Cellular Mechanisms of Synaptic Plasticity in Hippocampal Area CA1

Eric S. Guire

Doctoral Dissertation

Defended on April 10, 2006

Presented to the Neuroscience Graduate Program

and the Oregon Health & Science University

School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

School of Medicine

Oregon Health & Science University

CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. dissertation of

ERIC S. GUIRE

has been approved by the following:

[redacted]

Thomas Soderling, Mentor

[redacted]

Victor Derkach, Co-Mentor

[redacted]

Craig Jahr, Chair

[redacted]

Philip Stork, Member

[redacted]

Matthew Frerking, Member

[redacted]

Jacob Raber, Member

OHSU
Oregon Health & Science University

SCHOOL OF MEDICINE
OFFICE OF GRADUATE STUDIES

Mail code: L102 • 3181 S.W. Sam Jackson Park Road • Portland, Oregon 97239-3098
TEL: 503 494-6222 • FAX: 503 494-3400

May 3, 2006

Eric S. Guire
School of Medicine
Neuroscience Graduate Program
6438 N. Delware Avenue
Portland, OR 97217

Dear Eric,

I am pleased to inform you that, based on a nomination from your program, the Awards Committee of the School of Medicine Graduate Studies Program has selected your dissertation entitled "Cellular Mechanisms of Synaptic Plasticity in Hippocampal Area CA1" for the *2006 John A. Resko Outstanding Doctoral Dissertation Award.*

This award, which is co-sponsored by the John A. Resko Endowment Fund and the OHSU School of Medicine Alliance (http://www.ohsu.edu/sma/), consists of a certificate and a $1,000 honorarium. It will be presented at the School of Medicine Hooding Ceremony on Friday, June 2, 2006 at 9:30 am in the Arlene Schnitzer Concert Hall. I hope that you will be able to attend this ceremony to receive your award. If you are unable to attend, please contact Jim Bauer (somgrad@ohsu.edu) in the Office of Graduate Studies and we will make alternative arrangements for delivering the award.

The Alliance would also like to invite you to join other student awardees at their Spring Luncheon on Tuesday, May 16th, 11:30 am at the Waverly Country Club. You will soon receive a more formal invitation with additional details about this event from the Alliance President, Bonnie Nuttall. I certainly hope that your schedule will permit you to attend this luncheon.

Congratulations on this accomplishment and best wishes for the future.

Sincerely,

[redacted]

Christopher L. Cunningham, Ph.D.
Associate Dean for Graduate Studies
School of Medicine
Oregon Health & Science University

cc: Joseph Robertson, Cheryl Maslen, Peter Gillespie, Thomas Soderling, John Resko, Bonnie Nuttall

TABLE OF CONTENTS

Table of Figures

List of Common Abbreviations

4EBP1	eIF4E binding protein 1
μA	Microampere
μL	Microliter
μM	Micromolar
μs	Microsecond
Ω	Ohm
θ_M	LTP threshold
ABP	AMPAR binding protein
ACSF	Artificial cerebrospinal fluid
ADF	Actin depolymerizing factor
AKAP	A-kinase anchoring protein
AMPA	α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AMPAR	AMPA-type glutamate receptor
APV	D(-)-2-Amino-5-phosphonopentanoic acid; NMDAR antagonist
B.C.M. rule	Sliding modification rule of Bienenstock, Cooper, and Munro
ca	Constitutively active
$CaCl_2$	Calcium chloride
CalDAG	Calcium diacylglycerol
CaMK	Calcium/calmodulin-dependent protein kinase (I,II,IV,K)
CaMKIIN	CaMKII inhibitory protein
CaMKIINtide	Selective peptide inhibitor of CaMKII
CBP	CREB-binding protein
CREB	Cyclic AMP response element-binding protein
CYFIP	Cytoplasmic FMRP-interacting protein
DD	Dedepression
DHPG	Dihydroxyphenylglycine
DIV	Days *in vitro*
dn	Dominant-negative
DNA	Deoxyribonucleic acid
DP	Depotentiation
EGF	Epidermal growth factor
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid
eIF4E	Eukaryotic translation initiation factor 4E
E-LTD	Early-phase LTD
E-LTP	Early-phase LTP
EPSC	Excitatory postsynaptic current
EPSP	Excitatory postsynaptic potential
ER	Endoplasmic reticulum
ERK	Extracellular regulated kinase
fEPSP	Field excitatory postsynaptic potential
Flag	Epitope used for tagging recombinant proteins
FMRP2	Fragile-X mental retardation protein 2
FRAP	Fluorescence recovery after photobleaching

GAP	GTPase-activating protein
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GISP	Glycine-induced synaptic potentiation
GluR	Glutamate receptor subunit
HB-GAM	Heparin-binding growth associated molecule
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HFS	High frequency stimulation, such as 100 Hz or *theta*-bursts
HSPC300	Heat-shock protein C300
Hz	Cycles per second
IC_{50}	Inhibitory concentration, 50%
IgG	Immunoglobulin G
KCl	Potassium chloride
kDa	Kilodalton
kHz	Kilohertz
KN-62	General CaM Kinase inhibitor
KN-93	General CaM Kinase inhibitor
LFS	Low frequency stimulation
LIMK	LIM (Lin-11, Isl-1, and Mec-3) kinase
L-LTD	Late-phase LTD
L-LTP	Late-phase LTP
LTD	Long-term depression
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MEK	ERK-MAP kinase kinase
$MgCl_2$	Magnesium chloride
mGluR	Metabotropic glutamate receptor
mL	Milliliter
mM	Millimolar
mRNA	Messenger ribonucleic acid
msec	Millisecond
mV	Millivolt
myc	Epitope used for tagging recombinant proteins
n	Number
NaCl	Sodium chloride
NaF	Sodium fluoride
$NaHCO_3$	Sodium bicarbonate
NaH_3PO_4	Sodium phosphate, monobasic
NG-108	Neuroglioma cell line
nM	Nanomolar
NMDA	N-methyl-D-aspartate
NMDAR	NMDA-type glutamate receptor
NP40	Non-ionic detergent P40
NR2B	NMDA-type glutamate receptor subunit 2B
NSF	N-ethylmaleimide sensitive factor
p	Probability value

PAGE	Polyacrylamide gel electrophoresis
PAK	p21-activated kinase
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PI3K	Phosphoinositide-3 kinase
PICK	Protein interacting with C-kinase
PIX	Pak-interactive exchange factor
PKA	Protein kinase A
PKB	Protein kinase B (Akt)
PKC	Protein kinase C
PKI	Selective peptide inhibitor of PKA
PMSF	Phenylmethylsulphonylfluoride
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PP2B	Protein phosphatase 2B (calcineurin)
PPF	Paired pulse facilitation
PP-LFS	Paired pulse low frequency stimulation
PSD	Postsynaptic density
PSD-95	95 kD PSD protein
PTP	Post-tetanic potentiation
PVDF	Polyvinylidine fluoride
RasGRF	Ras guanine nucleotide-releasing factor
Ras N17	Dominant-negative form of Ras
rpm	Revolutions per minute
RyR3	Ryanodine receptor 3
SAP-97	Synapse associated protein-97
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SH3	Src-homology 3 domain
SL327	Selective MEK inhibitor
SNAP-25	synaptosome-associated protein 25
STO-609	Selective CaMKK inhibitor
STP	Short-term potentiation
TARP	Transmembrane AMPAR binding protein
theta-bursts	Brief 100Hz bursts of activity, occurring at 200msec intervals
theta-rhythm	Pattern of brain activity during certain states of consciousness
Tris	Tris Hydroxymethylaminoethane; buffer
U0126	Selective MEK inhibitor
VGCC	Voltage-gated calcium channel
WASP	Wiskott-Aldrich syndrome protein
WAVE	Wiskott-Aldrich syndrome protein verprolin homologous
WRP	WAVE-associated RacGAP protein
YFP	Yellow fluorescent protein

I dedicate this thesis to my unborn child,
who somehow managed to develop a complete human brain
in much less time than it took me
and my collaborators
to uncover just two small details of how the brain works.

Acknowledgements

Some perceive science as standing on the shoulders of giants. I believe this to be an unfitting description. Progress comes first through imitation, then through refinement. Most of the progress made in science, towards a better understanding of ourselves and the world around us, and towards enabling the relief of human suffering, comes from the interaction of scientists with one another. Indeed, the majority of paradigm-shifting breakthroughs, such as the discovery of the structure of DNA or the first description of membrane conductance changes that occur during an action potential, would not have been possible without the foundations that those discoveries rest upon. Nor would they have likely come to light had their discoverers not bounced these newly conceived ideas off of their friends and colleagues. This is the collaborative nature of science. I thus owe a debt of gratitude for my accomplishments as a PhD student to the collective works of those who came before me and to those who work around me. At the top of this list of course, are my mentors Tom Soderling and Victor Derkach, two brilliant scientists who have provided me with support that extends beyond my scientific training into my personal and family life. I have also appreciated the support other past and present members of Tom's lab who I have worked with (in particular, Michael Oh, John Schmitt, Gary Wayman, Monika Davare, Takeo Saneyoshi, Sean Nygaard, Debbie Brickey, and Coleen Atkins). Frequent discussions of ideas, data, and scientific publications in the lab have been incredibly valuable. My collaboration with John Schmitt was a salient reminder of the value in working with others towards a common goal. I have also greatly enjoyed the opportunity to work with Tom's son, Scott Soderling. What began as a simple experiment quickly evolved into an extensive and rewarding collaboration.

