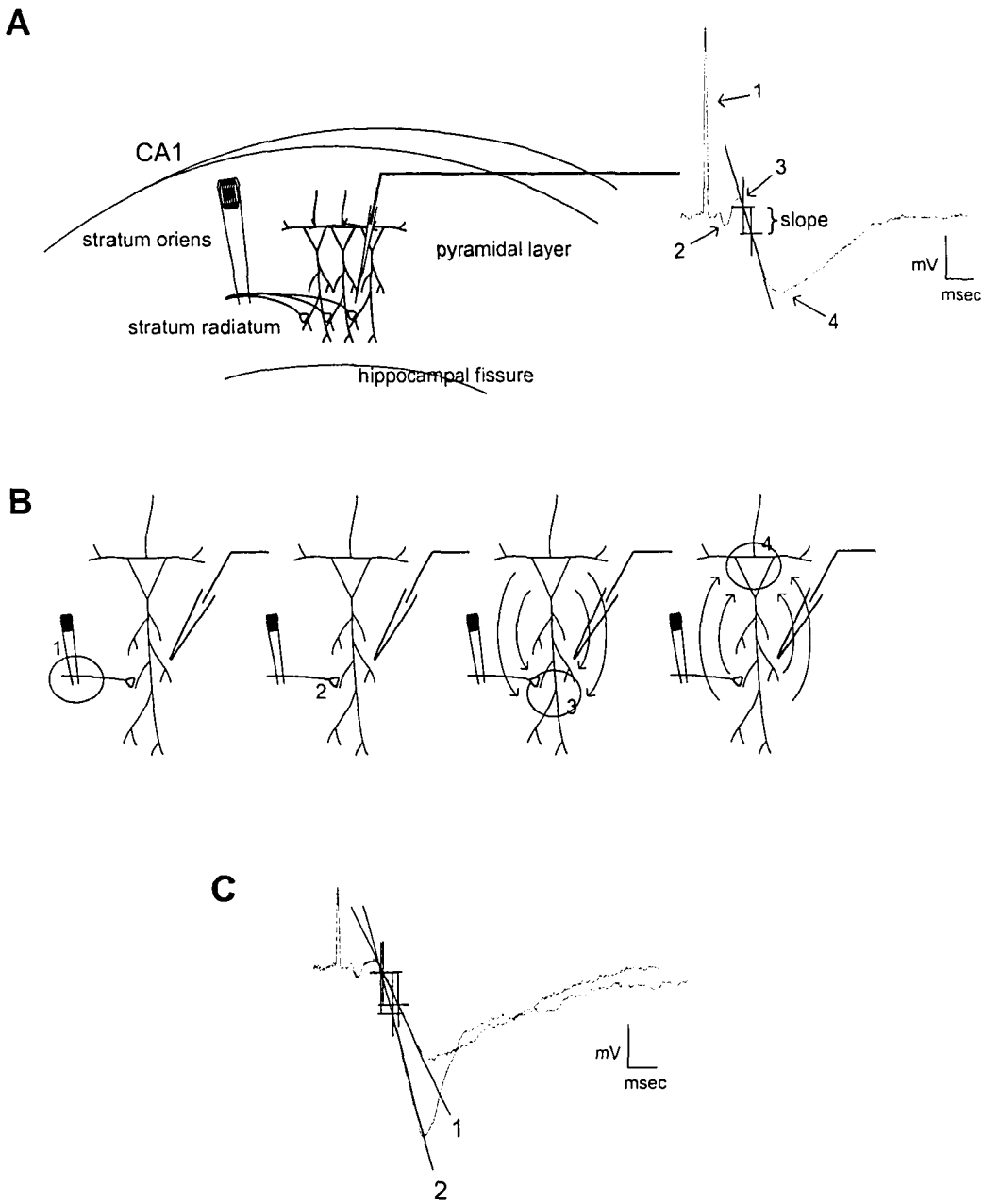

Figure 1.05: Schematic diagram of extracellular recordings in hippocampal area CA1. (A) Diagram of area CA1 in a hippocampal slice showing placement of stimulating and recording electrodes. Numbers in the sample fEPSP correspond to 1-4 in (B). **(B)** Schematic of pyramidal neuron showing location of events depicted in A (right): (1) stimulation artifact; (2) presynaptic fibre volley; (3) local depolarization of apical dendrites; (4) depolarization spreads to the soma. Arrows indicate the direction of current flow. **(C)** Field potential as recorded by an extracellular electrode placed near the proximal dendrites before (1), and after (2), LTP induction. Hashmarks denote measurement of inital slope which reflects the strength of synaptic transmission.

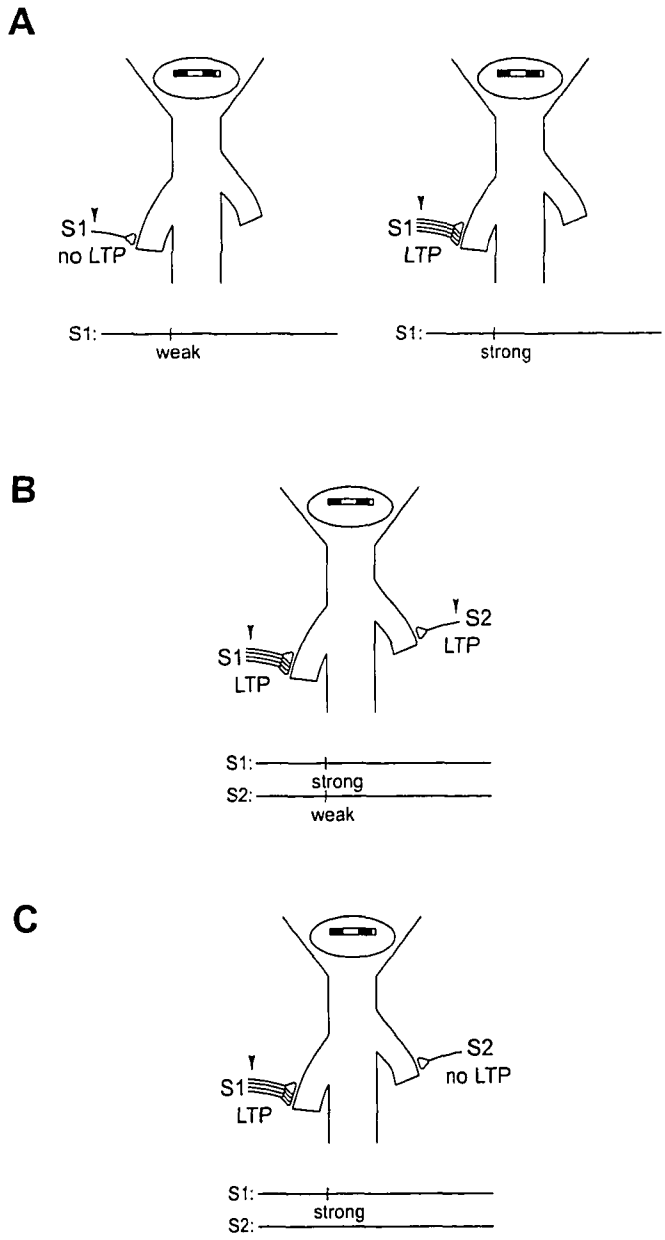

Figure 1.06: Characteristics of cooperativity, associativity, and specificity of long-term potentiation (LTP) in the mammalian hippocampus. (A) Cooperativity: a minimum number of presynaptic axons must be activated to induce LTP. **(B)** Associativity: simultaneous activation of weak inputs during LTP induction in the strong inputs induces LTP in both pathways. **(C)** Specificity: LTP is only expressed at inputs activated during LTP induction.

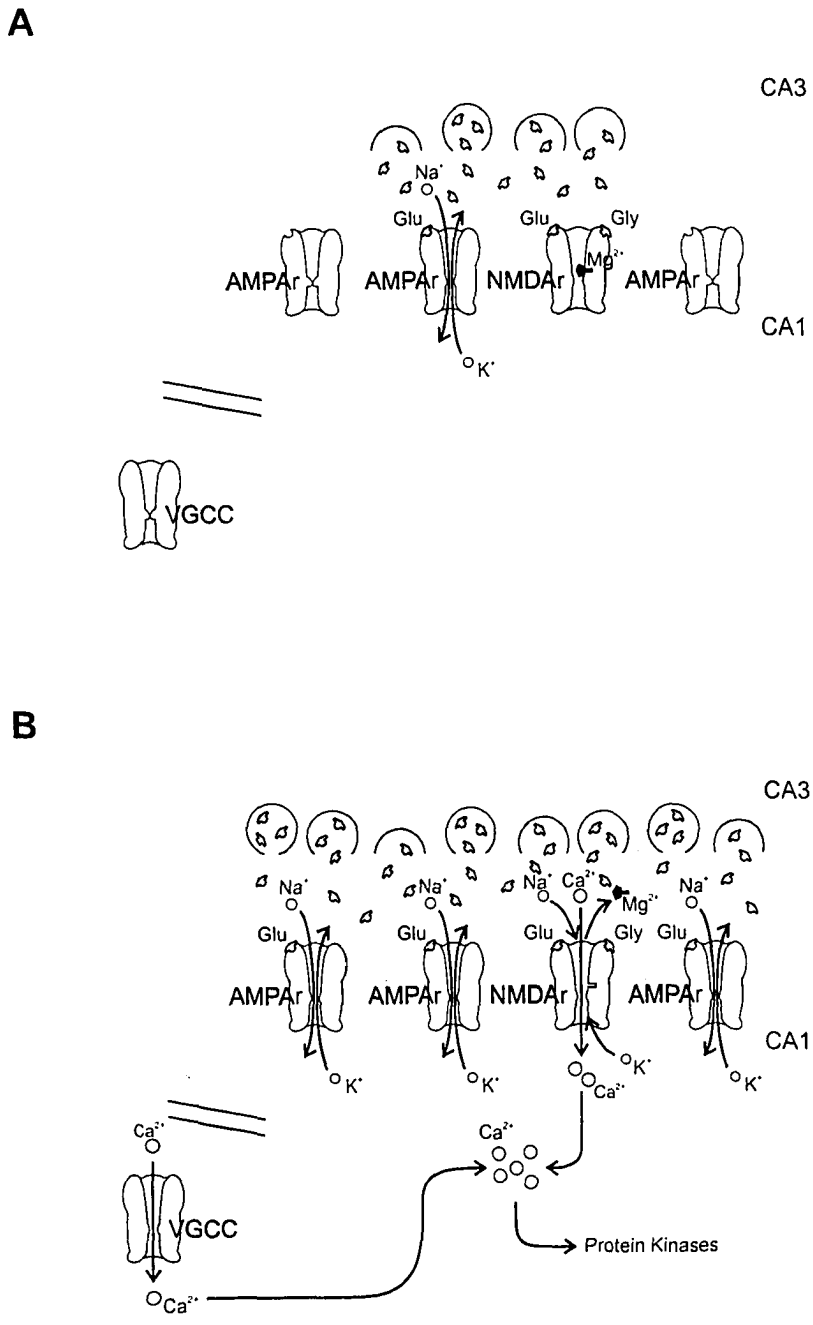

Figure 1.07: LTP induction at the CA3-CA1 synapse. (A) NMDA receptors are blocked by extracellular Mg^{2+} during basal synaptic transmission. **(B)** Strong activation of presynaptic inputs during LTP induction provides sufficient postsynaptic depolarization to remove the Mg^{2+} block from NMDA receptors. The resultant rise in intracellular Ca^{2+} (from NMDA receptors and VGCCs) leads to the activation of various protein kinases required for LTP expression.

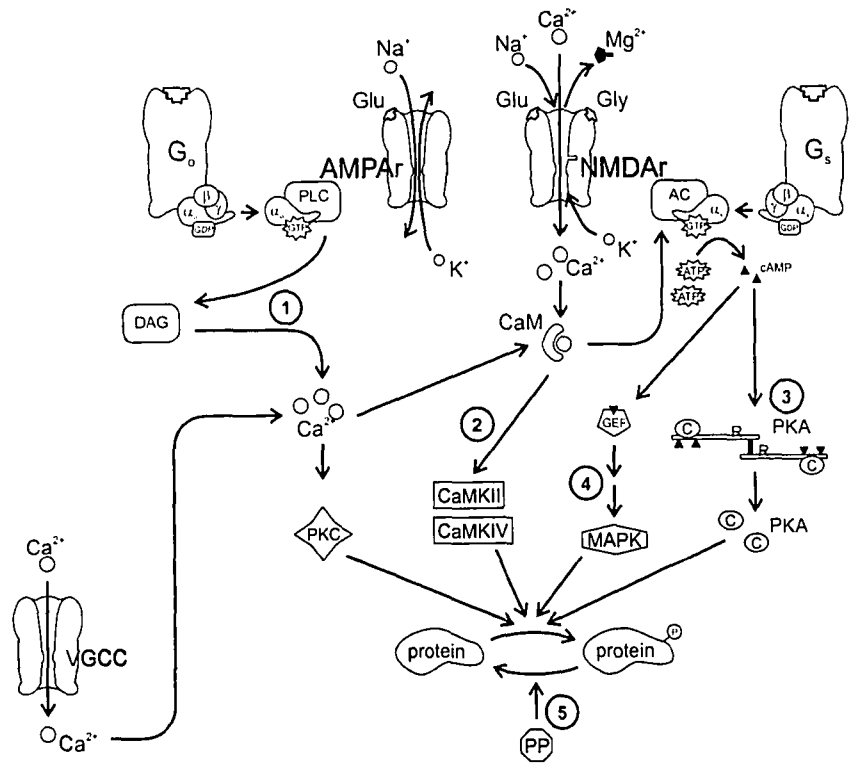

Figure 1.08: Simplified schematic of signal transduction cascades coupled to the influx in postsynaptic Ca^{2+} during LTP induction. (1) G_o-coupled receptors leads to PKC activation. (2) Downstream effectors of Ca^{2+}/calmodulin include CaMKII and CaMKIV. (3) Activation of G_s-coupled receptors and production of cAMP leads to PKA activation. (4) cAMP can also act to activate MAPK through GEF. (5) The activity of protein kinases are opposed by protein phosphatases that act to dephosphorylate their target proteins.

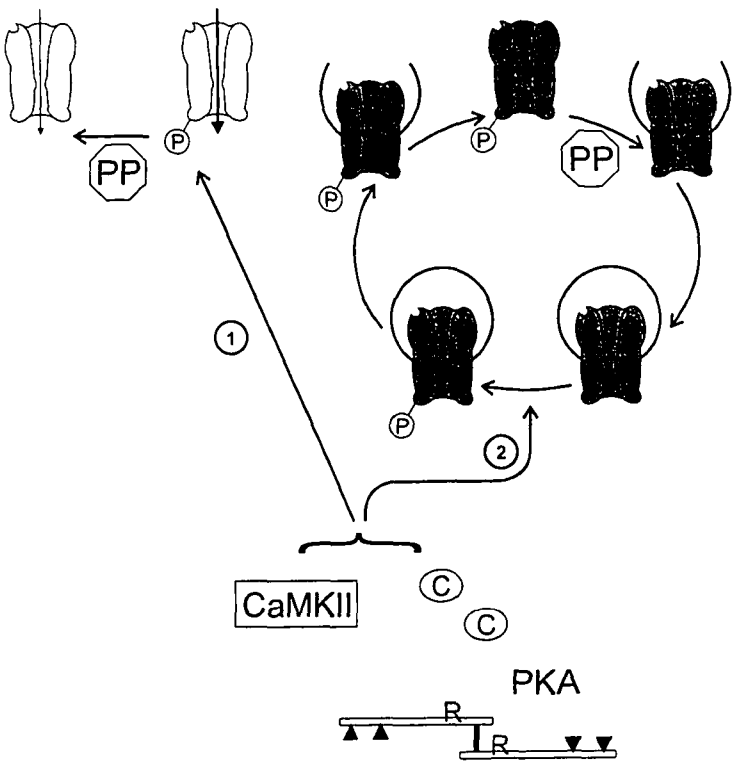

Figure 1.09: Potential mechanisms and sites of action of protein kinases underlying CA1-LTP expression. Early LTP expression involves PKA- and CaMKII-mediated (1) covalent modification of AMPA receptors, and (2) trafficking of AMPA receptors to the synaptic membrane. The actions of PKA and CaMKII are opposed by protein phosphatases (PP).

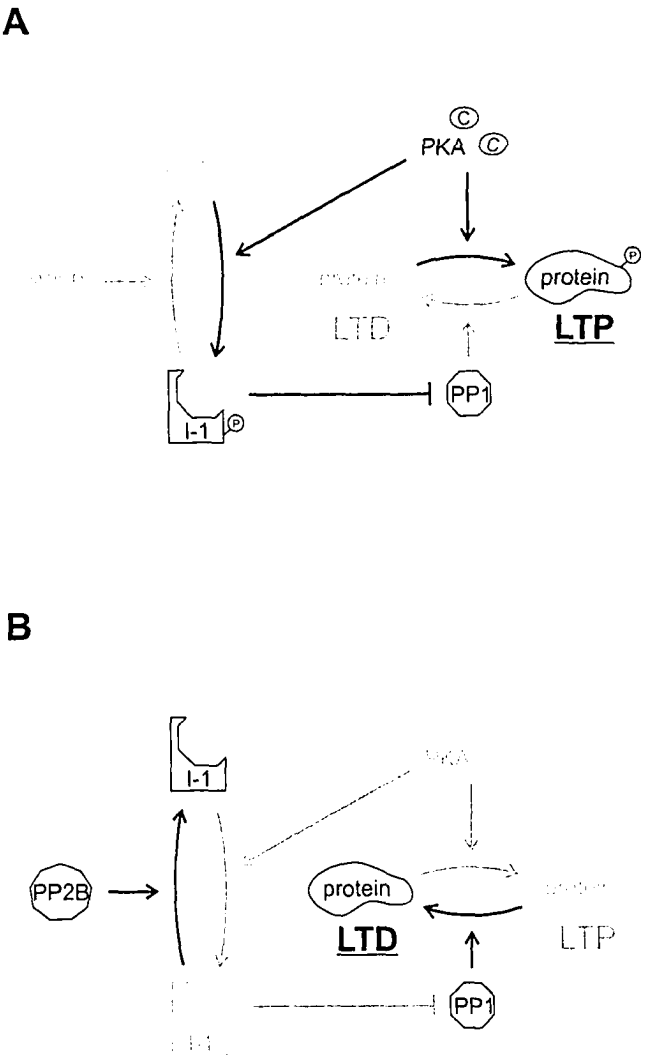

Figure 1.10: The relative balance of signaling through kinases and phosphatases determines the direction of changes in synaptic strength. (A) Targets of PKA phosphorylation during LTP include a PP1 inhibitor, I-1. **(B)** During synaptic depression, protein phosphatase activity (e.g., PP1) predominates. PP2B dephosphorylates I-1 to relieve inhibition of PP1.

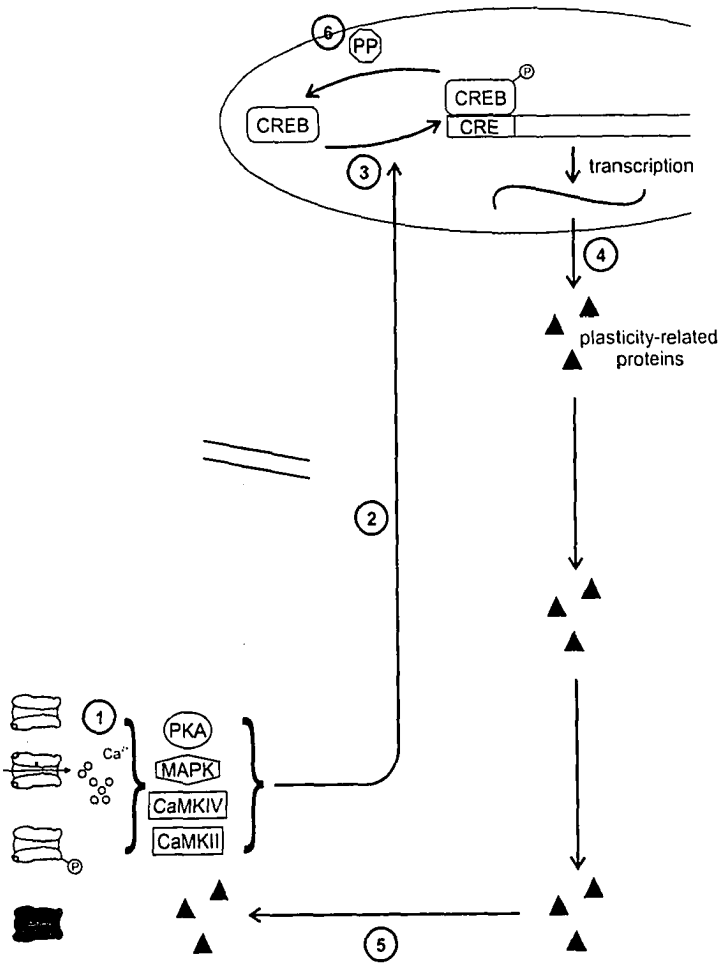

Figure 1.11: Potential mechanisms and sites of action of protein kinases underlying L-LTP. **(1)** Ca^{2+} influx during LTP induction activates protein kinases that **(2)** translocate to the nucleus to **(3)** phosphorylate transcription factors, such as CREB. **(4)** Newly synthesized proteins are **(5)** shipped back to activated synapses to stabilize L-LTP. **(6)** CREB phosphorylation is opposed by protein phosphatases.