Furthermore, I have benefited from working in an institute (the Vollum Institute) that successfully fosters collaboration and serves as an intellectual hub of activity. I also thank the members of my thesis committee (Phil Stork, Craig Jahr, and Matthew Frerking, in addition to Tom and Victor) for their wisdom, encouragement and guidance, and Jacob Raber for serving on my defense committee.

Finally, I feel the best science comes from a combination of earnest curiosity and a sense of duty, two traits that I value in my parents and continue to nurture in myself. A successful contribution to science also requires a life with sufficient freedom from burden to pursue one's intellectual interests. The good fortune I have enjoyed thus far is rooted in a happy and stable childhood in a free and privileged society, the guidance, example, and love of my parents (Patrick and Joette), and the teachings of many good teachers. Finally, I cannot overstate the importance of Mame and the role she has played in my adult life. She is a beautiful, loving, supportive, and caring wife (not to mention incredibly funny). Quite simply, she is wonderful. Together, we have realized the strength that comes from, and the value in, a true partnership.

Abstract

The coordinated activation of a number of neuronal activity-regulated kinases is required for the full expression of LTP. Modulating the efficacy of synaptic transmission, such as occurs in LTP and LTD, is thought to be a cellular mechanism for storing information in the brain. In particular, a variety of pharmacological and genetic studies have established important roles for CaMKII and ERK in excitatory synaptic plasticity, as well as hippocampal-dependent memory formation [1-3]. In hippocampal area CA1, CaMKII activity increases synaptic strength by two known mechanisms: modification of AMPAR channel properties [4] and regulation of AMPAR trafficking [5]. The ERK pathway also contributes to synaptic strengthening, in part through the regulation of mRNA translation [6], as well as through regulation of AMPAR trafficking mediated by the Ras pathway [5, 7]. As opposed to the classical routes for ERK activation in many cell types by growth factors, a calcium-dependent pathway upstream of Ras triggers the activation of ERK during NMDA receptor-dependent LTP in neurons, but precisely how this occurs remained unexplained. Previous investigations have focused on CaMKII as a potential mediator of calcium-dependent ERK activation in *via* the Ras GTPase activator SynGAP, but recent studies fail to support this hypothesis [8-11]. Interestingly, the CaMKK/CaMKI pathway was recently shown to mediate depolarization-induced activation of ERK *via* Ras in NG-108 cells [12]. Therefore, it was important to determine whether ERK can be similarly regulated by CaMKK/CaMKI in neurons, and to determine whether this pathway provides a biochemical link between the calcium-permeable NMDA receptor and ERK activation, which is required for the full expression

of LTP. This work and its findings are presented in chapter 2, and constitute the first half of this thesis.

In addition to kinases, small GTPases (G-proteins) such as Rac, Ras, and Rap likely play an important role in LTP and its counterpart LTD. However, the role of small G-proteins in LTP/LTD is less well characterized than that of CaMKII and ERK. Recent evidence suggests that activation of these G-proteins is somehow required for proper AMPAR trafficking during synaptic plasticity [7, 13], one of the primary mechanisms of LTP/LTD expression. Small G-proteins generally act as molecular switches for various cellular processes, including regulation of the actin cytoskeleton and regulation of gene transcription, *via* the binding and activation of multiple downstream effectors [14]. In LTP and LTD, these effectors and their signaling partners are believed to coordinate the cytoskeletal rearrangements underlying the morphological changes that accompany synaptic plasticity [15]. Interestingly, cytoskeletal dynamics may also contribute to activity-dependent AMPAR trafficking [16, 17]. Analyses of model organisms and gene mutations in inherited forms of mental retardation also implicate small G-proteins in the underlying alterations to synaptic plasticity and neuronal network wiring that accompany these cognitive disorders [18-21], which are commonly associated with abnormalities in dendritic spine morphology. Rac-1, as with other Rho-GTPase family members, is believed to contribute to the remodeling of actin in response to extracellular stimuli. Rac-1 couples to dynamic actin, in part, through its interaction with the scaffolding protein WAVE-1 [22, 23]. The importance of this pathway in learning and memory was recently demonstrated by genetic disruption of WAVE-1, which resulted in sensory motor

retardation and reduced learning in mice [24]. It was therefore of great interest to determine whether loss of WAVE-1 was also associated with alterations in dendritic spine morphology, neuronal connectivity, and synaptic plasticity. Furthermore, WAVE-1 was found to regulate a form of synaptic metaplasticity, which I have termed *capacitative metaplasticity*. On theoretical grounds, this form of synaptic plasticity may more accurately reflect the cellular process underlying learning and memory than LTP and LTD. This work and its findings are presented in chapter 3 and constitute the second half of this thesis.

Chapter 1

Introduction and Background Theory

1.1 On Synaptic Plasticity, Learning and Memory

The function of the nervous system is to communicate: to process, store, and transmit information about an organism and its environment on levels ranging from the molecular to the societal. The ability of an organism to adapt its behavior in response to environmental stimuli is essential for survival. The acquisition of such an adaptation is termed learning, and the process through which this learned information is stored is termed memory. A basic tenet of modern neuroscience is that memory has a physical correlate, which is measured as a corresponding change in the strength of synaptic connections between neurons. Changes in synaptic strength occur in a use-dependent manner, altering various network properties of the nervous system and thereby producing behavioral adaptations. The process whereby such experience-dependent changes in synaptic strength are acquired is termed neuronal activity-dependent synaptic plasticity. Accordingly, activity-dependent synaptic plasticity is believed to be a process that underlies learning. Understanding the cellular and molecular basis of synaptic plasticity is thus of great practical and intellectual interest to the field of neuroscience.

Relatively simple model organisms, as exemplified by the sea slug *Aplysia californica*, are capable of robust and experimentally accessible learning paradigms such as habituation, behavioral sensitization, and associative learning. Indeed, this model system was used by Nobel Laureate Eric Kandel and colleagues in pioneering studies on the cellular basis of learning and memory. Associative learning is an intriguing and commonly studied form of learning particularly applicable to higher organisms, and can be described as the encoding of cause-and-effect relationships by neural tissue. A set of

theoretical concepts formulated by Donald O. Hebb (1949) are usually applied in describing the neural basis of associative learning, and can be conveniently summarized with the saying "neurons that fire together, wire together".

The model system of choice for the majority of studies of mammalian learning and memory is the hippocampus. The most famous demonstration of the essential role of the hippocampus in memory formation came through studies of a patient known as "H.M.". Following surgical, bilateral lesions of the hippocampal formation (given to treat his epilepsy), H.M. lost practically all ability to form new memories (lasting more than a few seconds) while preserving his pre-existing memories. Extensive behavioral studies in rodents and other mammals have further established the role of the hippocampus in learning and memory, as well as defined additional physiological functions for this brain structure in cognition. The hippocampus and its surrounding tissues are widely interconnected with a diverse array of brain regions and play a role in both multimodal sensory information processing and memory consolidation. The hippocampus mediates complex associations, as well as encoding features of space and time. Accordingly, the hippocampus can be thought of as a sensory information "hub" in the brain (**Fig. 1.1**).

Figure 1.1) Hippocampal Connectivity in the CNS

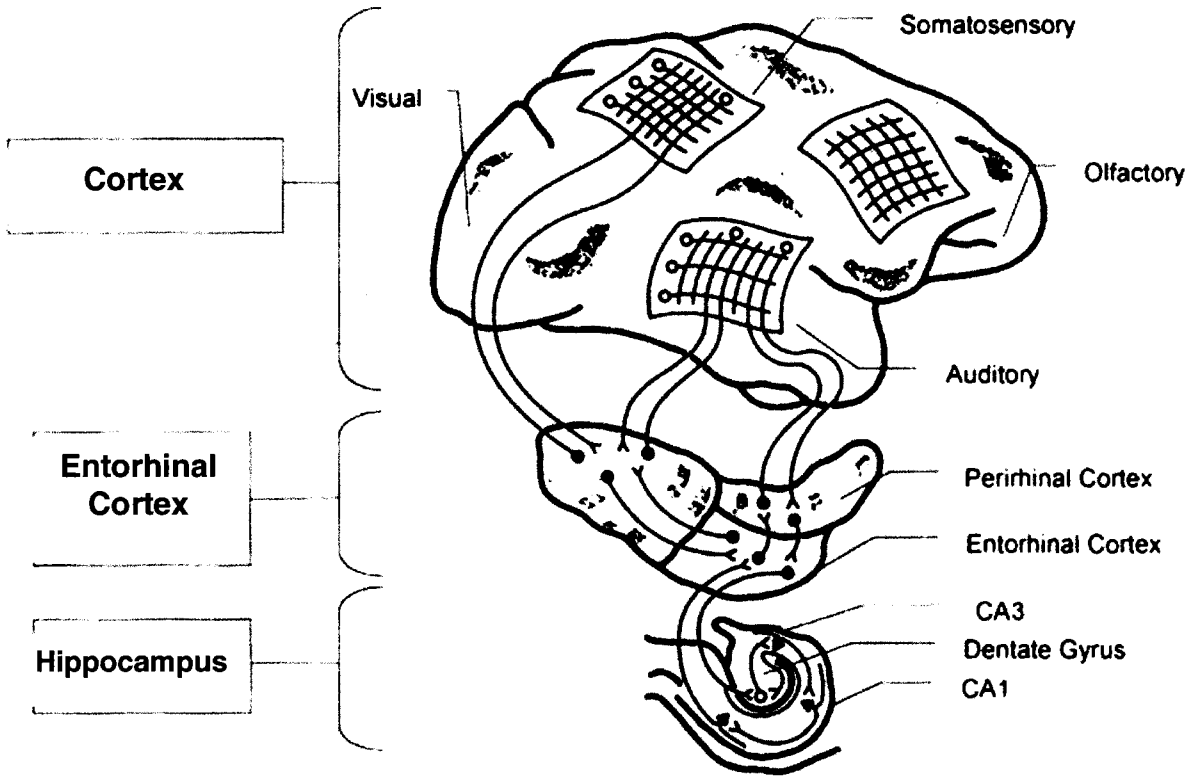


Fig. 1.1) Hippocampal connectivity in the CNS. Illustration of hippocampal connectivity with the primary sensory cortex *via* the perirhinal and entorhinal cortices. The hippocampus receives input from essentially all sensory cortices and the olfactory bulb. Outputs of the hippocampus in turn innervate the entorhinal cortex and sensory cortex, as well as other brain areas such as the lateral septum and amygdala. This extensive and diverse hippocampal interconnectivity with cortex is believed to subserve its role as a multimodal sensory information processor. Adapted from [1].