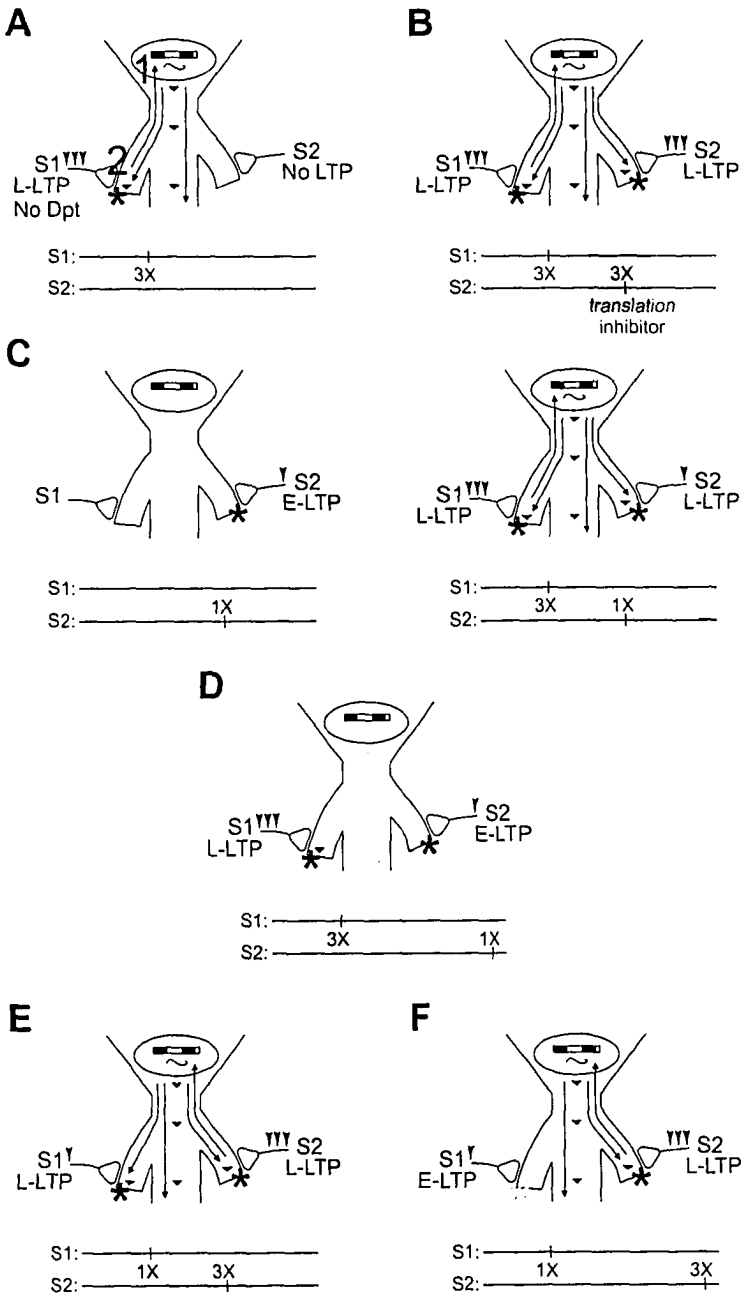

Figure 1.12: Schematic of the synaptic tagging hypothesis. (A) Input specific expression of L-LTP requires (1) signal to trigger somatic gene expression, and (2) generation of synaptic tag to permit capture of plasticity-related proteins at activated synapses. **(B)** Protein-synthesis dependent L-LTP can be elicited in the presence of translational inhibitors if it is paired with established L-LTP. **(C)** Weak stimulation that normally results in E-LTP can be transformed into L-LTP by capturing PRPs from previous L-LTP induction. **(D)** There is a limited period of PRP distribution. **(E)** E-LTP that is elicited prior to L-LTP can also be transformed into L-LTP by capturing PRPs. **(F)** The synaptic tag persists within a limited time period.

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CHAPTER 2:

HOMOSYNAPTIC AND HETEROSYNAPTIC INHIBITION OF L-LTP BY PRIOR SYNAPTIC ACTIVITY

INTRODUCTION

The hippocampus is critical for the initial storage of certain forms of long-term memory (for review, see Eichenbaum, 2000; Morris et al., 2003; Squire et al., 2004). Like most synapses in the nervous system, the strength of hippocampal synapses can be rapidly and persistently changed by brief bouts of patterned electrical activity (for reviews, see Malenka RC, 1994; Huang et al., 1996; Abraham and Williams, 2003). Long-term potentiation (LTP) is a well-studied form of activity-dependent synaptic enhancement that is well characterized in the hippocampus (Bliss and Lømo, 1973; Bliss and Collingridge, 1993). LTP can be divided into at least two temporal phases. The early phase of LTP (E-LTP) is commonly induced by a single train of 100-Hz stimulation, lasts for less than a few hours, and does not require translation or transcription (Reymann et al., 1985; Huang and Kandel, 1994). The persistence of LTP can be extended with multiple trains of 100-Hz stimulation to last for several hours in vitro (Andersen et al., 1977; Reymann et al., 1985; Huang and Kandel, 1994), and for many months in vivo (Douglas and Goddard, 1975; Abraham et al., 2002). Different types of L-LTP can be elicited by multiple trains of tetani depending on the temporal spacing and frequency of stimulation. While many different intracellular signaling cascades have been implicated in different types of L-LTP, they all share a common requirement for gene expression and de novo protein synthesis (Deadwyler et al., 1987; Frey et al., 1988; Nguyen et al., 1994; Bourtchouladze et al., 1994).

More recently, it has become apparent that the prior history of synaptic activity is an additional variable that can affect the plasticity of neural connections (Abraham and

Bear, 1996). For example, the duration of transient LTP induced at one set of synapses can be extended by capturing L-LTP associated gene products from previous synaptic activity at other synapses on the same postsynaptic neurons (i.e., heterosynaptic facilitation; Frey and Morris, 1997). On the other hand, various patterns of synaptic activity have also been found to impair subsequent LTP (Huang et al., 1992; Christie and Abraham, 1992; Fujii et al., 1996; Woo and Nguyen, 2002). However, in contrast to the heterosynaptic facilitation of LTP reported previously (Frey and Morris, 1997), inhibition of LTP by prior activity has typically been limited to the previously activated synapses (i.e., homosynaptic inhibition; Huang et al., 1992; Christie and Abraham, 1992; Fujii et al., 1996; Woo and Nguyen, 2002). For effective homeostatic regulation of synaptic weights, it has been theorized that metaplastic changes should be expressed cell-wide and should occur for all synapses terminating on the affected neurons (Bienenstock et al., 1982; Abraham and Bear, 1996; Turrigiano and Nelson, 2004).

I report here a novel form of heterosynaptic depression and cell-wide inhibition of subsequent L-LTP. Previously, I found that LFS at 5-Hz for 3 min produces a transient synaptic depression of fEPSPs in area CA1 of mouse hippocampal slices. Although this brief LFS did not persistently alter synaptic transmission, it decreased the stability of L-LTP induced later at the same synapses (Woo and Nguyen, 2002). I extend these findings by showing that these metaplastic effects of prior LFS also occur at synapses that did not contribute to postsynaptic activation (i.e., heterosynaptic inhibition). To gain a better understanding of the potential mechanisms underlying this novel metaplasticity, I examined the effect of prior LFS on LTP induced with different protocols. By manipulating the temporal spacing of synaptic stimulation, I revealed a differential

requirement for voltage-gated calcium channel (VGCC) activation during L-LTP induction. I show that prior LFS impairs both VGCC-dependent and VGCC-independent types of L-LTP. In contrast, transient E-LTP was not affected by prior LFS. Taken together, my results suggest that homosynaptic and heterosynaptic inhibition by LFS may be acting on more general processes that underlie cellular consolidation of long-lasting plasticity. Such inhibition of heterosynaptic plasticity may critically regulate the expression of L-LTP at multiple converging synaptic pathways.

MATERIALS AND METHODS

Hippocampal Slice Preparation

All experiments were conducted with female C57BL/6 mice (aged 10-14 wks, Charles River, Montreal, Canada) housed at the University of Alberta Animal Care Facility. Care and experimental procedures were in accordance with guidelines approved by the Canadian Council on Animal Care. Animals were euthanized by rapid cervically dislocated followed by decapitated. Their brains were removed and immersed in ice-cold (4°C) artificial cerebral spinal fluid (ACSF) bubbled with a "carbogen" mixture of 95% O_2, 5% CO_2. The ionic composition of the ACSF was the same as in previous studies (Nguyen and Kandel, 1997) consisting of (in mM): 124 NaCl, 4.4 KCl, 1.30 $MgSO_4 \cdot 7H_2O$, 1.0 $NaH_2PO_4 \cdot H_2O$, 26.2 $NaHCO_3$, 2.5 $CaCl_2$, and 10 D-glucose. Following a brief (~2 min) period to allow the brain to cool down, the hippocampus was dissected free from the surrounding 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, transverse slices (400 μm thickness) were cut on a manual tissue chopper (Stoelting, Wood Dale, Illinois). Slices were transferred using a fine paintbrush into a small glass Petri dish containing ice-cold exygenated ACSF until the entire hippocampus was sectioned. Hippocampal slices were then transferred onto a nylon mesh in an interface chamber where they were maintained at 28°C and perfused with oxygenated ACSF (1 ml/min). Slices were allowed to recover for at least 60 min before experiments commenced.

Electrophysiology

Extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded in stratum radiatum of area CA1 with a glass microelectrode (A-M Systems, Carlsborg, WA) filled with ACSF (electrical resistances: 2-4 MΩ). Extracellular stimulation of the Schaffer collateral pathway was accomplished with one (or two, where indicated) nickel-chromium (A-M Systems, Carlsborg, WA) bipolar stimulating electrodes (diameter 130um) placed on either side of a single recording electrode in s. radiatum (**Figure 2.02A**).

Evoked fEPSPs were amplified, digitized (DigiData 1200B Interface, Axon Instruments, Foster City, CA), and analyzed using Axon Clampex 7.0 (Axon Instruments, Foster City, CA). The test stimulus intensity was adjusted to produce basal fEPSP sizes that were 40% of maximal evoked fEPSP amplitude (Grass S48 Stimulator). Test stimuli were delivered to the Schaffer collaterals once per minute (0.08 ms stimulus duration). Two-pathway experiments were conducted with a 200 ms separation between stimulation through the two electrodes (stimulating electrodes "S1" and "S2"). To ensure that

fEPSPs evoked through each stimulating electrode resulted from activation of two independent synaptic pathways, I positioned the electrodes so that no paired pulse facilitation (PPF) was evident following sequential activation of S1 and S2. Interpathway PPF was assessed at various time intervals (40 ms, 50 ms, 75 ms, 100 ms, 150 ms and 200 ms) during baseline acquisition and at the end of experiments. Sample data from one experiment (i.e., one slice) is shown in **Figure 2.02B**.

Transient E-LTP was elicited by delivering 1 stimulus train (1s duration at 100-Hz; Huang and Kandel, 1994). Long-lasting late-phase LTP (L-LTP) was elicited by delivering 4 stimulus trains of 1s duration at 100 Hz with an interburst interval of 3 s ("compressed" protocol; Woo et al., 2003) or 5 mins ("spaced" protocol; Huang and Kandel, 1994). All three protocols used here (1X100Hz, 4X100Hz@3s, and 4X100Hz@5min) have been previously shown to require NMDA receptor activation for their induction (Woo et al., 2003). Low frequency stimulation (LFS) consisted of 5-Hz stimulation for 3 mins (Woo and Nguyen, 2002).

Drugs

Nifedipine (NIF; RBI), a selective L-type Ca^{2+} channel antagonist, was prepared fresh daily from 10 mM stock solution (DMSO, Sigma Aldrich) stored at -4°C (10 μM final concentration). Final concentration of applied DMSO was 0.01%. At this concentration, basal fEPSP slopes were not significantly affected (data not shown). Due to the sensitivity of nifedipine to light, experiments were performed in semi-darkness, and perfusate containers were protected from light with aluminum foil. Where indicated,

10 μM nifedipine was bath applied (following 10 min of stable baseline) for 15 min pre- and 10 mins post-tetanus.

Data and Statistical Analysis

fEPSP measurements were taken offline from two points during the initial fEPSP slope using Axon Clampex 7.0 (Axon Instruments, Foster City, CA). Raw data points were imported into Excel 2000 (Microsoft, Seattle, WA) for further analysis. Intial fEPSP slopes were calculated and expressed as a percentage of the averaged pre-treatment baseline. The latter was obtained by averaging 20 minutes of fEPSPs measured during baseline acquisition. Data are plotted as mean ± standard error of mean (SEM). Student's t-test was used to compare mean fEPSP slopes within paired data sets, with a significance level of $p < 0.05$ (denoted on graphs with an "*"). Data sets with more than two comparison groups were analyzed with ANOVA using GraphPad Instat 3.00 (GraphPad Software, San Diego, CA). Tukey-Kramer multiple comparisons test was completed if ANOVA analysis indicated a significant difference between groups ($p < 0.05$, denoted on graphs with an "*"). Kolmogorov-Smirnov, and Bartlett's tests were done to determine normality and to analyze standard deviations, respectively, of all test groups. Where indicated, Kruskal-Wallis test (non-parametric ANOVA) was applied accordingly. In all electrophysiological data, "n" indicates number of slices.

RESULTS

Differential Contribution of L-Type VGCCs to L-LTP

LTP induction at the hippocampal CA3-CA1 synapse requires postsynaptic calcium influx (Lynch *et al.*, 1983). There are two main routes of calcium entry into postsynaptic CA1 neurons: through N-methyl-D-aspartate (NMDA) receptors and through voltage-gated L-type calcium channels (VGCCs). Different signal transduction cascades can be activated depending on the frequency, number, and temporal spacing of the stimuli to elicit different forms of LTP (Grover and Teyler, 1990; Bading *et al.*, 1993; Huber *et al.*, 1995; Çavus and Teyler, 1996; Chen *et al.*, 1998; Morgan *et al.*, 1999). I began this study by characterizing the VGCC-dependence of different types of LTP. All three LTP protocols used here (E-LTP: 1X100Hz, "compressed" L-LTP: 4X100Hz@3s, and "spaced" L-LTP: 4X100Hz@5min) requires NMDA receptor activation for their induction (Woo et al., 2003), but their dependence on calcium influx through voltage-gated calcium channels is unknown.

Bath perfusion of the L-type calcium channel antagonist nifedipine (Nif; 10 μM) during tetanus revealed a differential involvement of VGCCs in LTP generated from the different induction protocols. Mean fEPSP values were measured 60 min after E-LTP induction in the presence of nifedipine (80 min, Nif: 142 ± 9%, n = 12) and in control slices that were not treated with nifedipine (80 min, Ctrl: 136 ± 12%, n = 6; **Figure 2.01B**). Statistical analyses showed that the difference between groups was not significant ($p > 0.05$). L-LTP induced with both the temporally compressed (ITI = 3 s; **Figure 2.01C**) and spaced (ITI = 5 min; **Figure 2.01D**) protocols elicited a stable and

persistent potentiation of fEPSP slopes in control slices that lasted 120 min post-tetanus (140 min, ITI = 3s: 214 ± 19%, n = 9; 155 min, ITI = 5 min: 227 ± 16%, n = 13). Bath application of nifedipine during L-LTP induction significantly impaired L-LTP elicited with the compressed protocol (140 min, ITI = 3s: 98 ± 19%, n = 8; $p < 0.001$). In contrast, blockade of L-type calcium channels did not affect L-LTP induced with the temporally spaced protocol (155 min, ITI = 5 min: 209 ± 27%, n = 6; $p > 0.05$). A summary histogram of the effect of nifedipine on E-LTP and L-LTP induced with the two protocols is shown in **Figure 2.01E**. Control experiments with bath application of nifedipine without inducing LTP were done to rule out harmful effects of nifedipine on basal synaptic transmission (Hirasawa and Pittman, 2003). Within-slice comparisons of fEPSP levels during (99 ± 4%) and 120 min after (92 ± 7%) drug application did not differ significantly from baseline values (91 ± 8%, n = 4; $F(2,9) = 0.4479$, $p = 0.6524$; **Figure 2.01A**).

These results indicate that L-type calcium channels are not required for transient E-LTP or L-LTP induced with a temporally spaced stimulation pattern. In contrast, L-LTP induced by compressing the same number of stimuli into a shorter time frame requires calcium influx through NMDA receptors (Woo et al., 2003) and L-type VGCCs. Previous experiments showed that low-frequency stimulation (LFS) at 5-Hz for 3 min impairs subsequently induced L-LTP (Woo and Nguyen, 2002). In those experiments, L-LTP was induced with the temporally spaced protocol. I wanted to investigate if LFS also impairs L-LTP induced with a compressed stimulation protocol, in the hopes of elucidating the mechanisms underlying this type of metaplasticity.

Transient Homosynaptic and Heterosynaptic Depression Evoked by Low Frequency Stimulation

Previous experiments demonstrated a homosynaptic inhibition of subsequently induced LTP by 5-Hz stimulation for 3 min (Woo and Nguyen, 2002). However, two-pathway experiments revealed a transient heterosynaptic depression in response to 5-Hz LFS. Two-pathway recordings were conducted using two stimulating electrodes (S1 and S2) positioned on either side of the recording electrode, all placed in stratum radiatum of area CA1 in mouse hippocampal slices (**Figure 2.02A**). **Figure 2.02B** shows sample traces and data from a representative experiment in which successive stimulation through S1 and S2 revealed no difference in the initial fEPSP slope in S2 at all time intervals tested (40 ms – 200 ms). A lack of interpathway paired-pulse facilitation was used to confirm the independence of the two inputs.

Low-frequency stimulation (LFS) at 5-Hz for 3 min produced a transient synaptic depression of fEPSPs in area CA1 (**Figures 2.02C and 2.02D**), both in the pathway receiving the conditioning stimulation (i.e., homosynaptic (Hom) inhibition; S1; 64 ± 4%, $n = 5$; $F(2,12) = 29.381$, $p < 0.0001$) and at other synapses converging on the same postsynaptic cells (i.e., heterosynaptic (Het) inhibition; S2; 69 ± 7%, $n = 5$; $F(2,12) = 6.893$, $p = 0.0102$). Post-hoc tests revealed a significant depression of initial fEPSP slope, as compared to baseline values, immediately following application of LFS (S1, Hom: $p < 0.001$; S2, Het: $p < 0.05$). Recovery to baseline values occurred within 10 min of starting LFS (S1, Hom: 106 ± 5%, $n = 5$; $p > 0.05$; S2, Het: 93 ± 3%, $n = 5$; $p > 0.05$).

Prior LFS Does Not Affect Homosynaptically- or Heterosynaptically-Induced E-LTP

Because LFS elicits both homo- and hetero-synaptic depression, I investigated whether the inhibitory effects of prior LFS also occurred at non-stimulated inputs. I examined the homosynaptic and heterosynaptic effects of prior LFS on E-LTP, and L-LTP induced with temporally compressed and spaced stimulation. To examine the effects of LFS on subsequently induced E-LTP, 5-Hz stimulation was applied to one pathway. Ten minutes after the beginning of LFS – a time point at which fEPSPs had recovered to control baseline values (Ctrl: 96 ± 4%, n = 7; Hom: 92 ± 2%, n = 6; Het: 95 ± 5%, n = 6; $F(2,16) = 0.3350$, $p = 0.7203$) – one train of tetanus was given to either the pathway that had received the 5-Hz conditioning stimulus (i.e., homosynaptic) or to a separate pathway (i.e., heterosynaptic). Prior LFS did not affect the amount of potentiation observed 60 min after E-LTP induction, as compared to controls that received no prior LFS (Ctrl: 119 ± 5%, n = 7; Hom: 119 ± 6%, n = 6; Het: 116 ± 13%, n = 6; Kruskal-Wallis ANOVA, KW = 0.2090, $p = 0.9008$; **Figure 2.03**). **Figure 2.03B** summarizes mean fEPSP measurements taken during baseline (a), just prior to E-LTP induction after recovering from LFS (b), and 60 min post-tetanus (c).

Prior LFS Impairs VGCC-Dependent and VGCC-Independent L-LTP in a Homosynaptic and Heterosynaptic Manner

I next examined the effect of prior LFS on VGCC-dependent L-LTP generated with the compressed stimulation protocol. Again, I waited 10 min after initial application of LFS for fEPSP values to return to baseline (Ctrl: 99 ± 1%, n = 12; Hom: 94 ± 2%, n =

7; Het: 99 ± 3%, n = 7; F(2,23) = 1.659, p = 0.2123). L-LTP induced in the homosynaptic (Hom: 108 ± 7%, n = 7) or heterosynaptic (Het: 97 ± 9%, n = 7) pathway following LFS decayed close to baseline by 120 min post-induction. In contrast, control slices that did not receive prior LFS remained significantly potentiated (Ctrl: 149 ± 7%, n = 12; F(2, 23) = 13.582, $p < 0.001$; **Figures 2.04A and 2.04C**). Post-hoc tests revealed a significant impairment of homosynaptic ($p < 0.01$) and heterosynaptic L-LTP ($p < 0.001$) compared to control slices that received L-LTP stimulus without prior LFS (**Figure 2.04C**).

As reported previously, prior 5-Hz LFS impairs homosynaptically L-LTP induced with the spaced stimulation protocol (VGCC-independent). I extend these findings by showing that prior LFS can also impair this type of L-LTP at other synapses converging on the same postsynaptic cells (heterosynaptic inhibition). Following a similar paradigm, I induced L-LTP with the spaced stimulation protocol in the homosynaptic or heterosynaptic pathway 10 min after LFS conditioning. L-LTP induced following LFS was significantly less than control slices that did not receive prior LFS at 120 min post-induction (Ctrl: 171 ± 11%, n = 10; Hom: 102 ± 15%, n = 7; Het: 124 ± 10%, n = 9; F(2,23) = 8.764, p = 0.0015; **Figure 2.04B**). Post-hoc analyses showed a significant impairment of homosynaptic ($p < 0.01$) and heterosynaptic groups ($p < 0.05$) compared to controls (**Figure 2.04D**). Heterosynaptically-induced L-LTP following LFS was slightly greater than homosynaptically-induced L-LTP following LFS, although the difference was not statistically significant ($p > 0.05$).

These results show that prior LFS impairs subsequently induced L-LTP in a homosynaptic and heterosynaptic manner. Maintenance of VGCC-dependent and

VGCC-independent L-LTP were similarly affected by prior LFS. Prior LFS produced significant impairments in the amount of initial potentiation elicited with the spaced L-LTP protocol (1 min; Ctrl: 318 ± 20%, n = 10; Hom: 226 ± 16%, n = 7; Het: 227 ± 22%, n = 9; $F(2,23) = 7.253$, $p = 0.0036$). Post-hoc analyses showed a significant impairment in both homosynaptic ($p < 0.05$) and heterosynaptic ($p < 0.01$) groups. This result is in contrast with previously published results that showed no effect of prior LFS on homosynaptic initial L-LTP elicited with a spaced tetraburst stimulation protocol (Woo and Nguyen, 2002). Initial levels of potentiation elicited with one 100-Hz train (Ctrl: 196 ± 13%, n = 7; Hom: 175 ± 6%, n = 6; Het: 196 ± 22%, n = 6; Kruskal-Wallis ANOVA, KW = 0.5496, p = 1.197) or the compressed tetraburst (Ctrl: 213 ± 8%, n = 12; Hom: 187 ± 7%, n = 7; Het: 186 ± 12%, n = 7; $F(2,23) = 3.004$, $p = 0.0693$) stimulation protocol were not affected by prior LFS.

DISCUSSION

My findings have revealed a novel form of homosynaptically and heterosynaptically expressed metaplasticity of L-LTP. I investigated the effects of prior low frequency stimulation (LFS) on voltage-gated calcium channel (VGCC) dependent and VGCC-independent forms of LTP. Prior LFS did not affect transient E-LTP (which does not require VGCC activation). In contrast, pre-conditioning with LFS impaired the maintenance of both VGCC-dependent and VGCC-independent L-LTP with a differential effect on initial levels of potentiation induced by the two L-LTP protocols. Prior LFS impaired both types of L-LTP in a homosynaptic and heterosynaptic manner.

Heterosynaptic Inhibition of L-LTP

Activity-dependent regulation of synaptic plasticity, or metaplasticity, can act to maintain the maximum flexibility of a given synapse. In order to effectively preserve the capacity for plasticity across multiple inputs to a neuron, metaplastic effects should be expressed in a cell-wide (i.e., heterosynaptic) manner (Bienenstock et al., 1982; Abraham and Bear, 1996; Turrigiano and Nelson, 2004). Heterosynaptic facilitation and reduced thresholds for inducing L-LTP have been previously reported (Frey and Morris, 1997). However, most studies examining metaplastic down-regulation of LTP have involved homosynaptic alterations (Huang et al., 1992; Christie and Abraham, 1992; Fujii et al., 1996; Woo and Nguyen, 2002). I report a novel form of metaplasticity, induced by LFS, that results in both homosynaptic and heterosynaptic inhibition of subsequent L-LTP. In contrast, E-LTP induced in homosynaptic or heterosynaptic inputs was not affected by prior LFS. Because prior LFS does not affect shorter-lasting facilitation, LFS may act to selective supress the expression of subsequent L-LTP elicited by multiple trains of high frequency stimuli. In this manner, prior LFS may prevent persistent synaptic facilitation from saturating synaptic strength in neural networks recruited for information storage.

LTP induction at the CA3-CA1 synapse requires a rise in postsynaptic calcium levels (Lynch et al., 1983; Malenka et al., 1988; Malenka et al., 1992). The main route of calcium entry into postsynaptic neurons is through NMDA receptors (Collingridge et al., 1983) although in some cases, this rise in intracellular calcium is amplified by concurrent activation of voltage-gated calcium channels (Grover and Teyler, 1990). NMDA receptor activation is associated with the activation of protein kinases whereas VGCC activation

has been linked to tyrosine kinases. Thus, multiple forms of LTP with different induction and maintenance mechanisms can be elicited depending on the stimulation protocol (Huber et al., 1995; Çavus and Teyler, 1996; Raymond and Redman, 2002). I found that prior LFS impaired the maintenance of both VGCC-dependent and VGCC-independent forms of L-LTP. Furthermore, inhibitory effects of LFS were evident in a non-input specific (i.e., heterosynaptic) manner. These data suggest that LFS may exert its metaplastic effects on L-LTP by acting on more general mechanisms of cellular consolidation of long-lasting plasticity.

Homosynaptic and Heterosynaptic Depression

What are the mechanisms underlying heterosynaptic regulation of L-LTP? In the present study, metaplastic effects are associated with a novel form of synaptic depression that is expressed in a heterosynaptic manner. Gunther Stent (1973) proposed a complementary postulate to Donald Hebb's to predict that synaptic connections would weaken when they are inactive at same time that the postsynaptic neuron is active (i.e., heterosynaptic depression). Heterosynaptic depression has been previously reported at synapses "silent" during strong postsynaptic depolarization, such as that associated with LTP induction (Lynch et al., 1977; Alger et al., 1978; Kerr and Abraham, 1993; Holland and Wagner, 1998; Wang and Wagner, 1999; Abraham et al., 2001). Voltage-gated calcium channels and intracellular calcium stores have been implicated in conveying the signal to silent synapses (Wickens and Abraham, 1991; Artola and Singer, 1993; Nishiyama et al., 2000). Although non-associative depression did not affect subsequent

LTP under the conditions of my experiments, similar mechanisms may underlie LFS-mediated heterosynaptic depression.

Various second messenger systems have been proposed to detect the moderate changes in Ca^{2+} associated with non-associative depression and to translate them into changes in synaptic efficacy. Protein phosphatases are preferentially activated by low frequency stimulation (Mulkey et al., 1993, 1994; Morishita et al., 2001), and significantly, prior LFS has been shown to engage protein phosphatases 1 and 2A (PP1 and PP2A, respectively) during homosynaptic inhibition of subsequent L-LTP (Woo and Nguyen, 2002). In addition, nuclear PP1 and PP2A activity can regulate the phosphorylation state of transcription factors that have been implicated in L-LTP expression, such as CREB (Bito et al., 1996; Genoux et al., 2002)

Functional Significance

Collectively, my data shows that prior low frequency stimulation can impair subsequently induced L-LTP in a non-input specific manner. Prior LFS does not affect transient E-LTP and may contribute to filtering of multiple episodes of synaptic activation that is associated with L-LTP expression. This form of synaptic plasticity may help encode behavioural events that require relatively transient neural representations that do not need to be preserved for extended periods of time (Morris RG, 2001). In addition, because repeated bouts of potentiation can result in a saturation of synapses, LFS-mediated inhibition may act to keep synaptic strengths within a functional range that is optimal for information storage. Thus, I speculate that synaptic activity at the theta (5-

Hz) frequency may, under some circumstances, "gate" or control the expression of future long-lasting plasticity across multiple synaptic pathways in a neural network.

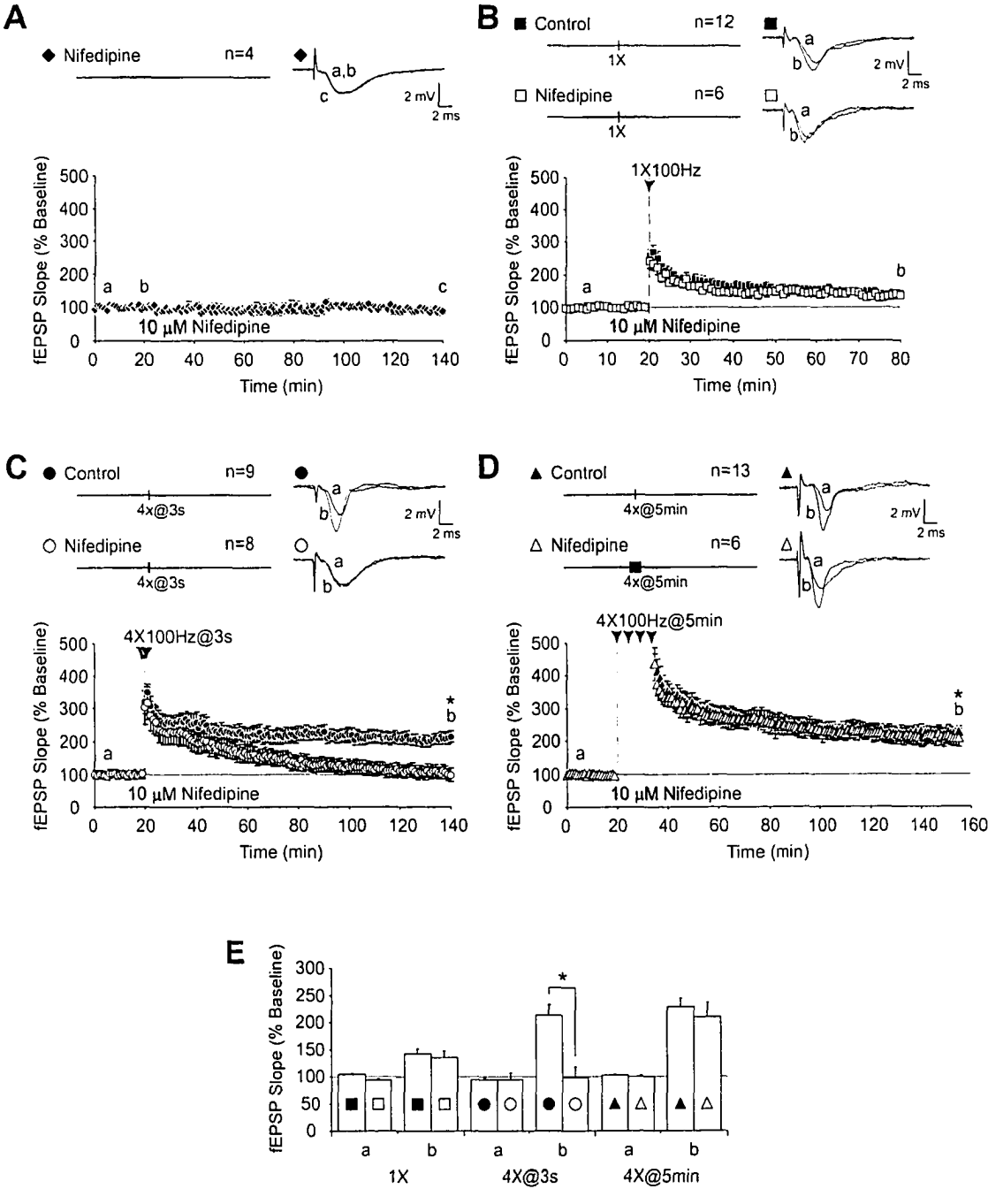

Figure 2.01: Differential requirement for L-type VGCCs in CA1-LTP. (A) 10 μM nifedipine application for 25 min does not affect basal synaptic transmission nor general health of hippocampal slices. **(B)** E-LTP elicited by a single 100-Hz tetanus does not require L-type VGCC activation. **(C)** L-LTP induced with a compressed four-train stimulation protocol requires VGCC activation. **(D)** L-LTP induced with a spaced four-train stimulation protocol does not require VGCC activation. **(E)** Summary histogram of mean fEPSP slopes during baseline (a), and 60 min after E-LTP induction or 120 min after L-LTP induction (b). Asterisks indicate statistical significance (*$p < 0.05$).

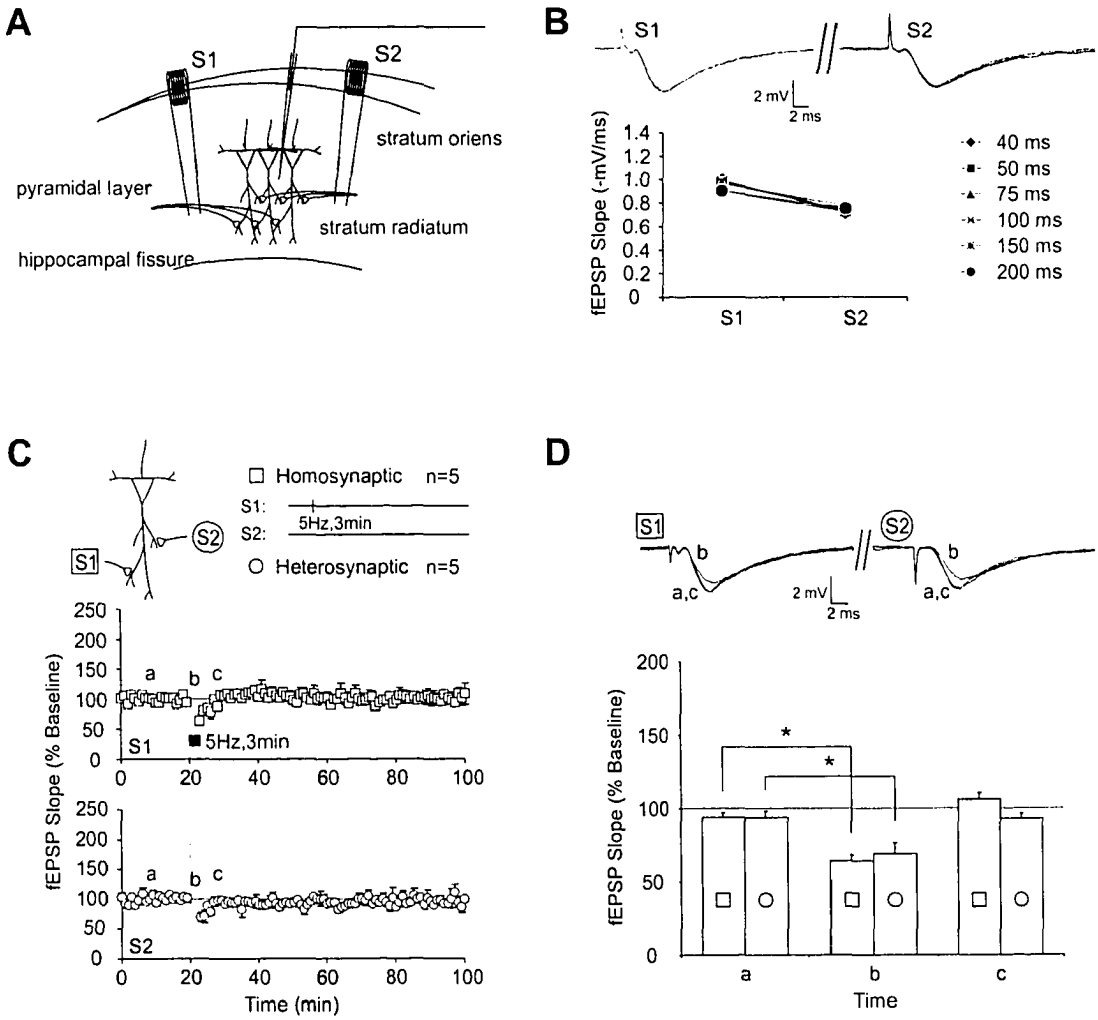

Figure 2.02: 5-Hz LFS produces a transient homosynaptic and heterosynaptic depression. **(A)** Schematic of area CA1 in a mouse hippocampus slice, showing positions of two stimulating electrodes (S1 and S2) and a single recording electrode placed in stratum radiatum. **(B)** Sample sweeps and analysis from a representative experiment showing a lack of inter-pathway paired-pulse facilitation. **(C)** Conditioning stimulation at 5-Hz for 3 min produces a transient depression in both homosynaptic and heterosynaptic pathways. **(D)** Sample sweeps and a summary histogram of mean fEPSP measurements during baseline (a), immediately after 5-Hz stimulation (b), and 10 min after initial 5-Hz stimulation (c). Asterisks indicate statistical significance (*$p < 0.05$).

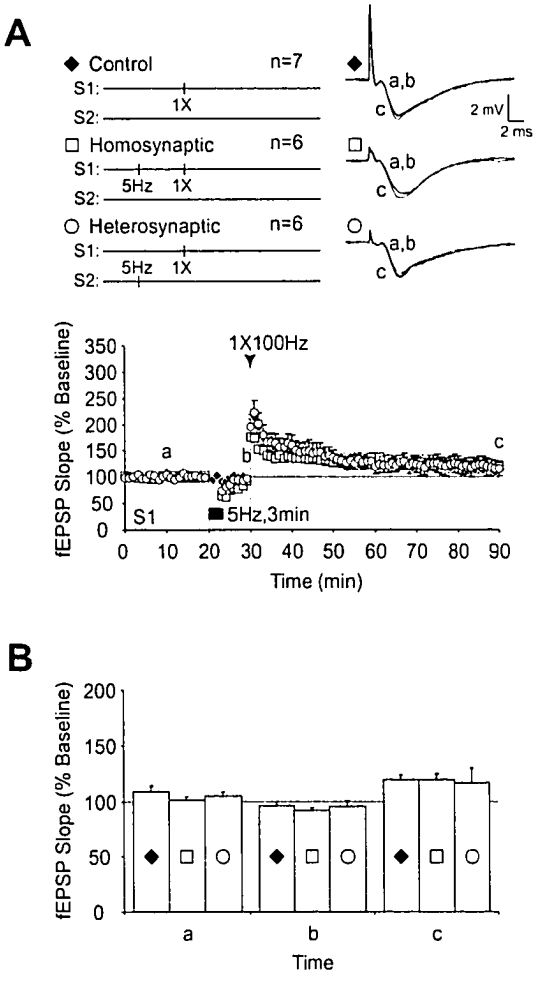

Figure 2.03: Prior low-frequency stimulation does not affect E-LTP. (A) E-LTP elicited by a single 100-Hz tetanus given to the homosynaptic (S1) or heterosynaptic (S2) pathway is not affected by prior LFS. **(B)** Summary histogram of of mean fEPSP slopes during baseline (a), following recovery from LFS (b), and 60 min after LTP induction (c).

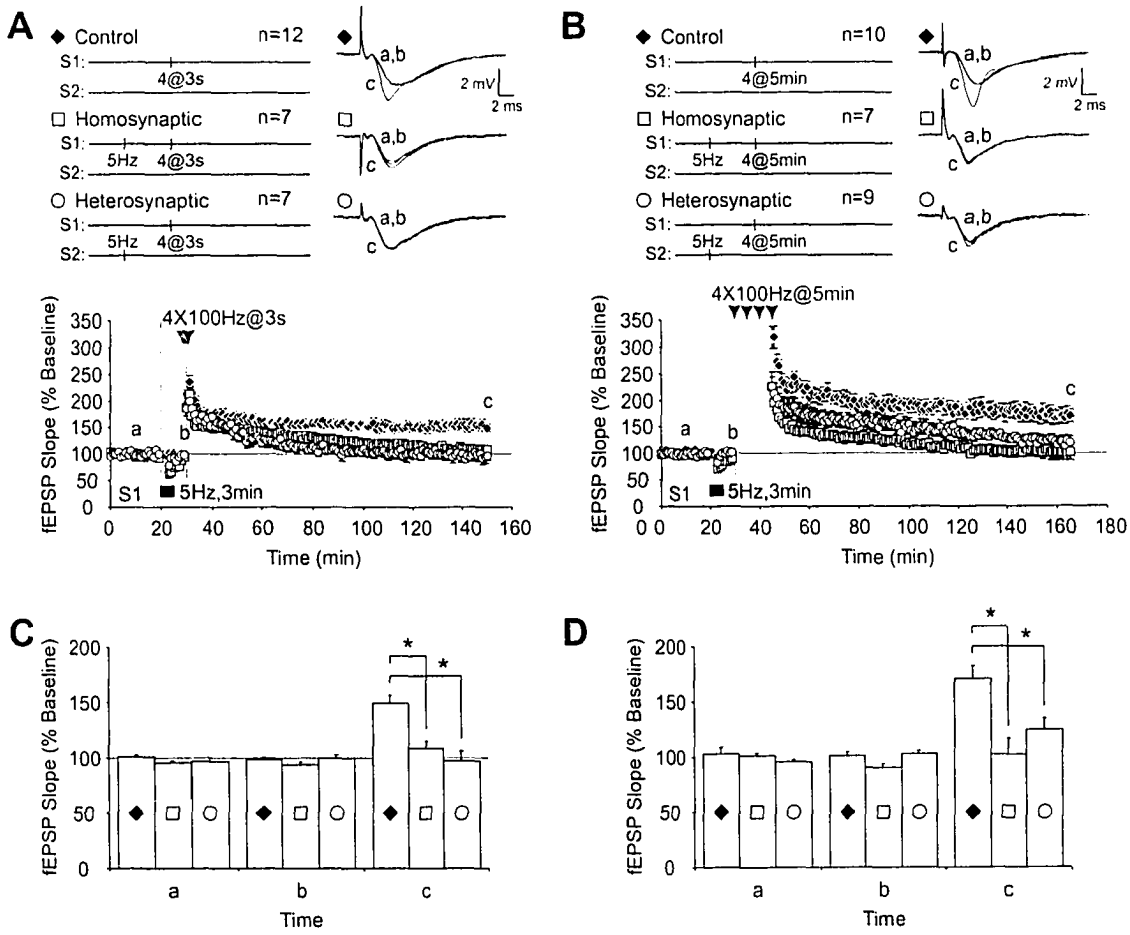

Figure 2.04: Prior LFS impairs homosynaptically and heterosynaptically induced L-LTP. **(A)** VGCC-dependent L-LTP induced with four temporally compressed 100-Hz trains of stimuli applied to the homosynaptic or heterosynaptic set of inputs is significantly impaired by prior 5-Hz LFS. **(B)** VGCC-independent L-LTP induced with four temporally spaced 100-Hz trains of stimuli applied to the homosynaptic or heterosynaptic set of inputs if significantly impaired by prior LFS. **(C)** and **(D)** summarize L-LTP data elicited with compressed and spaced, respectively, tetraburst L-LTP data. Columns represent mean fEPSP values during baseline (a), 10 min after beginning 5-Hz LFS (b), and 120 min after L-LTP induction (c). Significant impairments (*$p < 0.05$) in the level of potentiation at 120 min (c) are observed in groups that received prior 5-Hz conditioning stimuli.

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CHAPTER 3:

METAPLASTIC INHIBITION OF SYNAPTIC TAGGING AND CAPTURE OF L-LTP EXPRESSION

INTRODUCTION

Long-term potentiation (LTP) is a form of activity-dependent synaptic enhancement that is well characterized in the hippocampus (Bliss and Lømo, 1973; Bliss and Collingridge, 1993). LTP can be divided into at least two temporal phases. The early phase of LTP (E-LTP) lasts for less than a few hours in vitro, and does not require translation or transcription (Reymann et al., 1985; Huang and Kandel, 1994). Late-phase LTP (L-LTP) lasts for several hours in vitro (Andersen et al., 1977; Reymann et al., 1985; Huang and Kandel, 1994) and requires gene expression and de novo protein synthesis (Krug et al., 1984; Stanton and Sarvey, 1984; Deadwyler et al., 1987; Frey et al., 1988; Nguyen et al., 1994; Bourtchouladze et al., 1994).

Long-term potentiation displays input specificity. That is, it is expressed only at appropriately activated synapses. In contrast, transcriptional products may be shipped cell-wide. Because L-LTP requires transcription, a mechanism must exist to mark, or tag, active synapses in such a way as to correctly direct newly synthesized gene products from the soma and thus preserve the synapse specificity of LTP. The "synaptic tagging" model proposes that input-specific L-LTP expression requires: (1) gene expression and subsequent cell-wide distribution of newly synthesized plasticity-related proteins (PRPs); and (2) an activity-dependent synaptic tag that allows for synapse-specific capture of LTP-stabilizing proteins (Frey and Morris, 1997, 1998). Frey and Morris (1997) first provided evidence for the synaptic tagging theory in the rat hippocampus. They demonstrated that proteins synthesized in response to activation of long-term synaptic changes at one set of synapses could be successfully captured and utilized by another set

of synapses to express L-LTP, even if these synapses only experienced activation that is normally sufficient to produce only a transient potentiation (Frey and Morris, 1997, 1998). Because stimuli that are insufficient for inducing L-LTP on their own can nonetheless generate a synaptic tag, triggering L-LTP-associated transcription can result in a cell-wide decrease in the threshold for inducing long-term plasticity. This unregulated facilitation of synaptic strength can lead to a saturation of synaptic strength across neural networks of connections, thereby impeding the storage of new information. Thus, a mechanism should exist to regulate synaptic tagging and limit L-LTP expression. Indeed, various patterns of synaptic activity have been found to impair subsequent LTP (Huang et al., 1992; Christie and Abraham, 1992; Fujii et al., 1996; Woo and Nguyen, 2002). However, it is unknown whether prior synaptic activity can inhibit subsequent L-LTP by acting on synaptic capture.

Previously, I reported a novel form of homosynaptically and heterosynaptically expressed metaplasticity. Prior low frequency stimulation (LFS) impaired subsequently induced L-LTP, but not E-LTP. My data indicate that LFS does not affect L-LTP associated gene expression but may instead impair synaptic tagging that would otherwise permit capture of somatic gene products required for stabilizing LTP. Activity-dependent changes in gene expression can also confer immunity of LTP to reversal (depotentiation, Dpt) to heterosynaptically-activated inputs. This type of acquired immunity to Dpt is also impaired by prior LFS. In contrast, Dpt immunity arising from local protein synthesis is not affected by prior LFS, demonstrating that the effects of LFS on Dpt are specific to immunity mechanisms that involve synaptic capture. My study provides new

insight into how the synapse specificity of L-LTP and integration of synaptic events over time may be regulated at a cell-wide level by prior synaptic activity.