1.2 Hippocampal LTP and LTD

Memories can be very long lasting, and their effects can be measured minutes or decades after they have been acquired. Obviously, a comprehensive cellular model of learning and memory must satisfy this temporal durability, among other requirements. Timothy Bliss and Terje Lomo first discovered activity-dependent changes in synaptic strength that satisfied Hebb's postulate for associative learning, while artificially stimulating and recording responses within the hippocampal formation of rabbits [2]. In their discovery, they described for the first time a robust and long-lasting increase in synaptic strength, in response to patterned stimulation (a brief high-frequency tetanus) of the perforant pathway. They called this phenomenon Long-Term Potentiation (LTP). This seminal work formed the foundation for decades of work on the mechanisms and properties of LTP and its relationship to mammalian learning and memory. Several other labs have subsequently described a complementary property of excitatory synapses, which was termed Long-term depression (LTD): a long-lasting decrease in synaptic strength following low-frequency stimulation (1-3Hz). LTP and LTD together constitute what is now termed bidirectional synaptic plasticity, which are known to be associated with regulatory phosphorylation of the GluR1 subunit of the AMPA-type glutamate receptor (AMPAR) (**Fig. 1.2**). Indeed, the ability of synapses to change strength in both directions (bidirectional) is integral to modern theories of memory encoding [3-5]. Some of the most commonly used behavioral models (typically involving rodents) in studies of associative learning and memory are the Morris water and Barnes mazes, conditioned taste aversion, and cued and contextual fear conditioning. Generally, there is very good experimental agreement between the properties of learning and memory and those of

LTP/LTD in the brain regions that subserve these behavioral phenomena, with important exceptions, however. A more careful examination of one of these exceptions will be a topic of chapter 3, in which I propose the hypothesis that use-dependent *changes* in the *capacity* for bidirectional synaptic plasticity *ex vivo* may better reflect the capacity for learning and memory *in vivo* than simple measurement of LTP and LTD magnitudes (see Table 3.1).

Although various forms of synaptic plasticity occur in most brain structures, LTP was first described, and has been most extensively studied in the hippocampus. Beyond the obvious roles of the hippocampus in spatial and contextual learning, which makes it a good structure for examining the cellular basis of associative learning, the hippocampus is a highly ordered on the cellular level. This property facilitates electrophysiological studies. Furthermore, the hippocampus is also highly enriched with excitatory synapses, enabling the biochemistry underlying LTP and LTD to be more easily studied. Hippocampal LTP and LTD have emerged as the predominant cellular model of activity-dependent excitatory synaptic plasticity in the mammalian brain. With LTP and LTD, the postsynaptic response to a given presynaptic stimulus (usually an action potential) exhibits a stable variation in intensity following certain temporal presynaptic firing patterns. The post-synaptic response is typically measured as an excitatory post-synaptic potential (EPSP) or current (EPSC), using an electrode coupled to an amplifier. LTP and LTD have also been demonstrated following the correlation of presynaptic activity with post-synaptic depolarization, a phenomenon known as spike-timing dependent plasticity. The most extensively characterized forms of LTP and LTD (NMDAR-dependent,

associative) occur within the hippocampus at the Schaffer collateral-CA1 (CA3-CA1) pyramidal cell synapse, but essentially all excitatory pathways in the mammalian brain studied to date also express some form of activity-dependent synaptic plasticity akin to LTP or LTD. Moreover, these phenomena are not limited to excitatory synapses. Accordingly, many as yet undiscovered biochemical mechanisms of synaptic plasticity surely remain, and will undoubtedly be the subject of future investigations. The identification and characterization of two novel biochemical mechanisms of excitatory synaptic plasticity in hippocampal area CA1, and their relationship to learning and memory, will be the focus of this thesis (**Chapters 2 & 3**). These novel mechanisms pertain to the role of the Calcium Calmodulin-dependent Kinase Kinase/ Calcium Calmodulin-dependent Kinase I (CaMKK/CaMKI) cascade in LTP (**Chapter 2**), and the role of the cytoskeletal regulatory molecule WAVE-1 in LTP, LTD, and metaplasticity (**Chapter 3**).

Metaplasticity, or the plasticity of synaptic plasticity, is believed to be a necessary component of comprehensive cellular models of learning and memory [6-9], and may also serve as a homeostatic tuning mechanism during critical periods of developmental plasticity [10]. It is hypothesized to play a role in stabilizing networks, preventing their saturation over time, preventing seizures, and adjusting the activity thresholds for synaptic plasticity. One concept that laid the foundation for later models of metaplasticity was called the covariance rule. Simply stated, if the activities of the pre- and postsynaptic compartments of a synapse are positively correlated over time, synaptic strength increased, while negatively correlated activity leads to a decrease in synaptic strength. In

1982, Bienenstock, Cooper, and Munro further adapted this rule to render it more comprehensive [10]. The new rule they proposed models the relationship between correlation and synaptic strength non-linearly, introduces a threshold for synaptic depression in addition to synaptic strengthening, and most importantly, the threshold for LTP versus LTD induction varies as a function of the average activity of the postsynaptic neuron. This relationship is now called the B.C.M. or sliding modification rule (**Fig. 1.3**). Such flexibility or "plasticity" of the properties of synaptic plasticity itself is the hallmark of metaplasticity. While the sliding modification rule deals explicitly with the plasticity of the threshold for LTP induction, other forms of metaplasticity are possible, such as use-dependent changes in the magnitude of synaptic plasticity resulting from a given stimulus (see chapter3).

Figure 1.2) Biochemical Model of Bidirectional Synaptic Plasticity

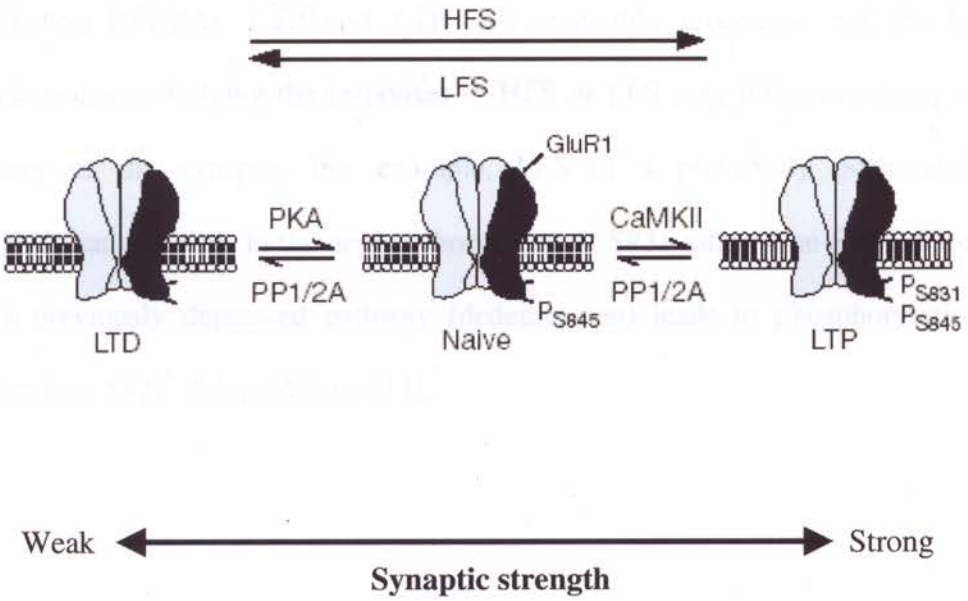


Fig. 1.2) Biochemical model of bidirectional synaptic plasticity. A graphical model depicting NMDAR-dependent bidirectional synaptic plasticity in hippocampal area CA1, and associated regulatory AMPA-receptor phosphorylations. High-frequency or *theta*-burst stimulation (HFS) of the Schaffer Collateral Pathway in hippocampal slices induces persistent synaptic strengthening (LTP) in area CA1. LTP in naïve slices is associated with phosphorylation of the GluR1 subunit of the AMPAR on S831 (CaMKII/PKC site). In contrast, low frequency stimulation (LFS) of the Schaffer Collateral Pathway induces persistent synaptic weakening (LTD). LTD of naïve slices is associated with dephosphorylation of S845 (PKA site), due to activation of protein phosphatases (including PP1/2A). LTP and LTD are reversible processes, but the biochemical mechanisms underlying the responses to HFS or LFS may differ according to the prior history of the synapse. For example, LFS of a previously potentiated pathway (depotentiation) leads to the dephosphorylation of S831 rather than S845. Similarly, HFS of a previously depressed pathway (dedepression) leads to phosphorylation of S845 rather than S831. Adapted from [11].

Figure 1.3) Metaplasticity and the Sliding Modification Rule

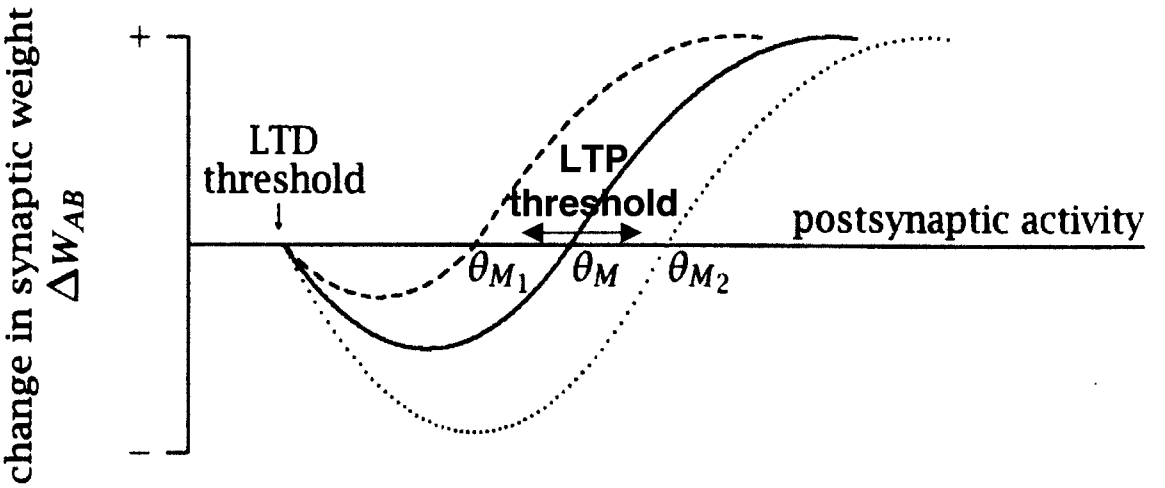


Fig. 1.3) Metaplasticity and the sliding modification rule. The sliding modification rule (also known as the B.C.M. rule) describes the relationship between the threshold for LTP/LTD induction and the average correlated network activity. The higher the average correlated activity in the postsynaptic neuron, the higher the amount of correlated activity that is required to induce LTP, and visa versa. (*i.e.* the threshold θ_M becomes larger and smaller). The relationship is described by the following equations:

$$\Delta W_{AB} = \phi(a_B, \bar{a}_B) s_B$$

and

$$\theta_M = \bar{a}_B^2,$$

where W_{AB} represents the synaptic strength or "weight" of a synapse between neurons A and B, ϕ is a non-linear function relating changes in synaptic weight to the correlated activity (a_B) of neurons A and B and the average correlated activity ($\bar{a}_B$); s_B is a local variable for the synapse AB; and θ_M is the modification threshold for LTP versus LTD. Adapted from [12].

1.3 Physiological Mechanisms of Synaptic Plasticity

Synaptic plasticity can be characterized by it various properties, such as duration (*e.g.* short or long forms), location (brain region, pre- or postsynaptic expression), and means of induction (type of activity). Here I provide a brief overview of some of these mechanisms. It is worth noting that there is considerable overlap between many of the mechanisms thus far described for synaptic plasticity. For example, there is generally a lack of distinct limits pertaining to the time durations that describe various forms of synaptic potentiation and depression. Furthermore, while much is known regarding the locus of expression (*i.e.* pre- *vs.* post-synaptic) for many of these forms, for some there is not a good scientific consensus, especially pertaining to the locus of induction. In my view, this lack of consensus may be confounded by an under-appreciated degree of communication and cooperation between the presynapse and postsynapse, which has been demonstrated to occur through the production and release of retrograde messengers [13], integrin-mediated signaling [14, 15], and enzymatic reactions in the extracellular matrix [16, 17].

Presynaptic plasticity is characterized by an increase or decrease in presynaptic efficacy, and occurs at many different types of synapses. Factors that influence presynaptic efficacy include calcium homeostasis and the actions of neurotransmitters and hormones on presynaptic receptors. The changes in synaptic efficacy occur mechanistically through the modulation of factors which influence the concentration and availability of neurotransmitter in the synaptic cleft, such as the concentration and compliment of neurotransmitters loaded into vesicles, the availability of readily releasable synaptic

vesicles and their release probabilities, and synaptic cleft geometry (*e.g.* synaptic cleft volume –while geometry is technically a synaptic property, the effects on postsynaptic physiology can resemble presynaptic changes). Furthermore, the availability (potentially sensitive to pH or enzymatic reactions), rates of diffusion, and uptake or release of neurotransmitter by glia also may affect activation of postsynaptic neurotransmitter receptors [18, 19]. While presynaptic plasticity mechanisms appear to contribute substantially to the modulation of excitatory synaptic efficacy on short time scales (milliseconds to minutes), a strong consensus exists favoring the post synapse as the primary locus of LTP and LTD in hippocampal area CA1.

At the Schaffer collateral-CA1 synapse, the primary final effector of LTP and LTD is the postsynaptic AMPAR, whose conductance upon activation by glutamate contributes the majority of the postsynaptic response. This occurs through two major mechanisms, changes in the number of AMPA receptors at active sites (**Fig. 1.4**), or changes in single-channel efficacy upon glutamate binding. Changes in AMPAR number occur through trafficking, which entails the endocytosis or exocytosis, lateral plasma membrane diffusion, and clustering of AMPARs at sites opposing the presynaptic release of glutamate [20-23]. Changes in single channel efficacy occur through rapid post-translational modification of the receptor by phosphorylation [11, 24], or by subunit recomposition [25], among other mechanisms. Regulatory phosphorlyation of the GluR1 subunit of the AMPAR has been well characterized at two sites: S831 and S845. At least in AMPARs composed of homomeric GluR1, phosphorylation of S831 results in an increase in single channel conductance [26], a property of AMPARs that has been

observed in dendrites following the induction of LTP [27, 28]. Phosphorylation of S845 results rather in an increase of open channel probability [29]. Current evidence supports a role of S845 dephosphorylation in LTD, and a role of S845 phosphorylation in regulating the levels of extrasynaptic surface AMPARs [30]. All of these changes to AMPARs may affect whole-cell or synaptic currents similarly, but often can be differentiated through the use of appropriate techniques.

Other potential means of regulating synaptic efficacy during synaptic plasticity exist. For example, as mentioned above, AMPAR subunit recomposition has recently been shown to occur in an activity-dependent manner at some synapses. This has generally been assumed to occur through trafficking mechanisms, although this assumption has not been tested. AMPAR kinetics can also be modulated (*e.g.* by ampakines), and the efficacy of the charge itself in contributing to the generation of an action potential can be modulated postsynaptically through regulation of dendritic cable properties (*e.g.* leak currents, the conductance of the spine neck, etc) and voltage gated ion channels. Good evidence exists that this latter mechanism can alter the threshold for action potential generation, as occurs with "pop-spike" or E-S potentiation [31]. Finally, potential alterations to AMPAR cooperativity/stochastics, and glutamate affinity are additional hypothetical mechanisms for regulating postsynaptic strength.

The temporal stages of synaptic plasticity in CA1 differ by mechanism and are generally divided into four stages. Facilitation occurs when two or more presynaptic action potentials are triggered within 10's or 100's of milliseconds of each other, and is believed

to result from an increase in presynaptic vesicle release probability due to a residual elevation of intracellular calcium levels from the first pulse at the time of the second pulse (the residual calcium hypothesis). Post-tetanic potentiation (PTP) occurs following high-frequency stimulation (such as a 100 Hz tetanus or theta-bursts) and generally lasts less than a minute. Short-term potentiation (STP) lasts roughly 10 minutes or so. The mechanism and locus of STP remains a topic of some debate, and probably results from a combination presynaptic and postsynaptic mechanisms. Long-term potentiation (and long-term depression) can be measured for up to as long as the recording preparation lasts, around 12 hours for acute brain slices or weeks *in vivo* (the limit of the durability of LTP seems, so far, to be a function of the stability of the recording preparation). LTP and LTD themselves are dived into two phases, early (E-LTP/D, lasting out to 1-2 hours) and late (L-LTP/D, lasting longer than 2 hours). Despite a differential dependence of the early and late forms of LTP/D on sensitivity to transcriptional inhibition by pharmacological or genetic techniques, the transition between them is smooth and indistinct (**Fig. 1.5**). Indeed, a failure of L-LTP/D results gradually from stimuli that are too weak or brief to induce sustained transcriptional activation, and mouse models which harbor deficits in the function of the transcription factor CREB, or its activation, have been shown to have selective deficits in L-LTP [32, 33]. Deficits in L-LTP also result from an inhibition of protein translation, but importantly, this latter manipulation can also produce significant impairment of the early phase of LTP [34-38]. This effect of translational inhibition on E-LTP differs among labs and experimental preparations, however.