Materials and Methods

Hippocampal slice preparation

All experiments were conducted with female C57BL/6 mice (aged 10-14 wks, Charles River, Montreal, Canada) housed at the University of Alberta Animal Care Facility. Care and experimental procedures were in accordance with guidelines approved by the Canadian Council on Animal Care. Animals were cervically dislocated and decapitated, and their brains removed and immersed in ice-cold (4°C) artificial cerebral spinal fluid (ACSF) bubbled with a "carbogen" mixture of 95% O_2, 5% CO_2. The ionic composition of my ACSF was the same as in previous studies (Nguyen and Kandel, 1997) consisting of (in mM): 124 NaCl, 4.4 KCl, 1.30 $MgSO_4 \cdot 7H_2O$, 1.0 $NaH_2PO_4 \cdot H_2O$, 26.2 $NaHCO_3$, 2.5 $CaCl_2$, and 10 D-glucose. The hippocampi were dissected free and transverse slices (400 μm thickness) were cut on a manual tissue chopper (Stoelting, Wood Dale, Illinois). Slices were transferred onto a nylon mesh in an interface chamber where they were perfused with carbogenized ACSF (1 ml/min). The temperature of the interface chamber was maintained at 28°C. Slices were allowed to recover for at least 60 min before experiments commenced.

Electrophysiology

Extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded in stratum radiatum of area CA1 with a glass microelectrode (A-M Systems, Carlsborg, WA) filled with ACSF (electrical resistances: 2-4 MΩ). Extracellular stimulation of the Schaffer collateral pathway was accomplished with two nickel-chromium (A-M Systems, Carlsborg, WA) bipolar stimulating electrodes (diameter 130um) placed on either side of a single recording electrode in s. radiatum.

Evoked fEPSPs were amplified, digitized (DigiData 1200B Interface, Axon Instruments, Foster City, CA), and analyzed using Axon Clampex 7.0 (Axon Instruments, Foster City, CA). The test stimulus intensity was adjusted to produce basal fEPSP sizes that were 40% of maximal evoked fEPSP amplitude (Grass S48 Stimulator). Test stimuli were delivered to the Schaffer collaterals once per minute (0.08 ms stimulus duration) with a 200 ms separation between stimulation through the two electrodes (stimulating electrodes "S1" and "S2"; This thesis, **Figure 2.02B**). To ensure that fEPSPs evoked through each stimulating electrode resulted from activation of two independent synaptic pathways, I positioned the electrodes so that no paired pulse facilitation (PPF) was evident following sequential activation of S1 and S2. Interpathway PPF was assessed at various time intervals (40 ms, 50 ms, 75 ms, 100 ms, 150 ms and 200 ms) during baseline acquisition and at the end of experiments.

LTP was elicited by delivering 1 stimulus train (1s duration at 100-Hz) to elicit E-LTP (Huang and Kandel, 1994), or 4 stimulus trains (1s duration at 100-Hz) with an intertrain interval of 3 s to induce L-LTP (Woo et al., 2003A). Low frequency stimulation (LFS) consisted of 5-Hz stimulation for 3 mins (Woo and Nguyen, 2002). I

used this same LFS to induce depotentiation (reversal of LTP; Barrionuevo et al., 1980; Stäubli and Lynch, 1990; Woo and Nguyen, 2002).

Drugs

Actinomycin D (Act D; Bioshop Canada, Burlington, ON), a transcription inhibitor, was added to ACSF to a final concentration of 25 μM from 25 mM stock in prepared in dimethylsulfoxide (DMSO, Sigma Aldrich). At 25 μM, Act D has been shown to block transcription by >70% in hippocampal slices (Nguyen et al., 1994). All drugs were bath applied. Experiments with actinomycin D were performed in dimmed-light conditions. Final concentration of applied DMSO was 0.01%. At this concentration, basal fEPSP slopes were not significantly affected (data not shown).

Data and Statistical Analysis

The initial fEPSP slope was measured and expressed as a percentage of the averaged pre-treatment baseline. The latter was obtained by averaging 20 minutes of fEPSPs measured during baseline acquisition. Data are plotted as mean ± standard error of mean (SEM). Student's t-test was used to compare mean fEPSP slopes within paired data sets, with a significance level of $p < 0.05$ (denoted on graphs with an "*"). Data sets with more than two comparison groups were analyzed with ANOVA. Tukey-Kramer multiple comparisons test was completed if ANOVA analysis indicated a significant difference between groups ($p < 0.05$, denoted on graphs with an "*"). Kolmogorov-Smirnov, and Bartlett's tests were done to determine normality and to analyze standard deviations, respectively, of all test groups. Where indicated, Kruskal-Wallis test (non-

parametric ANOVA) was applied accordingly. In all electrophysiological data, "n" indicates number of slices.

RESULTS

Synaptic Capture of L-LTP and Immunity to Depotentiation

According to the "synaptic tag" theory, input-specific L-LTP expression requires (1) a signal to trigger cell-wide delivery of plasticity-related proteins (PRPs); and (2) a synaptic tag that marks activated synapses to allow capture of LTP-stabilizing proteins (Frey and Morris, 1997, 1998). To probe the mechanism by which prior LFS inhibits subsequent L-LTP, we modeled our protocol on previous experiments that examined synaptic capture and L-LTP expression (Frey and Morris, 1997). A single tetanus of 100-Hz (1s duration) induces early phase, transient LTP (E-LTP) that decays within 1-2 hrs and requires only posttranslational modifications of existing proteins (Reymann et al., 1985; Huang and Kandel, 1994). Multiple trains of 100-Hz stimulation induce long-lasting LTP (L-LTP) that requires gene expression and de novo protein synthesis (Frey et al., 1988, Huang and Kandel, 1994; Nguyen et al., 1994). We used a protocol similar to that originally described by Frey and Morris (1997) whereby E-LTP at one set of inputs can be extended by pairing it with L-LTP established at a separate set of converging inputs.

E-LTP produced by a single 100-Hz tetanus decays to baseline values within 2 hours of induction (140 min: 101 ± 6%, n = 8) and can be reversed to baseline values, or depotentiated (Dpt), by subsequent low-frequency stimulation applied 5 min after tetanus

(S2, 110 min; 97 ± 6%, n = 5; **Figure 3.01A**). Pairing E-LTP (weak tetanic stimulation) at one set of inputs (S2 pathway) with established L-LTP (strong tetanic stimulation) at an independent set of inputs (S1 pathway) results in non-decremental LTP of the "weaker" pathway (S2, 170 min: 158 ± 9%, n = 8; **Figure 3.01B**). In addition to its prolonged expression, this LTP resembles L-LTP in its immunity to depotentiation. Within 55 min of depotentiating LFS, fEPSP measurements had recovered to potentiated levels and did not differ significantly from non-depotentiated values taken at a similar time-point (S2, 110 min; Ctrl: 147 ± 10%, n = 8; Dpt: 139 ± 9%, n = 7; $p < 0.05$; **Figure 3.01B**).

Pairing E-LTP and L-LTP within a 30 min time window results in L-LTP expression at both sets of synaptic inputs, consistent with previous studies (Frey and Morris, 1997; Barco et al., 2002). Moreover, successful synaptic capture by weak stimulation at S2 is evident as a persistent potentiation that is immune to depotentiation (Barco et al., 2002; Woo and Nguyen, 2002). Expression of stable L-LTP, and its immunity to Dpt, can be used to gauge successful heterosynaptic capture of L-LTP.

A Critical Period of Transcription is Needed for Synaptic Capture of L-LTP

Because prior LFS impairs L-LTP in a cell-wide manner, I hypothesized that it may be impairing transcription to prevent cell-wide distribution of plasticity-related proteins. As an initial step towards assessing whether LFS inhibits L-LTP-associated gene expression, I characterized the transcriptional requirements of synaptic capture. Previous experiments that paired weak tetanic stimulation with L-LTP have shown that L-LTP must trigger de novo protein synthesis for successful synaptic capture at pathways

experiencing E-LTP-inducing stimuli (Frey and Morris, 1997). However, translational processes can occur within the soma and in dendrites, and the process of synaptic tagging may itself require protein synthesis. Whereas Frey and Morris (1997) suggest that synaptic capture occurs independently of protein synthesis, more recent studies have reported a partial sensitivity to protein synthesis inhibition (Barco et al., 2002; Alarcon et al., 2004). Therefore, we used a transcriptional inhibitor, actinomycin D (Act D; 25 μM), to examine the effects of blocking transcription in the synaptic capture model. Strong tetanization (S1 pathway) in the presence of Act D produced LTP that decayed to baseline values within 2 hr (S1, 185 min: 104 ± 7%, n = 6). Weak tetanic stimulation of an independent pathway 30 min after strong tetanization of S1 also produced decremental LTP (S2, 185 min: 100 ± 6%, n = 6; **Figure 3.02A**). In contrast, application of Act D during the weak LTP tetanus in S2, but after strong tetani in S1, did not affect L-LTP expression in either pathway (170 min; S1: 153 ± 10%, n = 6; S2: 142 ± 4%, n = 6; **Figure 3.02B**). These results define a critical time period for transcription to be triggered following strong tetanic stimulation: from immediately after L-LTP induction to 20 min post-tetanus. Our data mirror previous findings indicating a specific time window for transcription in L-LTP expression (Nguyen et al., 1994).

Our results support the synaptic tag model in which L-LTP that is captured by weak tetanic stimulation does not depend on activating transcriptional processes itself. The duration of transient E-LTP induced at one set of synapses is extended by capturing L-LTP associated gene products produced by previous synaptic activity at other synapses on the same postsynaptic neurons. The experiments of **Figure 3.02B** show that

transcription is not required in the synaptic tagging process, and also shows that Act D application does not result in a non-specific run-down of LTP.

Prior LFS Does Not Affect Somatic Gene Expression Associated With L-LTP Expression

Pairing strong and weak tetanization allows for the dissociation of metaplastic effects on gene expression versus effects on synaptic tagging. If LFS inhibits L-LTP by impairing transcription, then synaptic capture by subsequent weak LTP stimuli should be unsuccessful because there will be no L-LTP gene products for those synapses to capture. In other words, application of LFS prior to L-LTP induction should exert effects that mirror those seen after applying actinomycin D during strong tetanization. Like Act D application during strong tetanization, prior LFS effectively blocked L-LTP expression induced homo- or hetero-synaptically 10 min after LFS (S1, 180 min; Hom: 103 ± 6%, n = 6; Het: 118 ± 7%, n = 6). However, unlike the results observed with Act D application, weak LTP stimulation given 30 min later (40 min after start of LFS) to S2 elicited stable and persistent potentiation 120 min later (S2, 180 min; Hom: 146 ± 13%, n = 6; Het: 142 ± 5%, n = 6; **Figures 3.03A and 3.03B**). In addition, these inputs acquired immunity to Dpt (**Figures 3.03A and 3.03B**). ANOVA revealed significant differences between levels of potentiation elicited by weak LTP stimulation (S2) with delayed Act D or prior LFS application, compared to Act D application during initial strong tetanus ($F(3,20) = 6.586$, $p = 0.0028$). Post-hoc tests indicate that significantly more potentiation was seen at 120 min with delayed Act D application ($p < 0.05$; filled circles) and both homosynaptic ($p < 0.01$; filled diamonds) and heterosynaptic ($p < 0.05$; filled squares) 5-

Hz conditioning, than with Act D application during strong LTP tetanus (filled triangles; **Figure 3.03C**). Student's t-tests confirmed that weak LTP applied to S2 acquired immunity to depotentiation despite 5-Hz pre-conditioning in the homosynaptic (S2, 110 min; Ctrl: 143 ± 8%, n = 6; Dpt: 139 ± 4%, n = 6, $p > 0.05$) and heterosynaptic pathways (S2, 110 min; Ctrl: 139 ± 7%, n = 6; Dpt: 138 ± 6%, n = 6, $p > 0.05$; **Figure 3.03D**).

Contrary to my original hypothesis, metaplastic effects of LFS are not mediated by inhibition of L-LTP-associated gene expression. Instead, LFS may be interfering with synaptic tagging.

Prior LFS Impairs Synaptic Capture of L-LTP Expression and Acquired Immunity to Depotentiation

We next asked whether LFS affects synaptic tagging. If prior LFS impairs tagging of activated synapses, then LFS given just prior to E-LTP tetanus in S2, after L-LTP stimuli in S1, should prevent S2 from capturing L-LTP expression. Indeed, we found that with this protocol, pairing of L-LTP and E-LTP stimuli no longer produced L-LTP in the weak (S2) pathway (**Figure 3.04A**). The inputs that received weak tetanization displayed only a transient potentiation (S2, 170 min; 109 ± 4%, n = 6), and remained at baseline values following depotentiating LFS (S2, 110 min: 106 ± 6%, n = 6). ANOVA of these experiments compared to slices that received weak LTP stimulation alone (S2, 170 min; 101 ± 6%, n = 8; **Figure 3.01A**), or weak LTP paired with prior L-LTP stimulation (S2, 170 min; 158 ± 9%, n = 8; **Figure 3.01B**) revealed a significant difference between the groups ($F(2,19) = 18.864$, $p < 0.0001$). Post-hoc tests showed that LTP elicited by weak LTP stimulation alone ($p < 0.001$; filled diamonds) or paired E-

LTP and L-LTP stimulation with LFS prior to E-LTP induction ($p < 0.001$; filled squares) were significantly lower than LTP seen with L-LTP and E-LTP pairing alone (filled circles; **Figure 3.04B**). **Figure 3.04C** summarizes depotentiation data.

Our results demonstrate that prior LFS can impair subsequent synaptic capture of L-LTP expression and acquired immunity to depotentiation. Our data also reveal a limited time window in which LFS can exert its metaplastic, anterograde inhibition of synaptic capture. LFS applied 10 min (**Figure 3.04A**), but not 40 min (**Figures 3.03A and 3.03B**), prior to E-LTP significantly impaired synaptic capture of L-LTP expression. This result extends previous work that showed that homosynaptic anterograde inhibition of L-LTP by prior LFS is effective when LFS is applied 20 min, but not 40 min, before L-LTP induction (Woo and Nguyen, 2002).

Prior LFS Does Not Affect Dendritic Protein Synthesis That Confers Local Immunity to Depotentiation

As demonstrated earlier (**Figure 3.01B**) and in previously published accounts (Barco et al., 2002; Woo and Nguyen, 2003), cell-wide distribution of L-LTP-transcriptional products can provide immunity to depotentiation. However, strong depolarization, such as that associated with L-LTP tetani, can also trigger dendritic protein synthesis and confer immediate, synaptically localized immunity to Dpt (Ouyang et al., 1999; Woo and Nguyen, 2003). Our experiments showed that prior LFS can impair synaptic capture of gene expression-mediated immunity to depotentiation. Do these effects extend to depotentiation mediated by local protein synthesis? Following LFS conditioning to one set of inputs, L-LTP tetani were given to either the same

(homosynaptic) or a separate (heterosynaptic) pathway. Five minutes after LTP induction, depotentiating LFS was given to the tetanized pathway (**Figure 3.05A**). We found that prior LFS did not affect the resistance of L-LTP to depotentiation (90 min; Hom: 131 ± 14%, n = 6; Het: 133 ± 9%, n = 6). Both groups recovered to mean potentiation values within 60 min that were comparable to control slices that did not receive LFS prior to L-LTP induction (90 min; Ctrl: 138 ± 10%, n = 6; $F(2,14) = 0.1395$, $p = 0.8709$; **Figures 3.05A and 3.05B**). Because LFS impairs synaptic capture of immunity to Dpt, these results suggest that the dendritic translation that underlies local immunity (Woo and Nguyen, 2003) is unaffected by prior LFS.

DISCUSSION

My findings have revealed a novel type of metaplasticity by low frequency stimulation (LFS) that acts to impair synaptic tagging and capture of long-lasting LTP. My results show that prior LFS impairs the expression of homosynaptically and heterosynaptically induced L-LTP but does not affect L-LTP-associated gene expression. Instead, prior LFS impairs synaptic tagging and capture of L-LTP expression. Prior LFS also blocked transcription-dependent, heterosynaptically transferred ("acquired") immunity of L-LTP to depotentiation. In contrast, Dpt immunity arising from local dendritic protein synthesis was unaffected by prior LFS. Metaplastic effects of LFS were anterograde (i.e., did not affect previously established L-LTP) and time-limited in their expression.

LFS Regulation of Synaptic Tagging and Capture of L-LTP Gene Products

The synaptic tag theory posits that input-specific L-LTP expression requires 1) gene expression and cell-wide distribution of newly synthesized plasticity-related proteins; and 2) an activity-dependent synaptic tag that allows for synapse specific capture of LTP-stabilizing proteins (Frey and Morris, 1997, 1998; **Figure 3.06B**). Once transcription and protein synthesis have been triggered by appropriate synaptic activity at a subset of inputs, a period of cell-wide, reduced threshold for L-LTP ensues. Transient E-LTP can be converted to persistent L-LTP by capturing plasticity-related proteins produced from previous synaptic activity at separate inputs (Frey and Morris, 1997; **Figures 3.01B and 3.06C**). Application of a transcriptional inhibitor, actinomycin D, during L-LTP-inducing stimuli prevented L-LTP expression at those inputs. Weak tetanic stimulation of an independent pathway 30 min later produced transient potentiation, presumably because there were no LTP-stabilizing gene products for those synapses to capture (**Figures 3.02A and 3.06D**). However, while LFS impaired L-LTP induced 10 min later, weaker tetanus given 40 min later remained successful in capturing L-LTP that was immune to depotentiation (**Figures 3.03A and 3.03B**). Thus, while L-LTP is impaired when induced within 40 min after LFS, the metaplastic effects of activating L-LTP-associated gene products – i.e., lowered threshold for inducing subsequent L-LTP – remains intact. These results demonstrate that L-LTP associated transcriptional processes and the expression of prolonged potentiation can be dissociated and differentially regulated by prior synaptic activity.

My data also suggest that prior LFS does not block L-LTP gene expression, but may instead impair the ability of activated synapses to capture LTP-stabilizing PRPs

(**Figures 3.06E and 3.06F**). Indeed, after inducing L-LTP at one set of inputs (S1), application of LFS prior to weak tetanic stimulation of an independent path (S2) prevented S2 from capturing persistent potentiation and immunity to depotentiation (**Figures 3.04A and 3.06F**). One caveat of this study is that I do not directly show heterosynaptic impairment of capture. This would involve strong tetanization of S1 followed by LFS in S1 (i.e., heterosynaptic relative to weak tetanus in S2) just prior to weak tetanus in S2. However, a major complication in interpreting these results arises from the fact that LFS would not be applied to "naïve" inputs, but rather to already potentiated inputs, owing to the initial L-LTP induction in S1 (i.e., depotentiation). Previous studies suggest that LTD and depotentiation are separate phenomena under the control of different regulatory mechanisms and that the nature of the signaling cascades induced by synaptic activity depends on the initial state of the stimulated synapses (i.e., naïve vs. potentiated; Katsuki et al., 1997; Lee et al., 2000; Jouvenceau et al., 2003). For example, homosynaptic inhibition of L-LTP by LFS at 5-Hz (900 pulses) requires NMDA receptor activation (Woo and Nguyen, 2002). In contrast, a similar LFS protocol (900 pulses at 2-Hz) given to potentiated inputs to induce depotentiation requires metabotropic glutamate receptor (mGluR) activation and is insensitive to NMDA receptor antagonists (Bashir and Collingridge, 1994).

I show that prior LFS impairs synaptic capture of L-LTP expression in a homosynaptic manner (**Figures 3.04A and 3.04B**), and I propose that heterosynaptic inhibition of L-LTP by prior LFS is mediated by similar mechanisms. Consistent with this interpretation, prior LFS impairs heterosynaptically induced L-LTP, but does not affect subsequent E-LTP from capturing stable potentiation and acquired immunity to

depotentiation (**Figures 3.03A and 3.03B**). These results demonstrate that this type of heterosynaptic inhibition does not interfere with L-LTP-associated transcription and supports the hypothesis that LFS regulates synaptic tagging and capture of plasticity-related proteins.

Synaptic capture of L-LTP likely encompasses many different processes, including cell-wide distribution of plasticity-related gene products, generation of the synaptic tag, and capture of PRPs at tagged synapses to enable transfer of L-LTP between distinct inputs (reviewed by Martin and Kosik, 2002; see also *Aplysia* work on long-term facilitation: Martin et al., 1997; Casadio et al., 1999). I propose that LFS likely inhibits synaptic capture of L-LTP by impairing synaptic tagging. The decay time-course of the synaptic tag has been previously estimated to be around 1-2 hr (Frey and Morris, 1997, 1998A). In contrast, LFS only impairs L-LTP when given within 40 min prior to L-LTP induction (**Figures 3.03A and 3.03B**). Therefore, the synaptic tag should outlive metaplastic effects of LFS on PRP distribution or capture processes. If these were the only mechanisms by which LFS acted, no deficits in L-LTP should be apparent. Another possibility that cannot be ruled out is that LFS inhibits subsequent L-LTP by impairing all three processes: tagging, cell-wide distribution of proteins, and capture of PRPs. My data suggest that LFS impairs activity-dependent tagging of synapses to prevent capture of LTP-stabilizing gene products. LFS may therefore act to regulate the synapse specificity of L-LTP during the period of reduced LTP threshold that occurs after LTP-related gene products have been activated (**Figure 3.06F**).

A Transient Form of LTP That is Immune to Depotentiation

Previously, I showed that L-LTP following LFS decayed with a similar time course as E-LTP elicited with a weaker tetanization protocol (within 1-2 hrs) (This thesis, Chapter 2). However, an important distinction between the two transient forms of potentiation reported here is in their different susceptibility to depotentiation. Whereas E-LTP reverses to baseline following Dpt stimuli, decaying L-LTP induced after LFS remained resistant to Dpt (**Figures 3.01A and 3.05A**). Multiple episodes of synaptic activity (i.e., 3 or more high frequency trains of stimulation) are required to induce L-LTP (Reymann et al., 1985; Huang and Kandel, 1994), and immunity of four-train LTP to depotentiation can be mediated by local dendritic protein synthesis or transport of somatic gene products to potentiated synapses (Woo and Nguyen, 2003). My results indicate that LFS interferes with synaptic capture of Dpt immunity that is mediated by somatic gene expression. Thus, immunity to depotentiation due to local protein synthesis (Woo and Nguyen, 2003) is spared by prior 5-Hz activity. Indeed, strong L-LTP tetani given in the presence of actinomycin D or in slices where the cell bodies have been severed, results in the expression of a decaying potentiation that is nonetheless resistant to depotentiation. My data indicate that Dpt immunity and synaptic capture of persistent potentiation are mediated by separate processes that may be subject to differential regulation by prior synaptic activity.