Figure 1.4) AMPAR Trafficking Model of Bidirectional Synaptic Plasticity

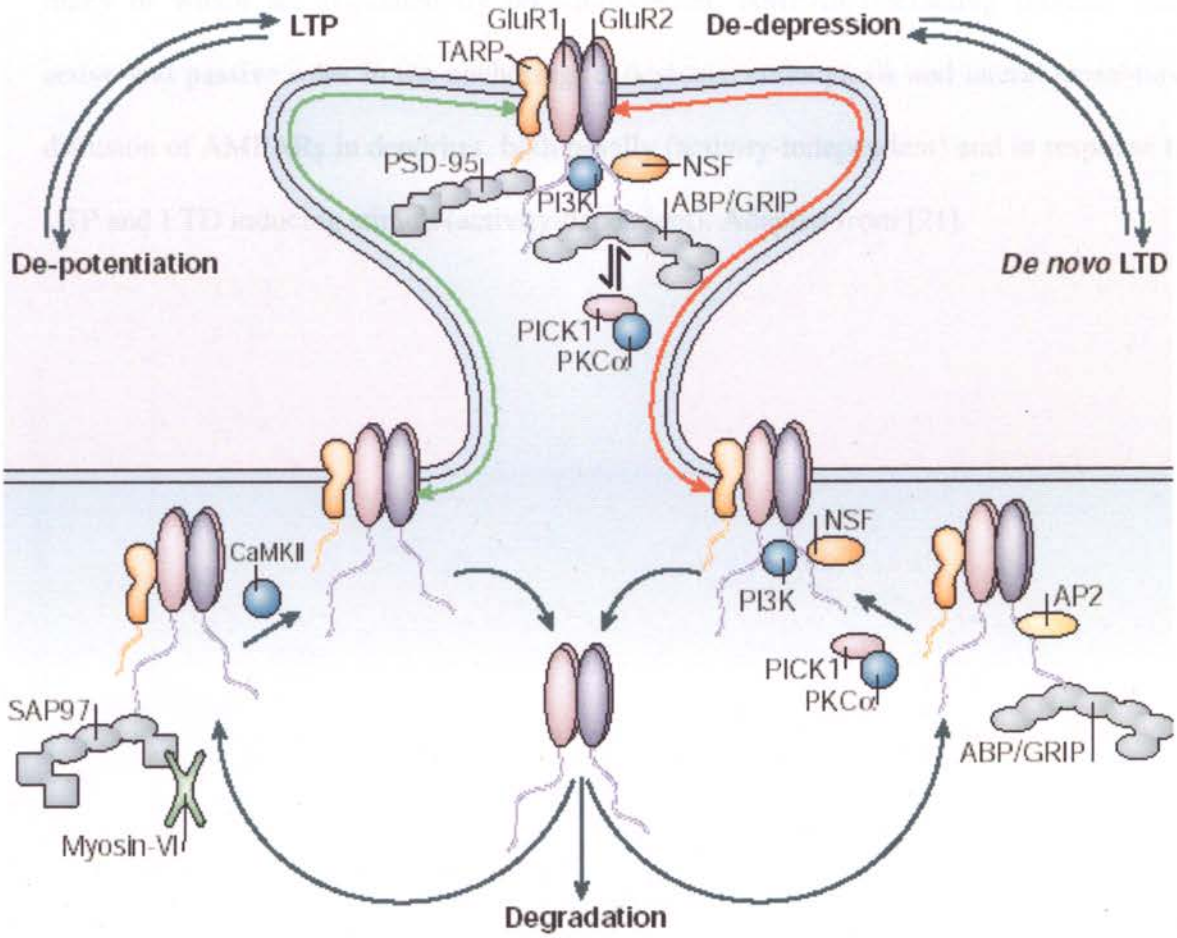
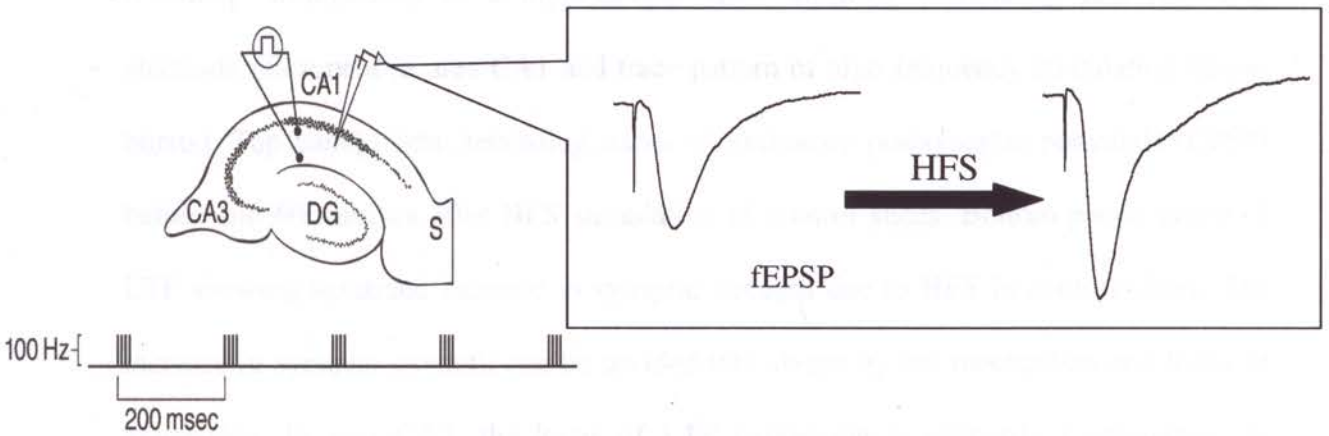


Fig. 1.4) AMPAR trafficking model of bidirectional synaptic plasticity. Depiction of AMPARs and their movements in spines during activity-dependent bidirectional synaptic plasticity. LTP and dedepression are believed to be associated with increases in the number of synaptically localized AMPARs, while LTD and depotentiation are believed to be associated with corresponding decreases. The discovery of proteins that directly bind to AMPAR subunits has facilitated the understanding of the molecular mechanisms of rapid AMPAR trafficking at synapses. Shown are a variety of these protein interactions, many of which are regulated by phosphorylation. AMPAR-interacting proteins play active and passive roles in the anchoring, exocytosis, endocytosis and lateral membrane diffusion of AMPARs in dendrites, both basally (activity-independent) and in response to LTP and LTD inducing stimuli (activity-dependent). Adapted from [21].

Figure 1.5) Temporal Stages of LTP



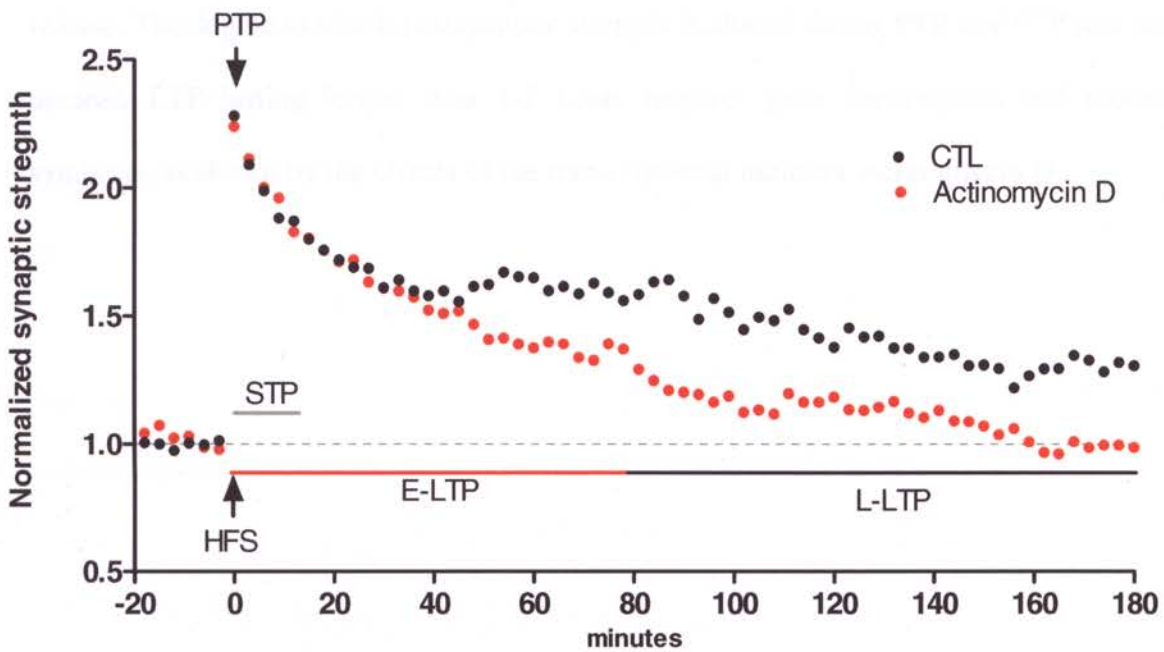


Fig. 1.5) Temporal stages of LTP. Following high-frequency stimulation (HFS) of the Schaffer collateral pathway in area CA1 with 100 Hz tetani or a trains of *theta*-bursts, a sustained increase in synaptic strength (LTP) results. Top panel, left: diagram of recording arrangement in a hippocampal slice, showing stimulating and recording electrode placement in area CA1 and trace pattern of high frequency stimulation (theta-bursts). Top panel, right: recording traces of excitatory postsynaptic potentials (EPSP) before and 60 minutes after HFS stimulation of control slices. Bottom panel: graph of LTP showing sustained increase in synaptic strength due to HFS in control slices. The increase in synaptic strength can be divided into stages by the mechanism and locus of expression. In area CA1, the locus of LTP expression is primarily postsynaptic. In contrast, PTP and STP are associated with an increase in presynaptic neurotransmitter release. The degree to which postsynaptic strength is altered during PTP and STP remains unclear. LTP lasting longer than 1-2 hours requires gene transcription and protein synthesis, as shown by the effects of the transcriptional inhibitor Actinomycin D.