What is the significance of this transient form of LTP that is immune to reversal? Immunity to Dpt may enable a cell to distinguish between synaptic changes resulting from repeated episodes of synaptic activity versus isolated bursts, such as those used to induce E-LTP (Zhou and Poo, 2004). In this way, sensitivity to Dpt can prevent

inappropriate stabilization of incidental synaptic modifications resulting from random, isolated episodes of synaptic activity. Thus, the prevailing immunity of transient L-LTP following 5-Hz pre-conditioning may allow those inputs to retain the *experience* of multiple episodes of synaptic activation, even if they do not result in long-lasting changes. This resistance to erasure may also be important in determining the capacity for inducing subsequent LTP at those inputs. Repeated induction of L-LTP in vitro at the same set of inputs requires several hours between episodes for recovery of functional plasticity (Frey et al., 1995). In contrast, conventional E-LTP may be reversed, and with a "reset" of the synaptic tags, be prepared quickly for subsequent LTP-inducing synaptic activity (Sajikumar and Frey, 2004). Therefore, further potentiation may be limited at synapses with decaying L-LTP until the effects on synaptic tagging wear off and synaptic strength returns to less potentiated levels.

Variable Persistence of LTP and Functional Significance

Collectively, my data suggest that LFS exerts metaplastic inhibition of subsequent L-LTP by impairing synaptic tagging that would otherwise allow for capture of somatic gene products necessary for stabilizing L-LTP expression. In contrast to the temporal summation of synaptic potentials that underlie associative LTP (i.e., over tens of milliseconds), metaplastic effects of LFS may regulate the integration of synaptic events over much longer periods of time (tens of minutes). Synaptic tagging allows the synapse, rather than the nucleus, to be the unit of long-lasting plasticity. However, newly synthesized gene products can, in principle, be transported to inactive as well as active synapses. These activity-dependent changes in gene expression have been proposed to

ready a cell to form lasting associations with other inputs (Frey and Morris, 1998; Morris, 2001; Morris et al., 2003) and they reduce the threshold for inducing subsequent L-LTP at all convergent inputs. During this period, L-LTP characteristics, such as persistence, stability, and immunity to reversal by subsequent low frequency activity (depotentiation), can be conferred by synaptic capture to the weakly stimulated pathway. By impairing synaptic tagging, I propose that low-frequency activity acts to preserve the synapse specificity of L-LTP expression. Furthermore, because LFS regulates L-LTP expression in a non-input specific manner, the maximum flexibility and capacity for synaptic plasticity can be maintained in the neuron as a whole.

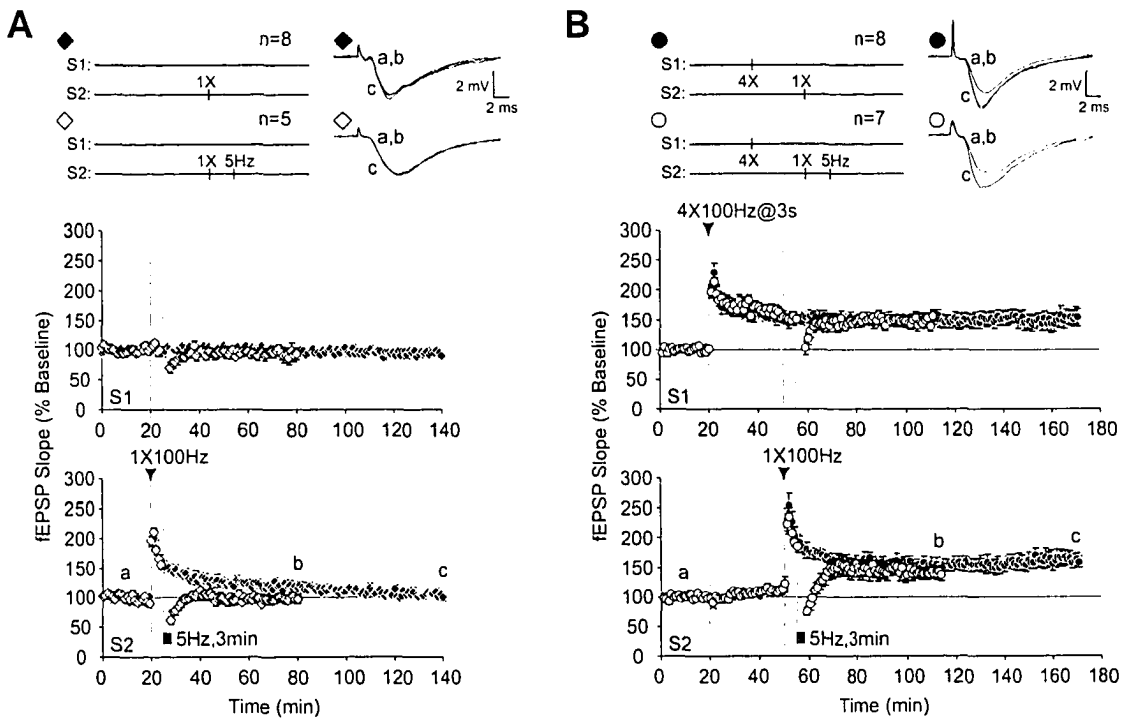

Figure 3.01: Successful synaptic capture of L-LTP by weak LTP stimuli can be assayed by prolonged potentiation and a newly acquired immunity to depotentiation (Dpt). (A) E-LTP induced by one train of tetanus is input specific, decays to baseline within 2 hr of induction (c), and is sensitive to Dpt (b). **(B)** One train of tetanus, when preceded by four-train L-LTP, results in potentiation that is persistent at 2 hrs post-tetnus (c). This potentiation is resistant to Dpt - follwing 5-Hz Dpt stimulation, fEPSPs gradually recover to potentiated levels (b).

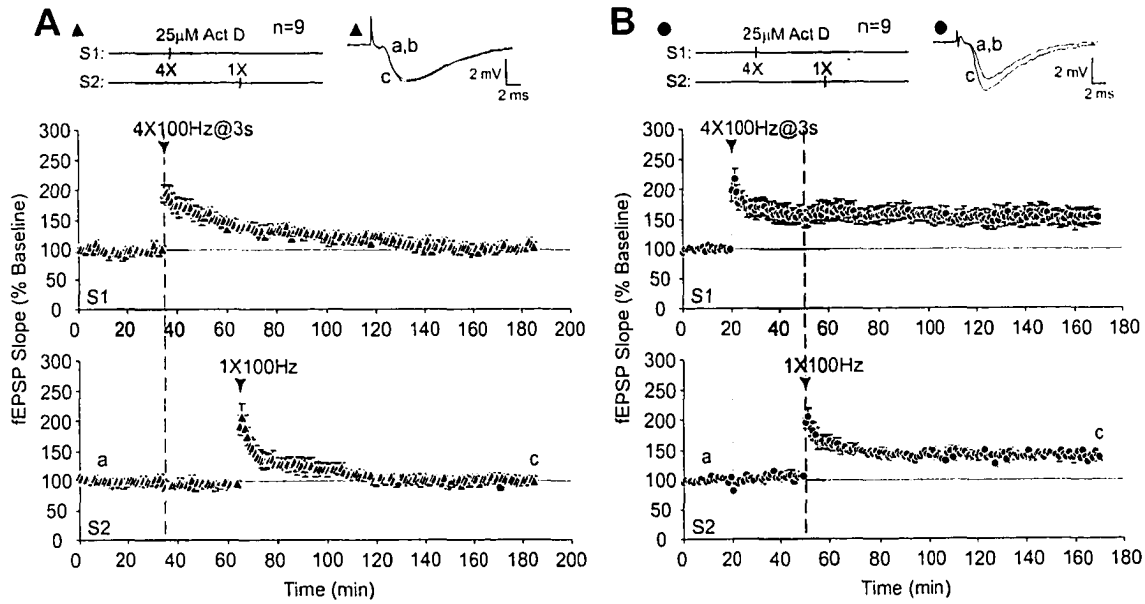

Figure 3.02: A critical period of transcription is needed for synaptic capture of L-LTP. (A) Application of Act D during four-train L-LTP induction prevented L-LTP expression in both strong (S1) and weak (S2) pathways. **(B)** Act D applied after strong tetanization, during the weak LTP tetanus, did not affect L-LTP expression in either pathway.

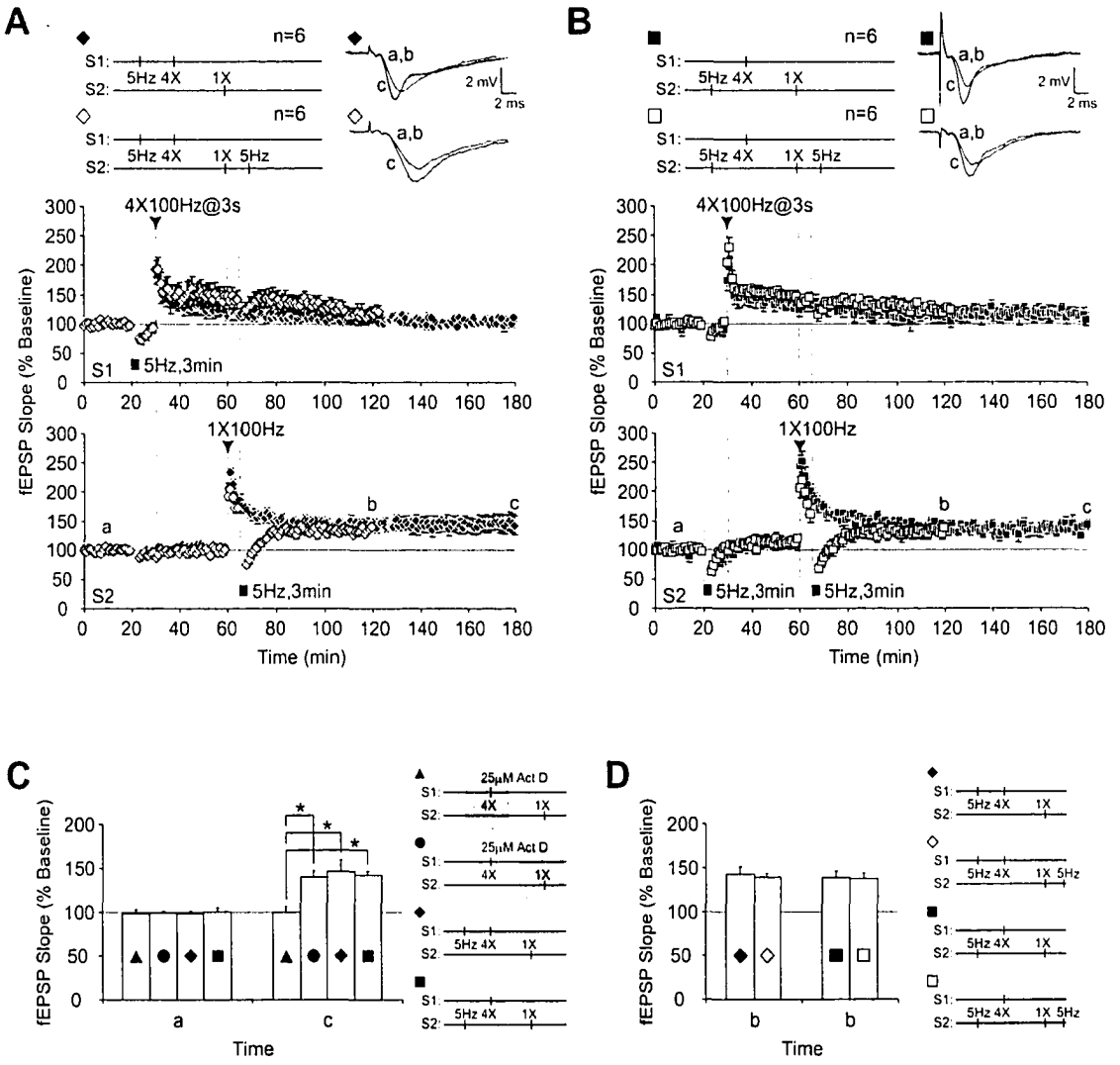

Figure 3.03: Prior 5-Hz stimulation does not affect transcription associated with L-LTP expression. (A) Weak LTP tetanus in S2 elicited prolonged potentiation and gained immunity to Dpt despite inhibition of four-train L-LTP by prior LFS. **(B)** Heterosynaptic inhibition of L-LTP by prior LFS did not affect subsequent synaptic capture by weak LTP tetanus. **(C)** and **(D)** summarize LTP and depotentiation data, respectively, from Act D and LFS experiments with significant differences marked by asterisks (*$p < 0.05$).

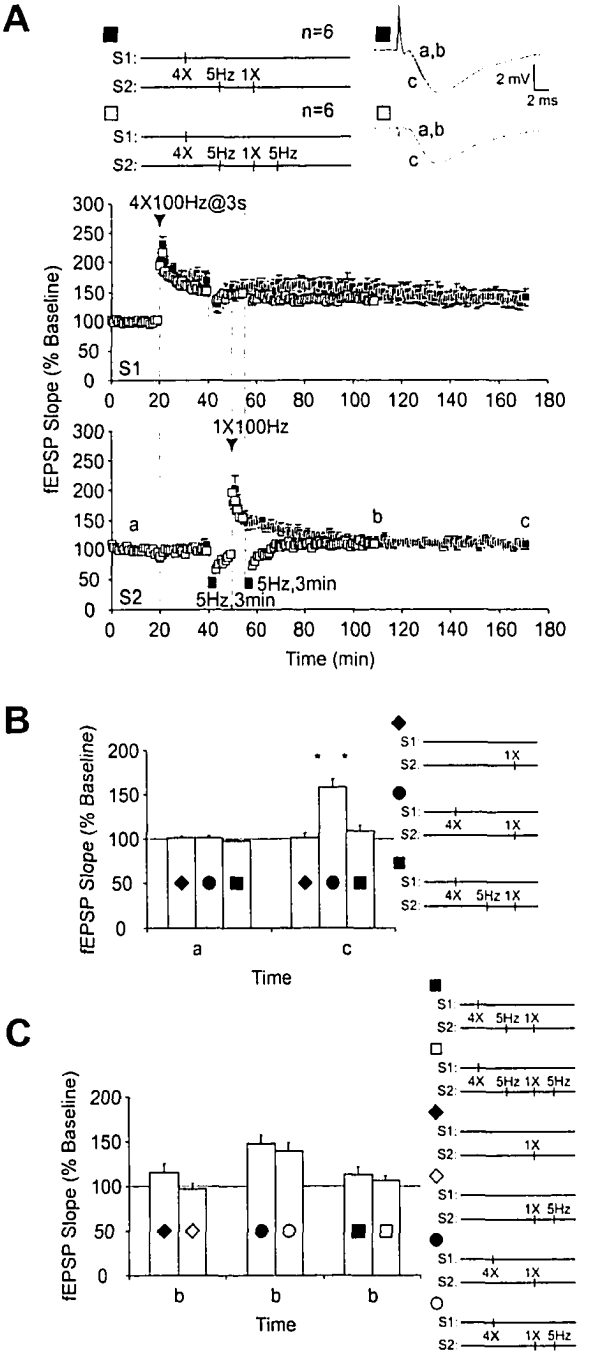

Figure 3.04: Prior LFS impairs synaptic capture of L-LTP expression and acquired immunity to Dpt. (A) LFS applied prior to weak LTP tetanus in S2 produced a transient potentiation that was sensitive to Dpt. **(B)** Mean fEPSPs in the pathway that received weak LTP stimulation (S2) were signficantly higher 120 min post-tetanus (c) in slices that received one train paired with four-train tetanus (filled circles), as compared to those that received one train alone (filled diamonds), or 5-Hz stimulation in between one- and four-train pairing (filled squares). **(C)** Mean fEPSP values of the weak pathway (S2) recovered to potentiated levels following Dpt if it was paired with four-train L-LTP (circles), but remained at baseline if one train of tetanus was given alone (diamonds), or if four- and one-train pairing was interrupted with 5-Hz LFS (squares).

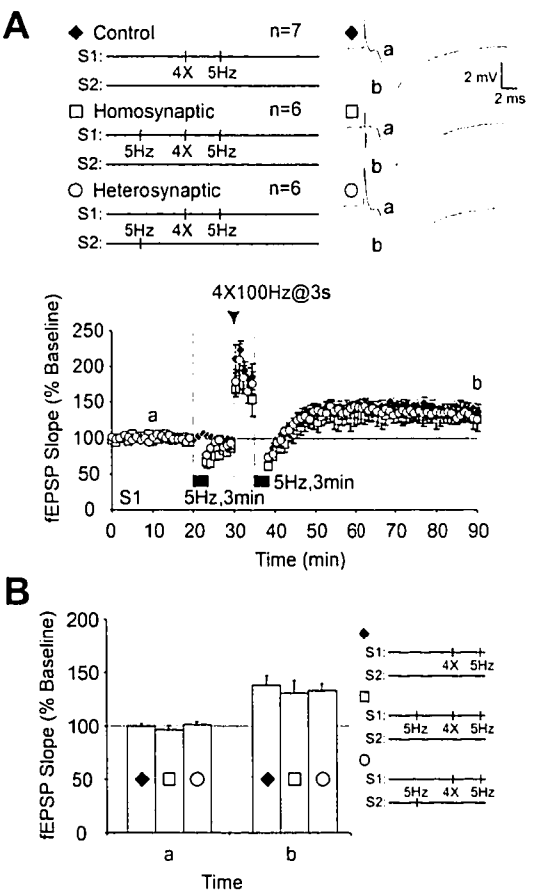

Figure 3.05: Prior 5-Hz stimulation does not affect four-train L-LTP's immunity to Dpt. (A) Mean fEPSP measurements in slices that received 5-Hz LFS prior to four-train L-LTP induction in the homo- or hetero-synaptic pathway, and in control slices that did not have prior 5-Hz conditioning were comparable 55 min after Dpt. **(B)** Summary histogram shows no significant difference in mean potentiated levels between groups ($p > 0.01$).

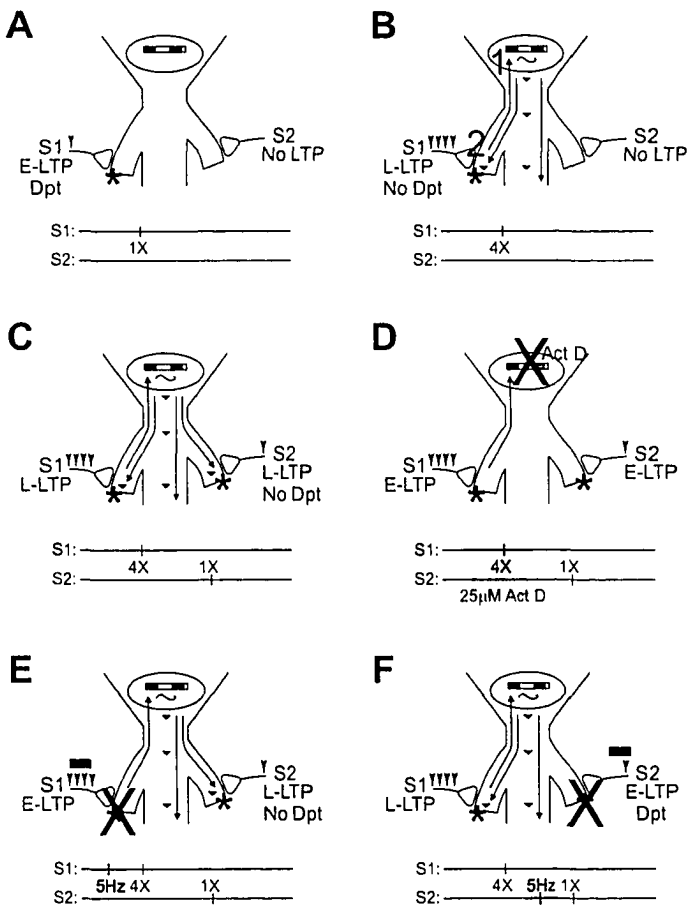

Figure 3.06: Schematic model of metaplastic effects of prior 5-Hz LFS on synaptic capture of L-LTP. (A) One tetanus train can generate a synaptic tag (*) but elicits only transient E-LTP. **(B)** Four tetanus trains 1) activate transcription (~); and 2) generate a synaptic tag (*) to capture plasticity-related proteins (PRPs; triangles). This produces input-specific L-LTP expression that is immune to Dpt. **(C)** Weak stimulation at S2 can capture PRPs activated by previous strong synaptic activity at S1 to gain persistant L-LTP expression and immunity to Dpt. **(D)** Blocking transcription with Act D impairs L-LTP expression in both strong and weak pathways. **(E)** Prior 5-Hz LFS does not affect transcription but impairs synaptic tagging and capture of L-LTP expression at S1. Subsequent weak stimulation of S2 can generate a synaptic tag and capture PRPs for L-LTP expression and immunity to Dpt. **(F)** 5-Hz LFS given just prior to weak stimulation of the S2 pathway prevents synaptic tagging, capture of L-LTP PRPs, and immunity to Dpt in S2.

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CHAPTER 4:

A CRITICAL ROLE FOR PKA IN SYNAPTIC TAGGING AND ITS REGULATION BY PRIOR SYNAPTIC ACTIVITY

INTRODUCTION

Activity-dependent changes in synaptic strength are believed to underlie information storage in the brain. Long-term potentiation (LTP) is a long-lasting increase in synaptic strength that occurs in response to brief, repetitive stimulation (Bliss and Lømo, 1973; Andersen et al., 1977). However, neurons in the central nervous system receive inputs from thousands of synaptic contacts, yet changes in the strength of synaptic connections can be input specific and localized to relatively small functional domains (Lynch et al., 1977; Andersen et al., 1977). To complicate things, long-lasting LTP (late phase LTP; L-LTP) requires gene expression and de novo protein synthesis, the products of which are presumed to be transported in a cell-wide manner (Stanton and Sarvey, 1984; Krug et al., 1984; Frey et al., 1988; Nguyen et al., 1994).

In order to preserve the input specificity of L-LTP, a mechanism to mark, or "tag", active synapses has been proposed that allows newly synthesized gene products to be captured and utilized at appropriately activated synapses (Sossin, 1996; Schuman, 1997; Frey and Morris, 1998). Frey and Morris (1997) first provided evidence for the synaptic tag hypothesis in the rat hippocampus. Paradoxically, protein synthesis-dependent L-LTP could be induced in the presence of a translational inhibitor if it was preceded by L-LTP at a separate set of inputs. They proposed that proteins synthesized in response to activation of long-term synaptic changes at one set of synapses could be successfully captured and utilized by another set of synapses to express L-LTP if a synaptic tag is generated by appropriate synaptic activation. In accord with this proposal, transient potentiation resulting from weaker synaptic activation could be prolonged to

resemble L-LTP if paired with established protein synthesis-dependent L-LTP at separate synaptic inputs (Frey and Morris, 1997, 1998A). Significantly, the nature and identity of the tag, and how it can be regulated by synaptic activity are largely unknown.

I show here that homosynaptic and heterosynaptic inhibition of L-LTP by prior LFS require protein phosphatase activity and concurrently impair signaling through the cAMP/PKA pathway in a cell-wide manner. Previous findings suggest that prior LFS impairs L-LTP by inhibiting synaptic tagging. Indeed, I find that pharmacological and genetic down-regulation of PKA signaling both impair synaptic capture. Moreover, pharmacological activation of cAMP/PKA signaling is sufficient to generate a synaptic tag to prolong the duration of synaptic facilitation. My results implicate a critical role for PKA in synaptic tagging that may be a novel control point in the consolidation of L-LTP. PKA-mediated signaling can be constrained by prior episodes of synaptic activity to regulate subsequent L-LTP expression and the integration of many synaptic events over time.

Materials and Methods

Animals

Experiments were performed on female C57BL/6 mice (aged 10-14 wks, Charles River, Montreal, Canada) housed at the University of Alberta under CCAC guidelines. Where indicated, female R(AB) transgenic mice and age-matched wildtype littermates of the transgenics were used (aged 12 – 14 mo). Transgenic animals were derived from two independent lines that were previously characterized for neural expression of the R(AB)

transgene (Clegg et al., 1987; **Figure 4.01**), hippocampal PKA activity, hippocampal synaptic physiology, and hippocampus-dependent long-term memory (Abel et al., 1997). Transgenic animals showed ~50% reduction in hippocampal basal PKA activity (Abel et al., 1997). For genotyping, tail DNA was analyzed by Southern blotting using a transgene-specific probe as described previously (Abel et al., 1997). R(AB) mice were housed at University of Pennsylvania and at University of Alberta under IACUC and CCAC guidelines.

Electrophysiology

Following cervical dislocation, transverse hippocampal slices (400 μm thickness) were cut and maintained in an interface chamber at 28°C. Artificial cerebrospinal fluid (ACSF) used for dissection and superfusion was consistent with previous studies and contained the following (in mM): 124 NaCl, 4.4 KCl, 1.30 $MgSO_4 \cdot 7H_2O$, 1.0 $NaH_2PO_4 \cdot H_2O$, 26.2 $NaHCO_3$, 2.5 $CaCl_2$, 10 D-glucose (Nguyen et al, 1994). Slices were allowed to recover for at least 60 min before experiments commenced. For experiments involving pre-incubation in okadaic acid (OA), slices were allowed to recover for 30 min before transfer to drug solutions.

Standard recordings of extracellular field excitatory postsynaptic potentials (fEPSPs) were accomplished with a glass microelectrode (A-M Systems, Carlsborg, WA; electrical resistances: 2-4 MΩ) filled with ACSF and placed in stratum radiatum of area CA1. Two bipolar nickel-chromium (A-M Systems, Carlsborg, WA) electrodes placed in stratum radiatum so as to stimulate fEPSPs (Grass S48 Stimulator) from two separate sets of inputs converging onto the same postsynaptic population of neurons (This thesis,

Figure 2.02B). A lack of inter-pathway paired-pulse facilitation (iPPF) following successive stimulation through the two electrodes (at 40 – 200 ms intervals) was used to confirm the independence of the two pathways. iPPF was assessed during baseline acquisition and at the conclusion of experiments. Test stimuli were given once per minute at a stimulus intensity that evoked fEPSP amplitudes of ~40% of the maximum response (0.08 ms pulse width). The "weak" LTP protocol consisted of a single 100-Hz train (1 s duration) and elicited transient E-LTP (Huang and Kandel, 1994). "Strong" tetanus to induce L-LTP consisted of four stimulus trains (100-Hz each) with an intertrain interval of 3 s (Woo et al., 2003). Low frequency stimulation (LFS) at 5-Hz for 3 min was used as the conditioning stimulation to induce metaplasticity and also to induce depotentiation (reversal of LTP; Barrioneuvo et al., 1980; Stäubli and Lynch, 1990; Woo and Nguyen, 2002).

Drugs

Forskolin (FSK, 50 μM; RBI), an adenylyl cyclase activator, and 3-isobutyl-1-methylxanthine (IBMX, 50 μM; RBI), a phosphodiesterase inhibitor, were used in conjunction to elicit cAMP/PKA-dependent chemical L-LTP (Chavez-Noriega and Stevens, 1992; Nguyen et al., 2000). Lower concentrations of FSK and IBMX (25 μM each) in combination with the transcriptional inhibitor actinomycin D (Act D; 25 μM; Bioshop Canada) were used in experiments that involved transient FSK/IBMX facilitation (**Figure 4.06**). KT-5720 (KT, 1 μM; Biomol) impairs PKA signaling by blocking activity of the catalytic subunits of PKA (Kase et al., 1987). FSK, IBMX, Act D, and KT were prepared as concentrated stock solutions (50 mM FSK, 50 mM IBMX,

25 mM Act D, 1 mM KT) in dimethylsulfoxide (DMSO, Sigma Aldrich) before dilution with ACSF to their final concentrations and bath applied. Sodium okadaic acid (OA; Sigma Aldrich) was prepared as a 1 mM concentrated stock solution in distilled water and diluted to a final concentration of 1 μM in ACSF. At this concentration, OA inhibits the protein phosphatases 1 and 2A (PP1 and PP2A, respectively; Ishihara et al., 1989; Cohen et al., 1990). Slices were pre-incubated in OA for 90 – 180 min before transfer back to the interface chamber where they were given 10 min to recover before experiments commenced. Preparation and experiments involving light-sensitive drugs were carried out in dim-light conditions.

Data and Statistical Analysis

The initial slope of the fEPSP was measured as an index of synaptic strength. Average "baseline" slope values were acquired over 20 min and graphs are plotted as the fEPSP slope expressed as a percentage of the averaged baseline. Student's t-test was used for statistical comparison mean fEPSP slopes within paired data sets, with a significance level of $p < 0.05$ (denoted on graphs with an "*"). Data sets with more than two comparison groups were analyzed with ANOVA. Tukey-Kramer multiple comparisons test was completed if ANOVA analysis indicated a significant difference between groups ($p < 0.05$, denoted on graphs with an "*"). Kolmogorov-Smirnov, and Bartlett's tests were done to determine normality and to analyze standard deviations, respectively, of all test groups. Where indicated, Kruskal-Wallis test (non-parametric ANOVA) was applied accordingly. All values shown are mean ± standard error of mean (SEM), with n = number of slices.

RESULTS

Homosynaptic and Heterosynaptic Inhibition of L-LTP by Prior Low-Frequency Stimulation Requires Phosphatase Activity

Previous experiments showed that prior low-frequency stimulation (LFS) at 5-Hz for 3 min impairs long-lasting LTP (L-LTP) subsequently induced in those same synapses (i.e., homosynaptic inhibition) and at other synapses converging on the same postsynaptic cells (i.e., heterosynaptic inhibition; This thesis, Chapter 2). Protein phosphatase activity is enhanced following low-frequency stimulation and LTD induction (Mulkey et al., 1993; Thiels et al., 1998) and protein phosphatases 1 and 2A (PP1 and PP2A, respectively) have been implicated in homosynaptic inhibition of subsequent L-LTP (Woo and Nguyen, 2002). It is unclear whether protein phosphatases can also play a role in heterosynaptic metaplasticity. I used a PP1 and PP2A inhibitor, sodium okadaic acid (OA; 1 μM), to examine the contribution of these phosphatases to the metaplastic effects of LFS on subsequent L-LTP.

I stimulated two independent pathways in stratum radiatum of area CA1 in OA-treated slices. LFS at 5-Hz was applied to one pathway followed by L-LTP-inducing tetanus in either the same pathway (i.e., homosynaptic, Hom) or at a separate set of inputs (i.e., heterosynaptic, Het). Pre-incubation of hippocampal slices in OA negated the impairing effects of prior LFS on subsequent L-LTP (**Figure 4.02A**). Two hours post-tetanus, mean fEPSP levels in slices that received LFS pre-conditioning (S2, 150 min; Hom: 142 ± 9%, n = 10; Het: 147 ± 9%, n = 8) did not differ significantly from slices that received L-LTP tetanus without prior LFS (S2, 150 min; Control: 151 ± 5%, n = 6;

$F(2,21) = 0.2943$, $p = 0.7481$). Control experiments, performed in OA-treated slices, show that the pre-incubation process did not affect the stability of L-LTP, baseline synaptic transmission, or general health of slices. A summary histogram of mean fEPSP slopes during baseline (a), 10 min after LFS (b), and 120 min after L-LTP induction (c) is shown in **Figure 4.02B**. These findings show that the PP1/PP2A are required for homosynaptic and heterosynaptic inhibition of L-LTP by prior LFS.

Prior LFS Impairs cAMP/PKA Signaling in a Homosynaptic and Heterosynaptic Manner

The expression of synaptic plasticity involves a balance between the opposing actions of protein kinase and protein phosphatase activity (Lisman J, 1989; Coussens and Teyler, 1996; Wang and Kelly, 1996; Blitzer et al., 1998). Stimulation at low frequencies (< 10 Hz) is associated with a predominance of phosphatase activity and decreased synaptic strength (Mulkey et al., 1993, 1994). In contrast, stimulation at high frequencies is associated with protein kinase activity and increases in synaptic strength (Malinow et al., 1989; Matthies and Reymann, 1993). In particular, the consolidation of E-LTP to L-LTP requires signaling through the cAMP-dependent protein kinase (PKA) pathway (Frey et al., 1993; Matthies and Reymann, 1993; Huang and Kandel, 1994; Abel et al., 1997; also, for review, see Nguyen and Woo, 2003). Because LFS selectively impairs L-LTP while leaving E-LTP intact (Woo and Nguyen, 2002; This thesis, Chapter 2), I hypothesized that LFS may impair expression of L-LTP by down-regulating cAMP/PKA signaling. Transient application of the adenylyl cyclase activator, forskolin (FSK; 50 μM), and phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX; 50 μM) has

been previously shown to induce long-lasting facilitation at CA3-CA1 synapses. This form of synaptic facilitation is mediated by the activation of PKA as it can be blocked by a PKA inhibitor, Rp-cAMPs (Nguyen et al., 2000; Woo et al., 2002A) and R(AB) transgenic mice that have reduced PKA activity show impaired FSK/IBMX-facilitation (Abel et al., 1997; Woo et al., 2002A). Indeed, I found that chemical LTP induced through activation of the cAMP/PKA pathway is significantly impaired in a cell-wide manner following LFS (**Figure 4.03A**). Brief application of FSK+IBMX produced a stable and persistent facilitation lasting 2 hr after drug application (150 min; Control: 143 ± 14%, n = 7). In accord with my previous results (This thesis, Chapters 2 and 3), LFS at 5-Hz for 3 min results in a transient homosynaptic and heterosynaptic synaptic depression. Mean fEPSP slopes recovered and did not differ significantly from baseline levels within 10 min of initial LFS (30 min; Hom: 89 ± 5%, n = 7; Het: 89 ± 4%, n = 7; Control: 96 ± 5%, n = 7; $F(2,18) = 0.7057$, $p = 0.5069$). Application of FSK+IBMX following LFS and recovery resulted in a smaller facilitation of fEPSPs than controls that did not receive LFS (45 min; Hom: 107 ± 7%, n = 7; Het: 99 ± 8%, n = 7; Control: 138 ± 8%, n = 7). Mean fEPSP slopes in slices that received prior LFS decayed to baseline within 2 hr of drug application (150 min; Hom: 102 ± 10%, n = 7; Het: 104 ± 8%, n = 7). ANOVA analysis indicated that mean fEPSP slopes were significantly less than controls that did not receive LFS (45 min: $F(2,19) = 7.069$, $p = 0.0054$; 150 min: ($F(2,18) = 4.664$, $p = 0.0233$). **Figure 4.03B** summarizes LTP data with post-hoc analysis of mean fEPSP values taken at baseline (a), after LFS just prior to FSK+IBMX application (b), following FSK/IBMX washout (c), and 120 min after initial application of FSK+IBMX (d). Prior LFS significantly impaired cAMP/PKA-mediated facilitation in both homosynaptic and

heterosynaptic pathways immediately following FSK+IBMX application (45 min; Hom: $p < 0.05$, Het: $p < 0.01$) and 2 hr after (150 min; Hom: $p < 0.05$; Het: $p < 0.05$).

In accord with previous reports, I show that pharmacological activation of the cAMP/PKA pathway is sufficient to elicit significant facilitation at CA3-CA1 synapses (Chavez-Noriega and Stevens 1992; Frey et al., 1993; Nguyen et al., 1994; Duffy and Nguyen, 2003). With prior LFS conditioning, long-lasting plasticity induced through chemical activation of cAMP/PKA signaling decayed to baseline values within 2 hr (**Figures 4.03A and 4.03B**; time-point "d"). These results mirror the decay of electrically induced L-LTP after prior LFS (This thesis, Chapter 2). Significantly, prior LFS also impaired FSK/IBMX facilitation during the initial development of facilitation (**Figures 4.03A and 4.03B**; time-point "c"), suggesting that cAMP/PKA signaling may be important during the initial stages of L-LTP expression.

Pharmacological Inhibition of PKA Blocks Synaptic Capture of L-LTP Expression and Acquired Immunity to Depotentiation

Earlier in my thesis, I showed that LFS impairs subsequently induced L-LTP by inhibiting synaptic tagging and capture of L-LTP stabilizing gene products (This thesis, Chapter 3). Given the inhibitory effects of LFS on cAMP/PKA signaling, does PKA play a critical role in synaptic tagging and capture of L-LTP? In area CA1 of hippocampal slices, a transient form of LTP (also known as early LTP, or E-LTP) can be induced with one high-frequency train of stimulation (typically 100-Hz, 1 s duration) that decays to baseline within 1-2 hr (Reymann et al., 1985; Huang and Kandel, 1994). With multiple trains of 100-Hz stimulation, late-phase LTP (L-LTP) is recruited and the duration of

potentiation can be considerably extended through transcription- and translation-dependent processes (Stanton and Sarvey, 1984; Krug et al., 1984; Frey et al., 1988; Nguyen et al., 1994). However, if transcription-dependent L-LTP is first established at one set of inputs (S1), weak tetanization at a separate set of inputs (S2) can generate a synaptic tag to capture plasticity-related proteins that have been mobilized in response to L-LTP in S1 (Frey and Morris, 1997). I paired multiple 100-Hz trains in S1 with a single tetanus to S2, 30 min later. Potentiation of the "weak" S2 pathway was persistent (S2, 120 min; Control: 152 ± 11%, n = 8; **Figure 4.04A**) and resistant to reversal by low-frequency stimulation given 5 min post-tetanus (depotentiation, Dpt; **Figure 4.04B**). After an initial depression below baseline (S2, 8 min; Dpt: 69 ± 8%, n = 6), mean fEPSP slopes recovered and stabilized at pre-depotentiated levels (S2, 60 min; Dpt: 149 ± 6%, n = 6). In contrast, application of a PKA inhibitor, KT-5720 (KT; 1 μM), during weak tetanus in S2 resulted in a transient potentiation of S2 inputs (S2, 120 min; 111 ± 7%, n = 8) that was significantly less than controls that did not receive the PKA inhibitor ($p < 0.01$). The resulting LTP induced with weak tetanus during KT application was sensitive to depotentiation. Initial levels of depression were comparable between control and KT treatment groups (S2, 8 min; KT: 71 ± 5%, n = 6; $p > 0.05$). However, mean fEPSP slopes in the KT treatment groups remained close to baseline values (S2, 60 min; KT: 102 ± 5%, n = 6) and were significantly lower than control slices that were not treated with KT ($p < 0.001$). Significantly, L-LTP in S1 was not affected by KT application, which started 10 min post-tetanus. Mean fEPSP levels in S1 of control and KT-treated slices did not differ significantly 2.5 hr post-induction (Control: 151 ± 11%, n = 8; KT: 140 ± 5%, n = 8; $p > 0.05$).

I show that PKA is required for synaptic capture of long-lasting potentiation by weak stimulation, consistent with previous studies (Barco et al., 2002). Heterosynaptic transfer of somatic gene products can confer immunity of LTP to depotentiation (Woo and Nguyen, 2003A). I demonstrate that capture of immunity to depotentiation is also dependent on PKA activity. Furthermore, PKA inhibitors applied after L-LTP induction does not affect previously established LTP, suggesting that there is a limited time period for the synaptic tag to be set following synaptic activity.

Impaired Synaptic Capture of L-LTP Expression in R(AB) Transgenic Mice

To further examine the role of PKA in synaptic tagging, I measured L-LTP in slices from mutant PKA mice [R(AB) transgenic mice] that have genetically reduced hippocampal PKA activity. R(AB) transgenics express an inhibitory form of a regulatory subunit of PKA (RIα) with a resultant ~50% reduction in basal PKA activity (Clegg et al., 1987; Abel et al., 1997; **Figure 4.01**). I examined synaptic capture using the previous protocol of pairing strong tetanization to one pathway (S1) followed by weak tetanization to another pathway (S2). Wildtype littermates showed robust long-lasting LTP in both input pathways (120 min; S1: 147 ± 10%, n = 6; S2: 142 ± 8%, n = 6). Transgenic mice showed comparable levels of potentiation in response to strong tetanization (S1, 120 min: 142 ± 9%, n = 10; $p > 0.05$). However, subsequent weak tetanization to S2 given 30 min later yielded LTP that decayed close to baseline levels within 120 min (109 ± 9%, n = 10) and was significantly lower than wildtype controls ($p < 0.05$; **Figure 4.05**).

Synaptic capture was impaired in mice with genetically reduced PKA activity, consistent with the pharmacological data obtained with KT-5720 and previously

published results (Barco et al., 2002). The pattern of LTP decay in the weak S2 pathway was comparable between the KT treatment group (S2, 60 min: 117 ± 7%, n = 8) and R(AB) transgenics (S2, 60 min: 122 ± 8%, n = 10; $p > 0.05$). Taken together, these results show that PKA is required for synaptic capture of L-LTP expression by weak tetanic stimulation.

Transient Activation of cAMP/PKA Signaling Can Generate a Synaptic Tag to Capture Long-Lasting Facilitation

Synaptic capture of L-LTP likely encompasses many different processes, including cell-wide distribution of plasticity-related gene products, generation of the synaptic tag, and capture of PRPs at tagged synapses to enable transfer of L-LTP expression between distinct inputs (reviewed by Martin and Kosik, 2002; Kelleher et al., 2004). While it is likely that prior LFS impairs synaptic tagging during subsequently induced L-LTP (This thesis, Chapter 3), the effects of pharmacological or genetic inhibition of PKA signaling are harder to narrow down. To distinguish the requirement for PKA in the distribution or capture process versus formation of a synaptic tag, I examined if activation of the cAMP/PKA pathway is sufficient to generate a synaptic tag. Because activation of PKA can generate long lasting facilitation on its own (Frey et al., 1993; Nguyen et al., 1994; **Figure 4.03A**), I used a transcriptional inhibitor, Act D, to eliminate transcriptional effects of PKA activation. Brief application of FSK+IBMX in the presence of a transcriptional inhibitor, Actinomycin D (Act D; 25 μM) results in a transient facilitation of fEPSPs that decays back to baseline within 120 min of FSK+IBMX application (175 min, S2: 103 ± 3%, n = 11). However, if L-LTP was first

established at one set of inputs, Act D + FSK+IBMX produced facilitation that persisted for 2 hr post-FSK+IBMX application (175 min, S2: 126 ± 7%, n = 9; **Figure 4.06**). Facilitation of fEPSPs by ActD + FSK+IBMX was significantly greater when drug application was paired with strong LTP tetanus as compared to slices that did not receive strong LTP tetanus prior to drug application ($p < 0.01$). A significant difference in mean fEPSP slopes was evident starting at 60 min post-FSK+IBMX application (115 min, S2; Control: 114 ± 2%, n = 11; L-LTP: 129 ± 4%, n = 9; $p < 0.01$). These results demonstrate that cAMP/PKA activation is sufficient to generate a synaptic tag that can capture prolonged facilitation from previously activated L-LTP.

DISCUSSION

Long-lasting LTP (L-LTP) in hippocampus CA1 requires gene expression and de novo protein synthesis, yet is expressed in an activity-dependent manner. The "synaptic tag" hypothesis proposes that L-LTP-associated gene products can only be captured and utilized at synapses that have been "tagged" by previous activity (Frey and Morris, 1997). My principal finding is a critical requirement for cAMP-dependent protein kinase (PKA) in synaptic tagging. Furthermore, PKA-dependent synaptic tagging can be constrained by prior episodes of synaptic activity to regulate subsequent L-LTP expression.

Activity-Dependent Regulation of Protein Phosphatase and Kinase Signaling

Using two-pathway extracellular field recordings, I show that protein phosphatases can be recruited by synaptic activity to regulate L-LTP expression in a cell-

wide manner. LFS-mediated homosynaptic and heterosynaptic inhibition of subsequent L-LTP was blocked by pre-incubation with okadaic acid, an inhibitor of protein phosphatases 1 and 2A (PP1 and PP2A, respectively). Although I did not directly measure hippocampal levels of PP1/PP2A activity following LFS, my results are in accord with previous studies which have shown increased PP1/PP2A activation in long-term depression (LTD; Mulkey at al., 1993; Thiels et al., 1998; Morishita et al., 2001) along with activity-dependent translocation of PP1 to synapses using a LFS paradigm identical to the one used in this study (5-Hz for 3min; Morishita et al, 2001). PP1/PP2A activity has also been linked to metaplasticity and decreased stability of L-LTP induced in the same set of inputs after low frequency stimulation (homosynaptic inhibition; Woo and Nguyen, 2002). I extend these findings by showing that PP1/PP2A activity can also regulate L-LTP expression at other synapses converging on the same postsynaptic cells (heterosynaptic inhibition).

The relative balance of signaling through protein phosphatases and kinases determines the direction of changes in synaptic efficacy (Lisman J, 1989; Coussens and Teyler, 1996; Wang and Kelly, 1996; Blitzer et al., 1998). My results suggest that LFS impairs signaling through the cAMP/PKA pathway, which has been implicated in the cellular consolidation of long-lasting potentiation (Matthies and Reymann, 1993; Frey et al., 1993; Abel et al., 1997). Although I did not directly assay PKA activity, chemical LTP induced with forskolin (FSK) in combination with 3-isobutyl-1-methylxanthine (IBMX) is mediated by activation of the cAMP/PKA cascade - FSK/IBMX-facilitation is blocked by PKA inhibitors and is impaired in transgenic mice that have reduced PKA activity (Abel et al., 1997; Nguyen et al., 2000; Woo et al., 2002). I found that

conditioning with LFS prior to induction of chemical LTP significantly attenuated its expression. Impairments in FSK/IBMX-facilitation were also evident immediately following drug application. Because prior LFS selectively impairs L-LTP while leaving early-LTP (E-LTP) intact (Woo and Nguyen, 2002; Chapter 2, this thesis), this suggests that early inhibition of cAMP/PKA signaling by prior LFS may be more important in regulating the consolidation of L-LTP.

The rapid impairment of PKA-dependent synaptic facilitation mirrors previously reported activation profiles of phosphatases following LFS. Pharmacological and biochemical studies show enhanced PP1 activity lasting 35-40 min following LTD induction *in vivo* and *in vitro* (Mulkey et al., 1993; Thiels et al., 1998). This temporal window of PKA inhibition and PP1/2A activation following LFS is consistent with previous studies of metaplasticity which revealed that LFS inhibits L-LTP for up to 20 – 40 min after LFS (Woo and Nguyen, 2002; Chapter 3, this thesis). Taken together, my data suggest that LFS-mediated metaplasticity requires protein phosphatases 1/2A and concurrently inhibits cAMP/PKA signaling to impair L-LTP expression in a cell-wide manner. Activity-dependent regulation of transcriptional and translational processes has been reported previously (for reviews, see West et al., 2002; Kelleher et al., 2004A). However, I demonstrate a novel role for protein kinases and phosphatases in regulating synaptic tagging and capture of long-lasting synaptic plasticity.