1.4 The Essential Role of Calcium and the NMDA Receptor in LTP and LTD

At the Schaffer Collateral-CA1 synapse, LTP and LTD induction occurs primarily through the activation of NMDA-type glutamate receptors (NMDARs), voltage-gated calcium channels (VGCCs), and metabotropic glutamate receptors (mGluRs). All three of these membrane proteins mediate transient increases in intracellular calcium levels under physiological conditions. Calcium entry *via* postsynaptic NMDARs is critically important for associative LTP and LTD in area CA1, where expression lies primarily in the dendrites of CA1 pyramidal cells [39, 40]. It has been known for some time that calcium is the central regulatory master molecule of synaptic plasticity, and CaMKII has received much attention as a conveyor of calcium signaling in LTP [41, 42]. Additionally, calcium regulated protein phosphatases are important mediators of LTD in area CA1 [43, 44]. Thus, intracellular calcium elevations are required for both LTP and LTD, and the determinant of whether LTP or LTD results from a given stimulus appears to be a function of both the magnitude and the spatial and temporal patterns of those intracellular calcium increases.

A comprehensive cellular and molecular model of learning and memory must satisfy certain requirements. This thesis will focus on the most extensively studied form of activity-dependent synaptic plasticity, NMDA receptor-dependent LTP and LTD. The NMDAR in particular has received attention for its ability to function as a molecular coincidence detector, a property that is implicit in associative memory formation. The NMDA receptor is able to accomplish this through a voltage-dependent block of calcium conductance by magnesium at resting membrane potentials [45]. Thus, full conductance

of calcium by the NMDA receptor requires simultaneous binding of its ligand glutamate, and post-synaptic depolarization to relieve magnesium binding from the channel. Such a situation occurs at a synapse when its activity is correlated with several other synapses on the same neuron over a short time duration (milliseconds). Following is a brief list of requirements satisfied by NMDAR-dependent LTP, which displays properties that are highly suggestive of an information storage device:

•**Coincidence detection:** simultaneous strong postsynaptic depolarization and the presence of glutamate in the synaptic cleft

•**Input specificity:** the synapses used to generate LTP are the same synapses that respond with an increase in synaptic strength

•**Persistent over time:** the limits of LTP duration, so far, appear to be a function of the stability of the recording preparation – the current record is more than a month *in vivo*

•**Naturalistic patterns of activity can induce LTP:** *e.g. theta*-bursts, which mimic the endogenous rhythmic bursting pattern within the hippocampus during exploratory behavior

•**Anterograde alteration:** interventions that block LTP inhibit memory formation (*e.g.* both require CaM Kinases, and both are blocked by NMDAR antagonists)

1.5 The Roles of CaM Kinases in LTP

As mentioned above, CaMKII is activated by NMDAR-mediated calcium influx during LTP. However, the CaMK family contains other members that are generally more sensitive to activation by calcium/calmodulin than CaMKII. The CaM Kinase family consists of 4 principal family members, CaMKK, CaMKI, CaMKII, and CaMKIV (**Fig. 1.6**), with each family member generally displaying activity towards a distinct set of cellular substrates despite having similar substrate recognition motifs as defined using synthetic peptides. The CaM Kinases share the trait of binding to calcium-bound calmodulin, which then relieves a tonic inhibition of kinase activity due to N-terminal autoinhibitory domains. CaMKK has 3 known cellular substrates, for each of which it functions as an upstream activator: CaMKI, CaMKIV, and in some cell types Akt (PKB). CaMKK and its substrates are collectively referred to as the CaMKK cascade. CaMKII on the other hand, is not activated by CaMKK, but rather functions as it's own "upstream" activator by undergoing calcium/calmodulin-dependent intersubunit autophosphorylation at T286 of its autoinhibitory domain. This event results in sustained calcium/calmodulin-independent activity. This property of CaMKII has drawn extensive attention for its ability to function as a molecular memory switch [42].

A critical role for the CaM Kinases in CA1 LTP is well established. CaMKII is by far the most extensively studied CaMK in NMDAR-dependent LTP, with a large body of evidence supporting its role. CaMKII appears to regulate several neuronal functions underlying LTP, including regulatory phosphorylation of the AMPA receptor subunit GluR1 at S831 [44, 46]. CaMKII has also been reported to mediate rapid activity-

dependent trafficking of AMPARs to synaptic sites in spines, which is believed to occur following LTP induction [47-50]. Much of the work implicating CaMKII in LTP has relied on use of the general CaMK inhibitors KN-62 and KN-93, which completely block LTP induction in slices from mice older than about 3 weeks of age [51, 52]. However, the lack of selectivity of these "KN" compounds, which function as calmodulin antagonists, among the CaM Kinase family members is often overlooked. Indeed the mechanisms whereby these KN compounds inhibit LTP induction probably include actions on all of the CaM Kinases.

Other work implicating CaMKII in LTP relies on αCaMKII knockout mice and an αCaMKII T286A transgenic mouse. Expression of αCaMKII is developmentally regulated, appearing around 1 week postnatally and reaching a maximum in adult brain. However, with the possible exception of fully matured mice (3 months or older), the loss of LTP in αCaMKII-deficient mice is incomplete in both hippocampus and visual cortex, with ~50% of wild-type LTP typically remaining [53, 54]. It has been suggested that βCaMKII may mediate the residual LTP in these α-CaMKII mutant mice, but it is equally possible that the CaMKK cascade performs this function. Furthermore, paired-pulse facilitation in the α-CaMKII knockout mice is deficient [55] and the synaptic input-output curve is less steep. These effects may result in weaker synaptic activation and thus produce in an indirect impairment of postsynaptic calcium-dependent processes during LTP induction in mutant mice, compared to their wild-type counterparts. Other studies address the role of CaMKII in LTP by overexpressing recombinant genetic constructs (which may have non-specific effects due to alteration of substrate specificity,

overexpression, and subcellular mislocalization), or by intracellular perfusion of inhibitor peptides such as AIP, which are not entirely specific for CaMKII, esp. at higher concentrations [56, 57], or by overexpression or intracellular perfusion of CaMKIIN/CaMKIINtide [51]. This last study used 5-10 μM CaMKIINtide and is perhaps the strongest evidence for CaMKII mediating the majority of CA1 HFS LTP, at least in animals or cultures 3-4 weeks of age. However, because the cellular function of CaMKIIN is not known, and the high concentration used, it might have non-specific effects on the CaMKK cascade. Indeed, it is also possible that the CaMKK cascade may not be completely independent of CaMKII activity in a cellular context. For example, CaMKII interacts with and phosphorylates the NMDAR [58, 59], and CaMKIINtide might therefore reduce calcium influx during LTP induction, which could impair activation of CaMKK and CaMKI. Thus, it remains to be determined whether the CaMKK cascade mediates a significant component of CaM Kinase-sensitive E-LTP. These common oversights may be one reason why a role for CaMKK and CaMKI in E-LTP has not been carefully considered. With the recent development and characterization of STO-609 [60], a selective, membrane-permeable inhibitor of CaMKK, we now have a convenient way of specifically testing the function of the CaMKK cascade in both the early and late phases of LTP in brain slices, while leaving CaMKII activity intact.

CaMKIV also plays a role in LTP, but its role appears to be limited to the late phase of expression [61, 62]. CaMKIV is primarily, but not exclusively, restricted to the nucleus [63-65], where it stimulates gene transcription required for maintenance of late-phase LTP through phosphorylation of the transcription factors CREB (cAMP response

element-binding protein) and CBP (CREB binding protein) [66, 67]. As CaMKK activity is required to fully activate CaMKIV, its role in L-LTP can be inferred from the studies that have implicated CaMKIV, and this was recently confirmed in a mouse lacking the βCaMKK isoform [68]. A role for the α-CaMKK isoform in LTP has not been assessed.