A Critical Role for PKA in Synaptic Capture and Tagging

I found that signaling through the cAMP/PKA cascade is critical for synaptic capture of L-LTP expression. If transcription-dependent L-LTP is first induced at one set

of synapses ("S1"), weak LTP induced at a second set of synapses on the same post-synaptic neurons ("S2") can be transformed to resemble L-LTP by generating a synaptic tag that enables them to capture L-LTP-stabilizing proteins that were destined for other synaptic sites (Frey and Morris, 1997; Barco et al., 2002; Chapter 3, this thesis). With successful synaptic capture, the previously transient LTP in S2 becomes persistent, lasting for 2 hr post-induction. In addition, whereas LTP induced by a single tetanic train is susceptible to activity-dependent erasure (depotentiation), pairing weak and strong LTP confers an acquired immunity to depotentiation to the weak S2 pathway (Barco et al., 2002; Woo and Nguyen, 2003; Chapter 3, this thesis).

I show that a PKA inhibitor, KT-5720, prevented synaptic capture of L-LTP expression and blocked acquired immunity to depotentiation. Furthermore, R(AB) transgenic mice that have impaired cAMP/PKA signaling show deficient synaptic capture when tested with the strong + weak LTP pairing protocol. LFS impairs synaptic capture of L-LTP expression (Chapter 3, this thesis), and I show in the current study that it also impairs cAMP/PKA signaling. These data demonstrate that synaptic capture of L-LTP expression can be impaired by electrical (i.e., LFS-mediated), pharmacological, or genetic down-regulation of PKA signaling. Collectively, these observations indicate that PKA is required for synaptic tagging and capture of L-LTP expression. Consistent with this interpretation, transient activation of the cAMP/PKA cascade is sufficient to generate a synaptic tag and capture persistent facilitation when paired with electrically induced L-LTP.

PKA-mediated phosphorylation of the cAMP response element-binding protein (CREB) transcription factor is a critical step in L-LTP consolidation from E-LTP

(Bourtchouladze et al., 1994; Impey et al., 1996; Matsushita et al., 2001). In the present study, I show that cAMP/PKA signaling also plays a critical role in synaptic tagging. This dual role for PKA in L-LTP is supported by pharmacological activation of the cAMP/PKA cascade that results in a prolonged synaptic facilitation. Previous studies have shown that this type of chemical LTP requires transcription and translation, and can occlude electrically induced L-LTP (Frey et al., 1993; Huang and Kandel, 1994). Significantly, several studies have shown that activation of transcriptional and translational processes alone is not sufficient to induce L-LTP, suggesting that during chemical LTP, PKA signaling may activate more than just transcriptional processes. Antidromic stimulation is sufficient for extracellular signal-regulated kinase (ERK) and CREB activation (Dudek and Fields, 2002) – two pathways associated with translational and transcriptional activation (Impey et al., 1998; Kelleher et al., 2004). However, antidromic stimulation does not elicit LTP (Dudek and Fields, 2002). Similarly, temporally restricted expression of constitutively active CREB (VP16-CREB) does not alter basal synaptic transmission in transgenic mice (Barco et al., 2002). These results show that in addition to transcription and translation, a synaptic tag is required to capture plasticity-related proteins for L-LTP expression. Consistent with a dual role for PKA in synaptic tagging and transcriptional control, postsynaptic activation of the PKA signaling cascade is sufficient for initiating persistent synaptic facilitation (Duffy and Nguyen, 2003).

Functional Significance

Acute activation of cAMP/PKA is critical for many types of long-lasting synaptic plasticity and long-term memory. However, several studies suggest that up-regulation of the cAMP/PKA pathway beyond a certain optimal range can also have deleterious effects on long-term memory. Genetic manipulations to enhance cAMP/PKA signaling by removing inhibitory constraints of $G_{i\alpha 1}$ on adenylyl cyclase produced impairments in hippocampus-dependent memory formation (Pineda et al., 2004). Significantly, ablation of $G_{i\alpha 1}$ in these mutants also resulted in 2-fold increase in basal adenylyl cyclase activity (Pineda et al., 2004). Pharmacological enhancement of cAMP/PKA signaling has also been targeted to improve spatial memory in young mice and to reverse age-related deficits in senescent mice (Barad et al., 1998; Bach et al., 1999; Hsu et al., 2002). However, pharmacological up-regulation of PKA activity is most effective at doses that amplify cAMP/PKA signaling without affecting basal cAMP levels, thus preserving the signal to noise ratio of cAMP/PKA signaling induced by synaptic activity (Barad et al., 1998; Bach et al., 1999). These results suggest that memory formation depends on a balance between mechanisms for increasing and decreasing cAMP/PKA signaling. My data suggest that PKA plays a critical role in synaptic tagging and in synapse-specific long-lasting potentiation in CA1 pyramidal neurons. Therefore, manipulations that produce a general increase in cAMP/PKA activation may result in a non-specific setting of synaptic tags that can occlude input-specific plasticity required for new memory formation. Consistent with this idea, saturation of LTP in vivo, with electrical stimulation or forskolin application, interferes with hippocampus-dependent memory (Moser et al., 1998; Pineda et al., 2004).

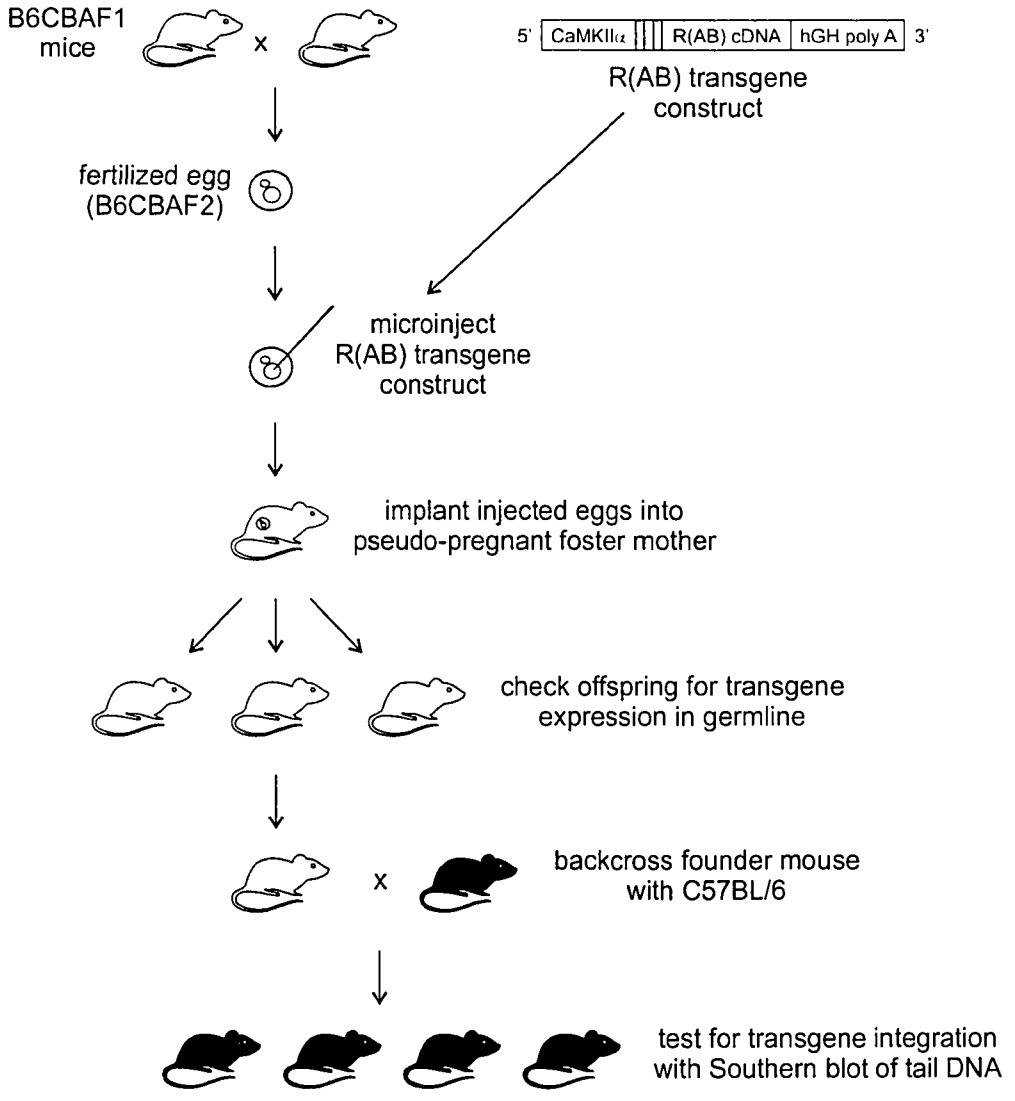

Figure 4.01: Generation of R(AB) transgenic mice. cDNA encoding an inhibitory form of RIα was placed in a hybrid construct under the control of the CaMKIIα promoter. The R(AB) transgene construct was microinjected into the pronuclei of fertilized (BL6CBAF2) eggs, and implanted into a pseudo-pregnant foster mother. Offspring were checked for transgene expression by Southern blot of tail DNA. Transgenic mice were backcrossed with C57BL/6 mice and transgene integration assessed with Southern blot.

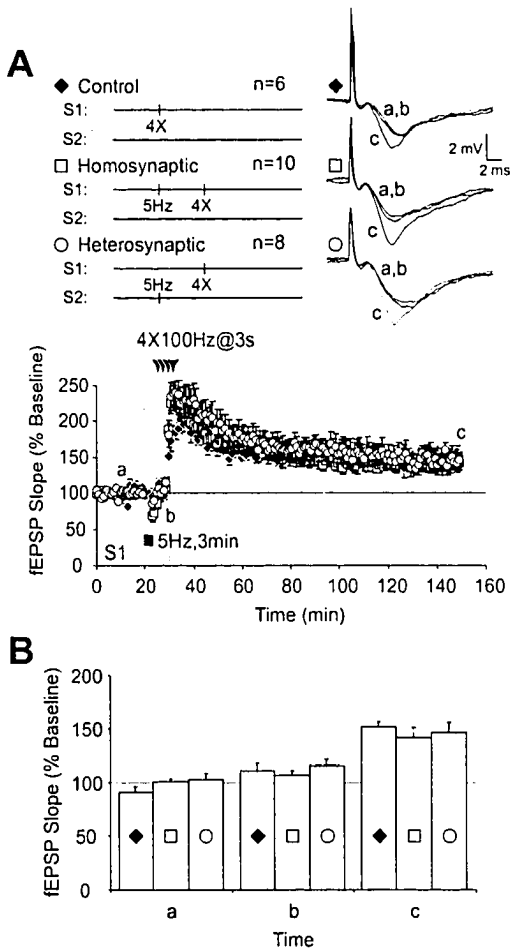

Figure 4.02: Homosynaptic and heterosynaptic inhibition of L-LTP by prior LFS requires PP1/PP2A activation. (A) Pre-incubation in okadaic acid negated impairing effects of LFS on subsequent induced L-LTP. **(B)** Summary histogram shows no significant difference in mean potentiated levels at 120 min post-tetanus (c) between slices that received LFS and slices that were subjected to the same pre-incubation protocol but did not receive LFS prior to L-LTP induction ($p > 0.01$). Mean fEPSP slopes are also given during baseline (a), and 10 min after initial LFS (b).

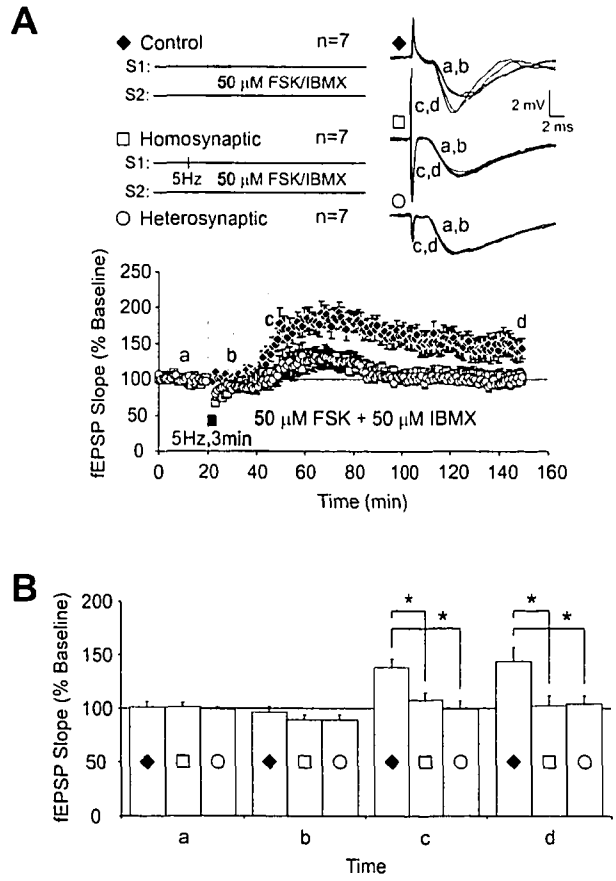

Figure 4.03: Prior LFS impairs homosynaptic and heterosynaptic cAMP/PKA signaling. (A) Application of FSK+IBMX generated a prolonged cell-wide facilitation that was significantly impaired by prior LFS. **(B)** Summary histogram of FSK+IMBX-mediated chemical LTP with and without prior LFS, during baseline (a), 10 min after initial LFS (b), following FSK+IBMX washout (c), and 120 min after initial application of FSK+IBMX. Significant differences are marked by asterisks (*$p < 0.05$).

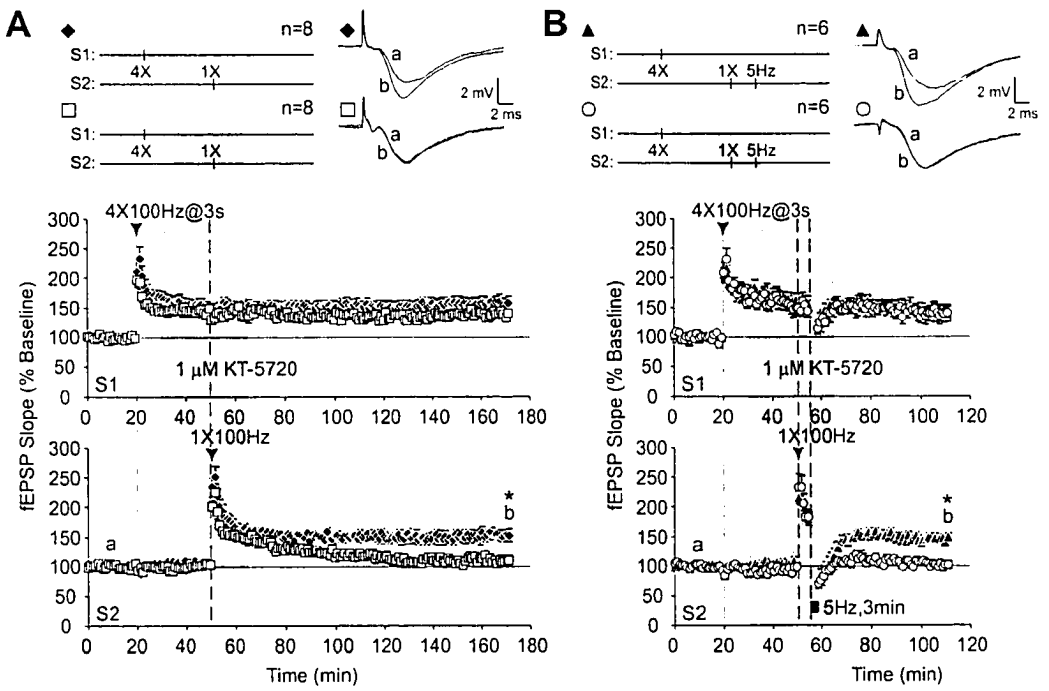

Figure 4.04: Synaptic capture of L-LTP and acquired immunity to depotentiation requires PKA activity. (A) Pairing one-train and four-train LTP resulted in L-LTP expression at both sets of inputs. Application of KT-5720 during one-train tetanus prevented L-LTP expression in the weaker pathway. **(B)** KT-5720 applied during one-train LTP induction in S2 prevented transfer of immunity to depotentiation. Asterisks denote statistical significance (*$p < 0.05$).

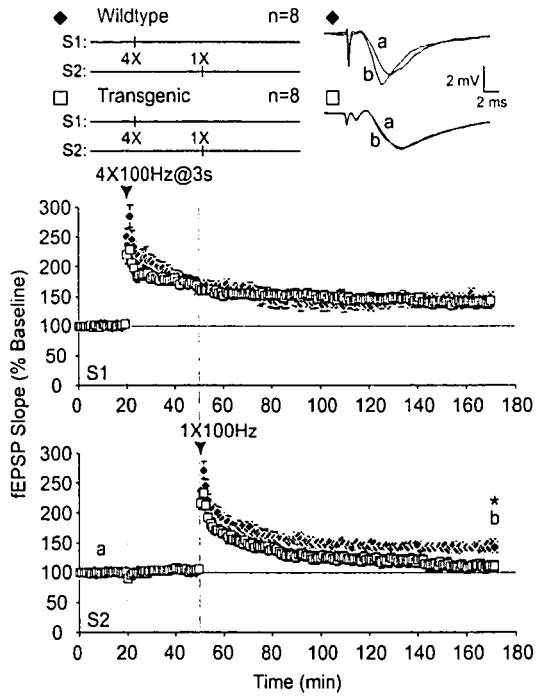

Figure 4.05: Synaptic capture of L-LTP is impaired in R(AB) transgenics that have ~50% reduced basal PKA activity. Pairing four-train and one-train LTP yielded persistent L-LTP at both sets of inputs. In contrast, R(AB) transgenics show significantly less potentiation following one-train LTP. Asterisk denotes statistical significance (*$p < 0.05$).

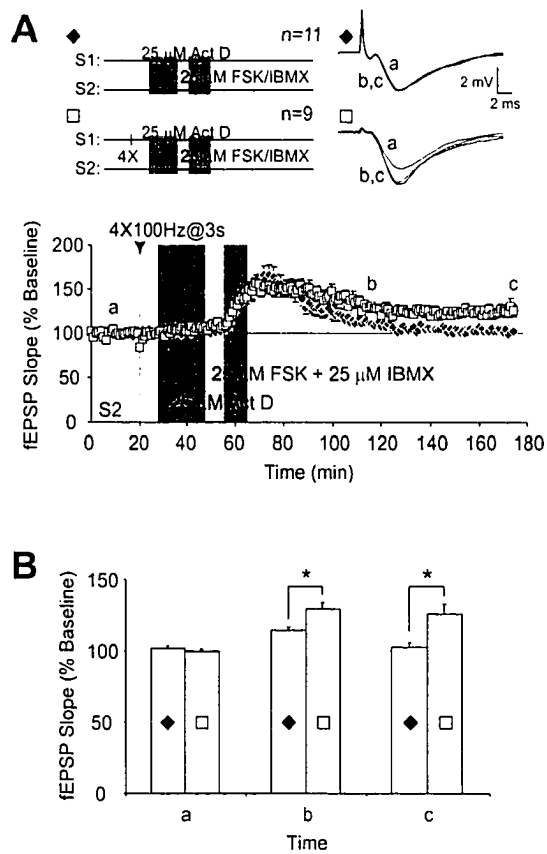

Figure 4.06: Pharmacological activation of the cAMP/PKA pathway can produce a synaptic tag that captures persistent synaptic facilitation. (A) Application of FSK+IBMX in the presence of actinomycin D produces a transient synaptic facilitation that can be prolonged by pairing drug application with four-train L-LTP. **(B)** Summary histogram shows a significant difference in mean potentiated levels between groups (*p > 0.05).

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CHAPTER 5:

GENERAL DISCUSSION

SYNAPSE SPECIFICITY OF LONG-TERM SYNAPTIC PLASTICITY: SUMMARY OF THESIS FINDINGS

Input specific, long-lasting synaptic plasticity has been proposed as a neural correlate of information storage in many brain regions and across many species, including long-term facilitation in *Aplysia* (Martin et al., 1997; Casadio et al., 1999) to L-LTP in honeybee mushroom bodies (Oleskevich et al., 1997), neocortical L-LTP in mammals (Kirkwood and Bear, 1993, 1994), and hippocampal L-LTP in mammals (Bliss and Lømo, 1973; Lynch et al., 1977; Andersen et al., 1977). Because neurons form thousands of contacts with each other, limiting plasticity to activated synapses maintains the fidelity of the information that is being encoded. L-LTP in the hippocampus is one cellular process through which new long-term memories may be encoded (Morris et al., 2003).

According to the synaptic tagging hypothesis, input specific L-LTP expression requires (1) transcription and synthesis of plasticity-related proteins (PRPs) that stabilize L-LTP expression; and (2) setting of an activity-dependent synaptic tag that enables capture of PRPs (Frey and Morris, 1998). Despite strong evidence for the existence of synaptic tags, its identification and the conditions that regulate its generation remain unknown.

Heterosynaptic Metaplasticity in Mouse Hippocampal CA1

The first objective of my research was to characterize a novel form of metaplasticity in hippocampal area CA1. As I report in *Chapter 2*, low-frequency stimulation (LFS) at 5-Hz for 3 min induces a transient, non-input specific synaptic

depression. However, not all types of synaptic plasticity are reflected by a prolonged change in synaptic efficacy, and LFS below the threshold for LTP induction can effect input specific changes in the synaptic state to inhibit subsequently induced LTP (Abraham and Tate, 1997). In parallel with the non-input specific synaptic depression that follows 5-Hz LFS, metaplastic inhibition of subsequent LTP by prior 5-Hz LFS was evident at homosynaptic and heterosynaptic inputs. Prior LFS impaired subsequent L-LTP induced at the same inputs that received the conditioning LFS (i.e., homosynaptic inhibition) and L-LTP induced at independent inputs converging onto the same population of postsynaptic cells (i.e., heterosynaptic inhibition). LTP induction requires a critical rise in postsynaptic Ca^{2+} that can occur through NMDA receptor or L-type voltage-gated Ca^{2+} channel (VGCC) activation (Grover and Teyler, 1990). To determine if metaplastic effects of prior LFS on L-LTP were dependent on the initial route of Ca^{2+} entry during L-LTP induction, I examined the effects of prior LFS on two forms of L-LTP that I characterized for VGCC-dependence. Prior LFS homosynaptically and heterosynaptically impaired L-LTP induced with a compressed tetraburst stimulation protocol (VGCC-dependent L-LTP), and L-LTP induced with a spaced tetraburst protocol (VGCC-independent L-LTP). L-LTP induced with both of these protocols is dependent on NMDA receptor activation (Woo et al., 2003), and prior LFS might selectively regulate NMDA receptor-dependent forms of LTP. However, I found that prior LFS does not affect NMDA receptor-dependent E-LTP. These results suggested that prior LFS might gate the cellular consolidation of L-LTP.