Figure 1.6) The CaM Kinase Cascade in LTP

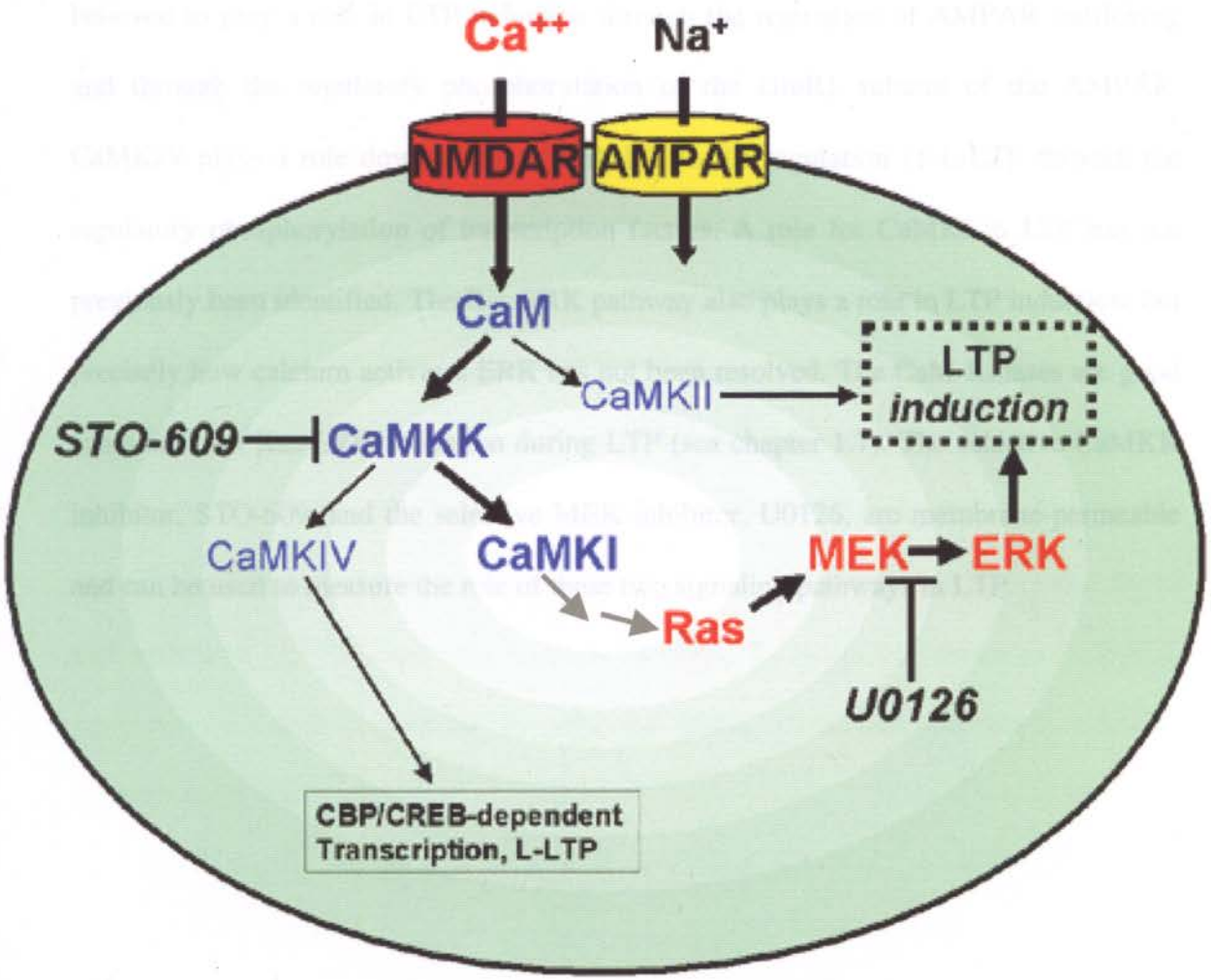


Fig. 1.6) The CaM Kinase Cascade in LTP. The role of CaM Kinases in LTP. Calcium entry *via* the NMDAR, during LTP induction, binds to calmodulin. Calcium-bound calmodulin then activates all four CaM Kinases. CaMKI and CaMKIV are substrates of CaMKK, whose phosphorylation further increases their activities. CaMKII activity is believed to play a role in LTP induction through the regulation of AMPAR trafficking and through the regulatory phosphorylation of the GluR1 subunit of the AMPAR. CaMKIV plays a role downstream of CaMKK in the regulation of L-LTP through the regulatory phosphorylation of transcription factors. A role for CaMKI in LTP has not previously been identified. The Ras-ERK pathway also plays a role in LTP induction, but precisely how calcium activates ERK has not been resolved. The CaM Kinases are good candidates for Ras-ERK activation during LTP (see chapter 1.7). The selective CaMKK inhibitor, STO-609, and the selective MEK inhibitor, U0126, are membrane-permeable and can be used to measure the role of these two signaling pathways in LTP.

1.6 Cellular Functions of CaMKI

A role for cytosolic CaMKI in either early- or late-phase LTP has not previously been addressed, but CaMKI has been shown to modulate other processes relevant to neuronal plasticity such as cytoskeletal organization [69], cell morphology [70], and axonal growth cone motility [71]. Furthermore, CaMKI was recently shown to mediate extracellular signal-regulated kinase (ERK) activation in response to membrane depolarization in NG-108 cells [72]. Despite the identification of CaMKI contemporaneously with CaMKII, only a few cellular substrates have thus far been identified, including synapsin, myosin II regulatory light chain (MRLC) [69], Numb [73], and the translation factor eIF4GII [74]. Indeed, the regulatory phosphorylations of MRLC and Numb further suggest a role for CaMKI in the reorganization of actin cytoskeleton. As opposed to CaMKII and CaMKIV, activation of CaMKI does not result in calcium/calmodulin-independent activity, but phosphorylation of CaMKI at T177 in its activation loop by CaMKK is required for its full activation [75, 76], and may regulate its ability to recognize substrates [73]. Four isoforms of CaMKI, encoded by separate genes, have thus far been identified (α,β,γ, and δ).

1.7 MAPK-Dependent LTP

In hippocampal area CA1, patterned elevations of postsynaptic intracellular calcium *via* NMDAR activation results in protein phosphorylation cascades that are critical for both the induction and expression of LTP. In addition to the CaMK cascade, the ERK cascade also plays an important role in LTP. Both signal transduction cascades are required for full expression of LTP and for proper hippocampal-dependent learning and memory [77]. However, one elusive bit of knowledge has been: how is the MAPK pathway is activated by calcium during NMDAR-dependent LTP? The ERK family of MAP (mitogen-activated protein) kinases are activated by growth factors, neurotransmitters, and hormones, as well as by the NMDA receptor, voltage-gated calcium channels, mGluRs and AMPARs in neurons [78-81]. Activation of ERK during neuronal depolarization or NMDA receptor stimulation requires elevation of intracellular calcium, and it appears to be mediated through the classical Ras/Raf/MEK/ERK cascade [82, 83]. Molecules that may couple NMDAR-mediated calcium influx to ERK activation in neurons include SynGAP, CalDAG-GEF's, and RasGRF1, among others (**Fig. 1.7**). Recent studies highlight a role for Ras-GRF1 (Ras-guanyl-nucleotide releasing factor) in coupling the NMDA receptor to Ras and ERK activation in neurons [84, 85]. Phosphorylation of S916 of Ras-GRF appears to play a role in its physiological functions and serves as a readout for activation [86-88]. However, a calcium sensitive molecule, likely acting *via* RasGRF upstream of Ras-dependent ERK activation during LTP, has not yet been conclusively identified.

Induction of LTP in area CA1 activates ERK, and inhibitors of the ERK pathway (*e.g.*, PD098059, U0126) suppress E-LTP on the order of 50%, an effect that appears rapidly (often within seconds, depending on the stimulus) following LTP induction [31, 78, 89] (**Fig. 1.8**). CaM kinases were first proposed to mediate calcium-dependent ERK activation in some cell types nearly a decade ago. This work implicated CaMKIV, which is homologous to CaMKI, upstream of depolarization-induced ERK activation in PC12 cells [66]. However, this study used cotransfection of constitutively active forms (ca) of CaMKK and CaMKIV, and it has since been discovered that overexpression of caCaMKIV results in the mislocalization this normally nuclear protein to the cytoplasm, where it may function similarly to cytoplasmic, activated CaMKI. Furthermore, subsequent studies using a CaMKIV knockout mouse [61] and a dominant-negative CaMKIV (CaMKIVdn) mouse [62] found that activity-dependent activation of ERK was normal in hippocampal neurons. Other studies have implicated, retracted, and refuted roles for CaMKII in calcium-dependent Ras activation *via* SynGAP [90-92], a protein that negatively regulates Ras activity by stimulating hydrolysis of bound GTP. Current evidence supports a model where CaMKII phosphorylation negatively impacts Ras activation *via* SynGAP *in vitro*; however it remains unclear whether regulation of SynGAP by CaMKII translates into significant attenuation of ERK activation in the cellular context of neurons [91-94]. Consequently, since neither CaMKII nor CaMKIV appear to mediate CaMK-dependent (*i.e.* KN-sensitive) ERK activation, CaMKI is a likely candidate for regulation of ERK activation during LTP. This hypothesis was recently made quite compelling by the discovery that CaMKK and CaMKI function upstream of depolarization-induced ERK activation in NG-108 cells, a pathway that was

found to regulate neurite outgrowth [72]. Because of the key role of ERKs in both CA1 LTP and hippocampal-dependent learning and memory, it will be important to determine whether this recently identified crosstalk between CaMKK/CaMKI and ERK in NG-108 cells also occurs in hippocampal neurons, and if so whether it plays a role in LTP. Yet another possible mediator of CaMK-dependent ERK activation in neurons is Akt, but this seems less likely to play a role in E-LTP since activation of Akt, but not induction of LTP, was blocked by inhibitors of PI3K [95], and since the activation of Akt by NMDAR activation requires 30-60 minutes or more [96].

Other signaling molecules have been proposed to mediate activation of ERK during LTP, in particular PKA and PKC. PKA is capable of mediating ERK activation in hippocampal slices *via* Rap1 [97]. Developmentally however, a disconnection is seen between the effects of inhibition of PKA with the inhibitory peptide PKI and inhibition of MEK activity with PD098059 in the generation of LTP in area CA1: PKI alone was effective at suppressing LTP in slices obtained from one week old rats whereas PD098059 and KN-93, but not PKI, were effective in slices from rats 4 weeks or older [51](supplemental data). This finding raises the likelihood that the role of PKA in E-LTP lies outside the ERK-MAPK pathway, and that the cellular pool of ERK regulated by PKA may not participate in the generation of postsynaptic E-LTP. PKC also has been suggested to be an upstream activator of ERK in LTP [98], but a role for PKC in LTP induction appears to be upstream of NMDAR function [99]. Further complicating these biochemical studies of ERK's role in LTP, the signaling cascades that participate in LTP may differ not only with age, but also with the induction stimulus, the stimulus intensity, recording

conditions, and electrode placements. This was apparent in a recent study, performed in mice, of ERK's role in LTP: the sensitivity of CA1 LTP to inhibitors of MEK, the upstream activator of ERK, was lost during high-intensity tetanus-induced LTP (100Hz), but not high-intensity *theta*-bursts LTP [31]. However, given that the CaMK inhibitors KN-62 and KN-93 completely block LTP in response either HFS or *theta*-burst (in mature slices) and potently attenuate ERK activation, the role of CaM Kinases in ERK-dependant LTP is explicit.