Activity-Dependent Regulation of Synaptic Tagging

My second objective was to determine the locus of LFS-mediated metaplastic effects on L-LTP (*Chapter 3*). L-LTP requires transcription (Nguyen et al., 1994) and I hypothesized that prior LFS could exert homosynaptic and heterosynaptic inhibition of L-LTP by blocking postsynaptic gene expression. I began by developing a stimulation protocol that would allow me to dissociate effects of LFS on synaptic tagging versus effects on transcription. I found that persistent L-LTP could be generated in the presence of a transcriptional inhibitor, actinomycin D (Act D), if it was preceded by established L-LTP. Moreover, L-LTP induced in the presence of Act D was triggered by stimulation that normally only results in transient potentiation. It has previously been shown that pairing "weak" (i.e., E-LTP-inducing) with "strong" (i.e., L-LTP-inducing) stimulation enables L-LTP expression at the weaker set of inputs due to the generation of a synaptic tag and capture of PRPs (Frey and Morris, 1997; **Figure 3.06C**). Because L-LTP expression could be captured in the presence of Act D, these results also suggest that generation of the synaptic tag and capture of PRPs does not require transcription.

Next, I showed that synaptic capture of L-LTP expression by weak stimulation was prevented if Act D was applied during strong stimulation. This suggests that stable L-LTP expression elicited with weak stimulation in the presence of Act D is likely due to captured transcriptional products already in circulation. Therefore, if prior LFS impairs L-LTP by inhibiting gene expression, no transcriptional products should be available for capture by subsequent weak stimulation. Contrary to this hypothesis, I found that LFS applied homosynaptically and heterosynaptically prior to strong stimulation did not prevent synaptic capture by subsequent weak stimulation (**Figure 3.06E**). These results

showed that metaplastic effects of LFS are not mediated by blocking transcription. In addition, homosynaptic and heterosynaptic inhibition by prior LFS is reversible and limited in duration because synaptic capture by weak stimulation was possible 40 min after initial application of LFS. Thus, these results suggested that prior LFS decreases the stability of subsequent L-LTP by impairing synaptic tagging and capture of PRPs. Consistent with this hypothesis, LFS applied prior to weak stimulation (after establishing L-LTP at separate inputs) prevented capture of stable L-LTP expression (**Figure 3.06F**). Although LFS impaired synaptic tagging in an anterograde manner, it did not affect L-LTP that was previously established in a separate pathway.

LTP can be reversed (depotentiated, Dpt) in an activity-dependent manner by LFS (Zhou and Poo, 2004). Transfer of somatic transcriptional products ("acquired immunity) and local protein synthesis ("local immunity") can confer immunity to depotentiation (Woo and Nguyen, 2003). My results suggest that synaptic tagging and capture occurs within minutes of synaptic activation as successful acquired immunity was evident when challenged with depotentiating LFS applied 5 min post-tetanus. Consistent with a role in blocking synaptic tagging and capture, prior LFS selectively impaired acquired Dpt immunity that is mediated by somatic gene expression. In contrast, even though L-LTP induced after LFS is not persistent, it retains immunity to Dpt. Because acquired immunity is impaired, this suggested that local protein synthesis-mediated immunity is not affected by prior LFS. Taken together, my results in *Chapter 3* support the notion that separate processes mediate L-LTP-associated transcriptional processes and the expression of persistent potentiation. Moreover, transcription (e.g., gene products that stabilize L-LTP and confer immunity to Dpt), translation (e.g., local protein synthesis that

mediates Dpt immunity), and synaptic tagging can be differentially regulated by prior synaptic activity.

Critical Role for PKA in Synaptic Tagging

The third objective of my thesis was to determine the role of protein kinases and phosphatases in the metaplastic effects of 5-Hz LFS and synaptic tagging (*Chapter 4*). I found that homosynaptic and heterosynaptic inhibition of L-LTP by prior LFS requires the activity of protein phosphatases 1/2A. Protein kinases balance the activity of phosphatases to gate the expression and stability of long-lasting plasticity (Blitzer et al., 1995; Bito et al., 1996) and PKA has been implicated in the consolidation of long-lasting plasticity (Nguyen and Woo, 2003). I found that prior LFS impairs a form of PKA-dependent chemical LTP in a homosynaptic and heterosynaptic manner, suggesting that prior LFS impairs L-LTP by inhibiting activation of cAMP/PKA. Given the effects of LFS on synaptic tagging (*Chapter 3*), I hypothesized that PKA may play a critical role in synaptic tagging. Consistent with this idea, I presented evidence that genetic and pharmacological inhibition of PKA prevents synaptic capture of L-LTP. Pairing strong and weak stimulation at separate inputs results in stable L-LTP expression at both inputs. However, weak stimulation in the presence of a PKA inhibitor, KT-5720, elicited transient LTP. To examine the effects of genetic downregulation of PKA activity, R(AB) mice were tested because they express substantially lower hippocampal PKA activity due to transgenic overexpression of an inhibitory isoform of RIα (Abel et al., 1997). Synaptic capture in transgenics, as assayed with the strong + weak pairing protocol, was significantly impaired when compared to their wildtype littermates. These results show

that PKA is necessary for synaptic tagging and capture of L-LTP expression as it can be impaired by electrical (i.e., LFS-mediated; *Chapter 3*), pharmacological, or genetic downregulation of PKA signalling (*Chapter 4*). To examine the criterion of sufficiency, I first established a protocol to transiently activate cAMP/PKA signaling without triggering L-LTP stabilizing gene expression. Brief application of forskolin + 3-isobutyl-1-methylxanthine (FSK/IMBX) in the presence of Act D (FSK/IBMX+Act D) elicited a transient facilitation of fEPSPs. Pairing FSK/IBMX+Act D with previously established L-LTP elicited persistent facilitation in the heterosynaptic pathway, demonstrating that cAMP/PKA activation is also sufficient to generate a synaptic tag that is capable of capturing stable L-LTP expression.

Summary of Conclusions

In summary, my data support the following conclusions:

1. Prior LFS impairs subsequently induced L-LTP in a homosynaptic and heterosynaptic manner.
2. LFS selectively impairs L-LTP, while leaving E-LTP intact, by regulating synaptic tagging and capture of L-LTP expression.
3. Metaplastic effects of LFS require PP1/PP2A activation and involve a down-regulation of cAMP/PKA signaling.

4. PKA is necessary for synaptic tagging and capture - electrical (i.e., LFS-mediated), pharmacological, and genetic downregulation of PKA signalling impair synaptic tagging and capture.

5. PKA is sufficient for synaptic tagging and capture - pharmacological activation of cAMP/PKA signaling can generate a synaptic tag to capture prolonged facilitation.

Collectively, my results suggest that PKA plays a critical role in synaptic tagging and in synapse-specific, long-lasting potentiation in CA1 pyramidal neurons. Moreover, PKA-mediated signaling can be constrained by prior episodes of synaptic activity to regulate subsequent L-LTP expression and the integration of many synaptic events over time.

A Critical Role for PKA in Synaptic Tagging and its Regulation by Prior Synaptic Activity: An Amalgamation of Results

Heterosynaptic Metaplasticity

Previous studies showed that LTP elicited with a particular pattern of stimulation could give rise to transient or persistent LTP depending on the history of activation of the neuron (Frey and Morris, 1997; Barco et al., 2002; This thesis, Chapter 3). Activated synapses are thought to generate a synaptic tag that enables capture of protein products required for the establishment of long-term synaptic plasticity (Frey and Morris, 1997). In this manner, triggering transcription-dependent late-LTP at one set of inputs leads to

heterosynaptic facilitation of L-LTP induction at other synaptic sites. This type of cell-wide metaplasticity is consistent with the heterosynaptic nature of changes in the threshold for LTP induction as proposed by the BCM theory of metaplasticity early in development (Bienenstock et al., 1982). My findings complement the discovery of heterosynaptic facilitation by establishing that the threshold for LTP induction can also be reduced in a heterosynaptic manner. Prior LFS impaired subsequent L-LTP induced at both homosynaptic and heterosynaptic inputs. Because stimulation electrodes were placed in random configuration, this suggests that prior LFS results in a cell-wide impairment of synaptic tagging, although additional experiments will be required to determine if this phenomenon is limited to the apical dendrites of CA1 pyramidal neurons. In addition, further experiments are required to determine if metaplastic effects of LFS reflect a shift in the threshold for L-LTP induction or constitute a general inhibition of L-LTP. Previous experiments have shown that LTP induced by brief 5-Hz stimulation shifts the threshold for subsequent homosynaptic LTP induction such that the original 5-Hz protocol is rendered insufficient for induction, but stimulation protocols using higher frequencies remain intact (Moody et al., 1999). In accord with the BCM theory, metaplastic effects of prolonged 5-Hz LFS may not affect LTP elicited with stronger induction protocols that involve higher stimulation frequencies.

Synaptic Tagging in Long-term Synaptic Plasticity

The molecular nature of the tag remains unknown, but previous research has shown that formation of the synaptic tag requires NMDA receptors and PKA (Barco et al., 2002). My results confirm and extend these findings by showing that PKA is

necessary and sufficient to generate a synaptic tag, and the tagging process can be regulated in an anterograde manner by synaptic activity through the recruitment of protein phosphatases 1/2A. Metaplastic effects of 5-Hz LFS on synaptic tagging are time limited and impair L-LTP that is induced 10 min after, but not 40 min after initial LFS (Woo and Nguyen, 2002; This thesis, *Chapter 3*). LFS and PKA inhibitors did not impair previously established L-LTP and suggest a limited time window during which the newly set synaptic tag is sensitive to disruption. Consistent with this interpretation, recent work in the Frey lab shows that synaptic tags can be reset in an activity-dependent manner after LTP induction by applying depotentiating LFS at 1-Hz (Sajikumar and Frey, 2004). Moreover, retrograde resetting of synaptic tags was ineffective when LFS was applied beyond 5 min post-tetanus (Sajikumar and Frey, 2004). These data complement my findings, which showed that Dpt immunity could be successfully acquired within 5 min post-tetanus. Together, these results support a rapid generation of a synaptic tag and capture of pre-existing PRPs that occurs within minutes of synaptic activation. Although the study by the Frey lab did not investigate the specific role of protein phosphatases in resetting the synaptic tag, depotentiation with LFS at 1-Hz has previously been shown to require PP1/PP2A activity (O'Dell et al., 1994). Collectively, these data demonstrate that the balance of PKA and PP1/PP2A can regulate synaptic tagging in an anterograde and retrograde manner.

Protein phosphatases are recruited by low-frequency stimulation to induce long-term depression (LTD; Mulkey et al., 1993, 1994; Morishita et al., 2001). Significantly, synaptic tagging and capture can also be observed with LTD (Kauderer and Kandel, 2000; Sajikumar and Frey, 2004A). Like LTP, LTD can be divided into different

temporal and mechanistic phases – a transient, protein-synthesis independent "early" phase (E-LTD) and long-lasting LTD (L-LTD) that requires protein synthesis. In experiments analogous to the original LTP synaptic tagging experiments (Frey and Morris, 1997), it was shown that L-LTD induction at one input enables the capture of L-LTD at an independent set of inputs receiving either L-LTD-inducing stimulation in the presence of protein synthesis inhibitors, or stimulation that normally elicits E-LTD (Kauderer and Kandel, 2000; Sajukumar and Frey, 2004A). Thus, like LTP, induction of L-LTD can be heterosynaptically associated following long-lasting synaptic plasticity.

Surprisingly, recent work shows that L-LTP and L-LTD can also be heterosynaptically associated (Sajikumar and Frey, 2004A). L-LTP stimulus at one set of inputs followed by E-LTD stimulus at a separate set of inputs generates L-LTD at the latter inputs. Conversely, if L-LTD stimulus is followed by an E-LTP stimulus, E-LTP is converted to L-LTP. These results suggest that "cross-capture" of transcriptional and translational products resulting from the induction of long-term synaptic plasticity enable stabilization of both L-LTP and L-LTD (Sajikumar and Frey, 2004A). Consistent with this model, similar translational mechanisms have been implicated in both LTP and LTD (Gallagher et al., 2004; Hou and Klann, 2004; Kelleher et al., 2004; Govindarajan et al., 2004). It is believed that the nature of synaptic activity determines the direction of change in synaptic efficacy by generating LTP or LTD synaptic tags that mediate capture of L-LTP- or L-LTD-associated PRPs, respectively (Sajikumar and Frey, 2004A).

Numerous studies have demonstrated that PKA is required for L-LTP expression (Frey et al., 1993; Matthies and Reymann, 1993; Huang and Kandel, 1994; Abel et al., 1997; reviewed in Nguyen and Woo, 2003), and my results implicate PKA in synaptic

tagging. Given the possibility that LTP and LTD PRPs are generally distributed after induction of long-term changes in synaptic efficacy, it follows that LTD should involve a downregulation of processes that could generate a LTP synaptic tag. Interestingly, several lines of evidence suggest that, in addition to protein phosphatase activation, LTD also involves a concurrent downregulation of PKA signaling. Low-frequency stimulation promotes PP1 translocation to synaptic sites (Morishita et al., 2001) and induces conformational changes in cytoskeletal and scaffolding proteins (Okamoto et al., 2004) that disrupt postsynaptic targeting of PKA (Gomez et al., 2002). LTD is also correlated with a selective PP1/PP2A-mediated dephosphorylation of AMPA receptors at the PKA, but not CaMKII, phosphorylation site (Lee et al., 1998, 2000; Kameyama et al., 1998).

Previous work has shown that the balance of PKA and PP1/PP2A activity regulates many aspects of long-lasting synaptic plasticity, including positive and negative regulation of transcription factors, such as CREB (Bito et al., 1996), and covalent modifications of AMPA receptors at sites that regulate channel properties (Banke et al., 2000; Lee et al., 2000) and receptor trafficking (Lee et al., 1998, 2000; Kameyama et al., 1998). My results support and extend this view of PKA/PP1 function by showing that PKA-mediated synaptic tagging may be a novel control point for L-LTP expression that can be regulated in an activity dependent manner (**Figure 5.01**).

THEORETICAL IMPLICATIONS OF RESEARCH FINDINGS

The mammalian hippocampus is a critical component of a neural system involved in the initial storage of declarative memory. Hippocampal synaptic activity and plasticity

are believed to represent a continuous and automatic encoding of attended experiences (Morris et al., 2003). Stimulus elements and the context of experiences are associated in relational hippocampal networks that are linked by common features to allow for flexible inferential memory expression (Eichenbaum, 2004). The associative properties of LTP make it an attractive candidate cellular mechanism for this type of information storage.

Temporal summation of synaptic activity at converging inputs that are closely associated in time (i.e., tens of milliseconds) elicits associative LTP at both sets of inputs (Levy and Steward, 1979; Barrionuevo and Brown, 1983). During L-LTP, transcription and translation permit the integration of synaptic events over much longer periods of time (tens of minutes). Induction of transcription-dependent late-LTP at one set of inputs results in a period of cell-wide reduced threshold for subsequent long-lasting plasticity (Frey and Morris, 1997; Barco et al., 2002; Sajikumar and Frey, 2004A; *This thesis, Chapter 3*). From a behavioural perspective, this period may reflect a process of rapid consolidation through which events that are ordinarily remembered only transiently, can be stabilized for longer periods by significant events occurring around the same time (Morris and Frey, 1997).

The synaptic tagging hypothesis was initially proposed as a solution to explain input specific expression of protein synthesis-dependent L-LTP (Frey and Morris, 1997). In the elegant study by Frey and Morris (1997), they demonstrated that L-LTP established at one set of inputs could heterosynaptically facilitate L-LTP at other inputs on the same population of postsynaptic neurons. These studies highlighted a potential problem with synaptic tagging and input specific expression of long-lasting plasticity. Paradoxically, because synaptic activity that is subthreshold for inducing long-lasting

plasticity can nonetheless generate synaptic tags, they can also result in *reduced* specificity of long-lasting plasticity when multiple synaptic events are closely related in time. By regulating synaptic tagging, 5-Hz LFS may act to restore input specificity to L-LTP during periods of reduced threshold for long-term plasticity. In addition, homosynaptic and heterosynaptic inhibition of L-LTP expression by prior LFS may also act to prevent saturation and stabilize synaptic strengths within a dynamic range that is optimal for information storage (Abraham and Bear, 1996).

CONCLUSION AND FUTURE DIRECTIONS

In my thesis, I demonstrate that PKA is necessary and sufficient to generate a synaptic tag that enables capture of input specific L-LTP expression. Long-term synaptic plasticity in different brain regions and across many species is characterized by a common requirement for cAMP/PKA signaling, and transcription and translation. Long-term synaptic plasticity in the mammalian hippocampus (Andersen et al., 1977; Huang and Kandel, 1994), visual cortex (Kirkwood et al., 1993; Beaver et al., 2001), and lateral amygdala (Huang et al., 2000; Schafe et al., 2000) require PKA and protein synthesis. These requirements are also evident in invertebrates including *Aplysia* (Martin et al., 1997; Casadio et al., 1999), honeybees (Oleskevich et al., 1997), and *Drosophila* (Livingstone et al., 1984; Feany et al., 1995; Tully et al., 1994), where the original memory mutants with dysfunctional PKA signaling first implicated PKA in learning and memory (Dudai et al., 1976). It remains to be determined if PKA plays a similar role in input specific long-term synaptic plasticity in these systems.

Specific phosphorylation and dephosphorylation events fit well with previous experimental evidence regarding the nature of the synaptic tag (Frey and Morris, 1998; Martin and Kosik, 2002; Kelleher et al., 2004A). PKA has also been implicated in synaptic tagging in input specific facilitation of the sensorimotor synapse in *Aplysia* (Martin et al., 1997; Casadio et al., 1999). However, the downstream target of PKA to generate the tag is unknown. The mechanism through which synaptic capture occurs and the identity of captured proteins also remains to be elucidated. Initially, protein synthesis dependent L-LTP was believed to reflect purely transcriptional requirements. More recently, translation independent of transcription has been discovered from dendritically localized mRNAs (Ouyang et al., 1999). Although translation of pre-existing mRNAs can stabilize L-LTP initially, transcriptional products are required to consolidate more permanent changes (Otani et al., 1989; Kelleher et al., 2004). My results with Act D support this view and show that synaptic capture of transcriptional products are required to stabilize L-LTP. Significantly, the identity, and spatial and temporal limits on the availability of PRPs have yet to be identified. In addition, the process through which PRPs are captured at active synapses is unknown.

The recent demonstration of cross-capture between L-LTP and L-LTD suggest that separate synaptic tags exist for LTP and LTD. If the LTP tag is mediated by kinase activity, it follows that the LTD tag may involve dephosphorylation of specific substrates. In addition, just as the phenomena of LTP and LTD encompass many different forms and are supported by a rich variety of signaling molecules and processes, there are also likely multiple candidate synaptic tags.

Last, but not least, it will be critical to develop an in vivo model of synaptic tagging in order to examine these issues in the context of animal behaviour (Hassan and Frey, 2004).

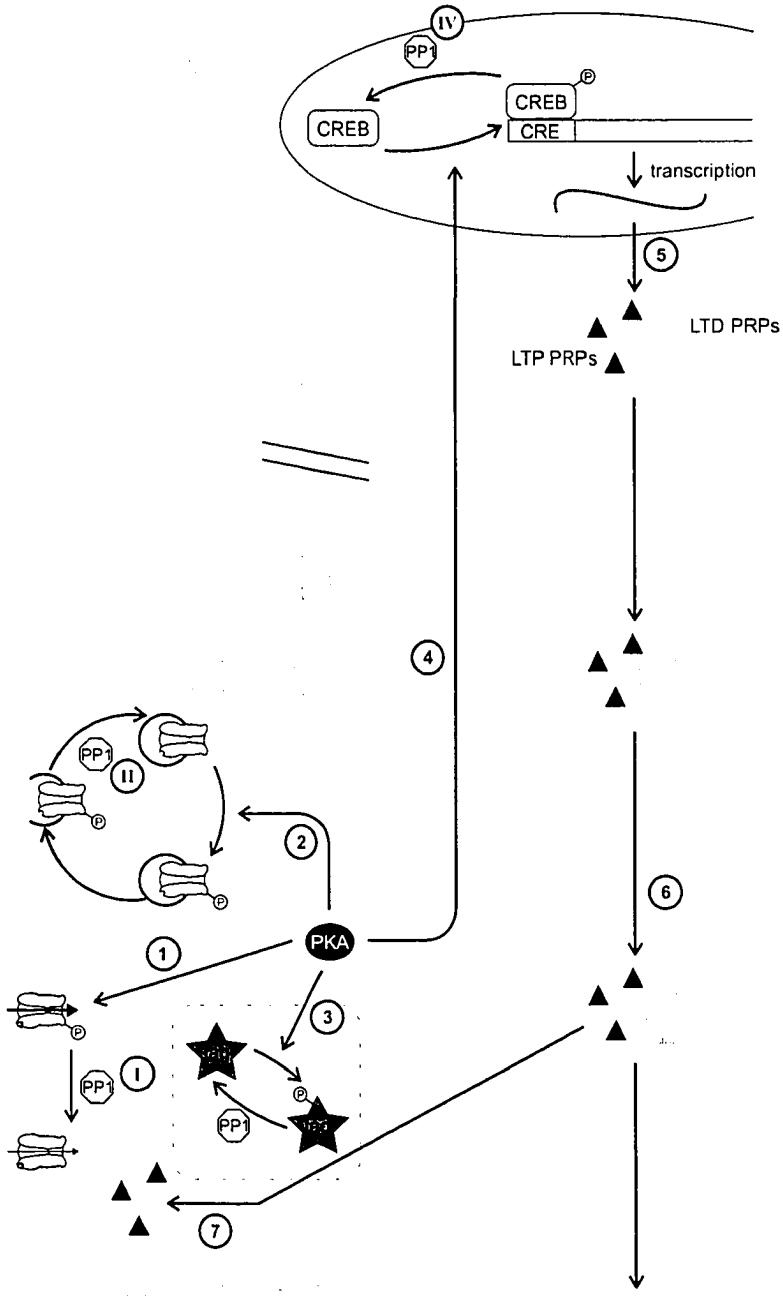

Figure 5.01: Emerging model in L-LTP expression in hippocampal CA1: regulation by PKA and PP1/PP2A. PKA activation during L-LTP covalently modifies AMPA receptors to (1) alter AMPA receptor channel conductance and (2) regulate receptor trafficking to the synaptic membrane. I found that PKA activity sets a synaptic tag that enables capture of L-LTP stabilizing PRPs (3). (4) PKA can translocate to the nucleus where it phosphorylates transcription factors to trigger CRE-mediated gene expression. (5) L-LTP- and L-LTD-stabilizing PRPs are synthesized and (6) distributed cell-wide. A PKA-dependent synaptic tag (3) permits capture of LTP PRPs that stabilize long-lasting plasticity at active synapses (7). PKA-mediated changes in AMPA receptor channel properties (I), AMPA receptor trafficking (II), and CREB phosphosphorylation (IV) are all opposed by PP1/PP2A activity. My results show that PP1/PP2A can be recruited by low frequency activity to inhibit synaptic tagging and impair L-LTP expression (III).

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