Despite the well-characterized role of the ERK pathway in LTP, precisely how ERK modulates hippocampal physiology during LTP is less clear. However, there are a few known examples: the potassium channel Kv4.2 is inhibited when phosphorylated by ERK2, decreasing outward potassium conductance. In this model, PKA and Rap1-mediated ERK activation results in increased dendritic excitability and enhanced action potential backpropagation during some forms of LTP [31, 97]. Other known targets of ERK are factors that regulate protein synthesis: RSKs, MSKs, CREB, eIF4E, and 4EBP1. The cAMP-specific phosphodiesterase PDE4 has also been identified as a substrate and possible mediator of ERK's effects in learning and memory [100]. Perhaps most relevant to this thesis, however, is the finding that activity in the Ras/ERK pathway can potentiate synaptic AMPAR responses [89, 101], and this effect is mutually occlusive with CaMK's effects on AMPAR function [101]. These effects include CaMK- and Ras-mediated changes in synaptic rectification in neurons overexpressing GluR1, and thus are consistent with a role for the ERK pathway in the regulation of AMPAR trafficking during activity-dependant plasticity.

Given the considerations raised in sections 1.5 and 1.6, a direct examination of the role of CaMKK and CaMKI in MAPK-dependent E-LTP was warranted, and will be the subject of chapter 2 of this thesis.

Figure 1.7) Potential Biochemical Routes for ERK Activation by Calcium During LTP

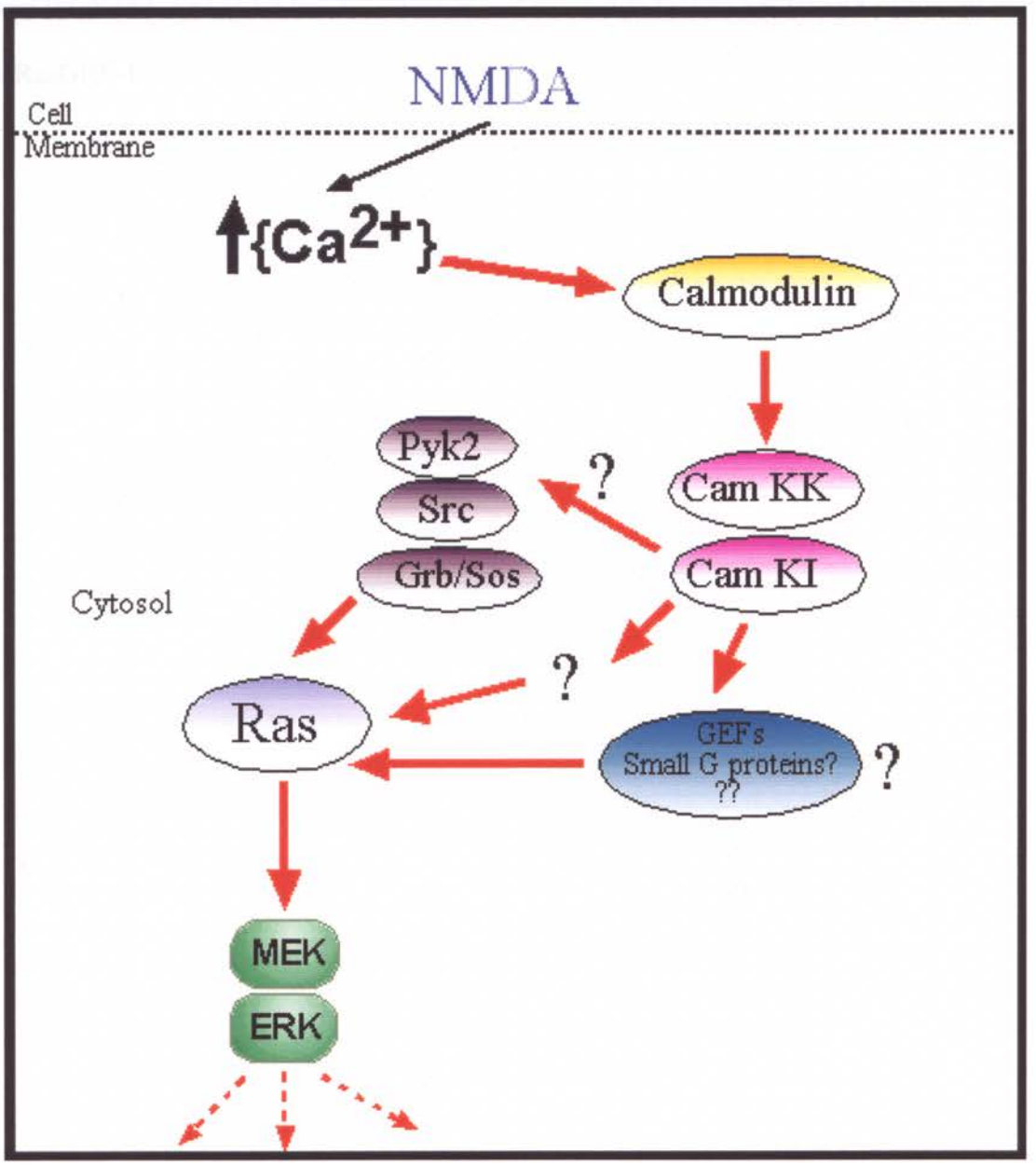


Fig. 1.7) Potential biochemical routes for ERK activation by calcium during LTP. Schematic of potential biochemical routes for cytoplasmic ERK activation, downstream of Ras, during NMDAR-dependent LTP. NMDAR activation results in significant elevations of intracellular calcium. Elevated levels of intracellular calcium activate the CaMKK cascade. A number of pathways linking calcium to the Ras-ERK pathway have been proposed, including the Src cascade and exchange factors for small G-proteins such as RasGRF-1.

Figure 1.8) ERK Activation is Required for Full LTP Induction

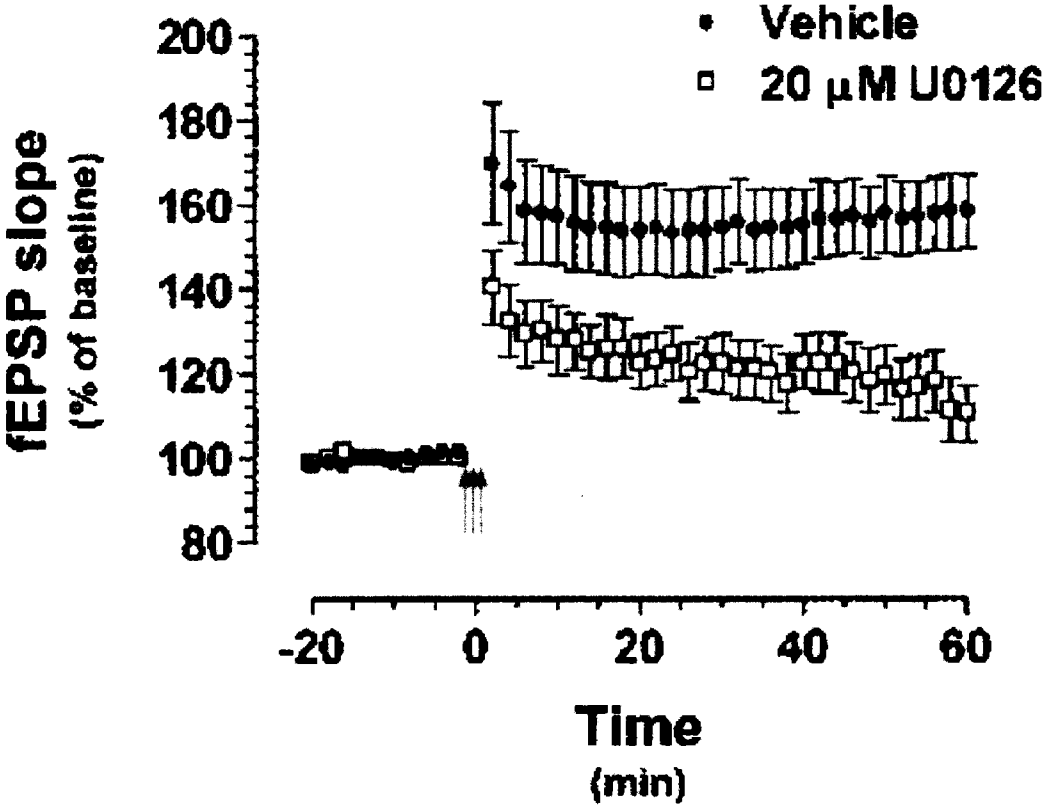


Fig. 1.8) ERK Activation is required for full LTP induction. Hippocampal field recordings from area CA1 of acute mouse hippocampal slices, demonstrating the requirement for the ERK pathway in LTP. Preincubation of hippocampal slices with the MEK inhibitor U0126 (open squares), results in a partial suppression of LTP in response to *theta*-bursts (arrows), compared to vehicle (closed circles, DMSO). For further details see [47].

1.8 Role of the Actin Cytoskeleton in Neuronal Plasticity and Learning & Memory

Actin filaments are highly dynamic in dendritic spines with an average turnover rate of approximately 44 seconds [102]. Actin polymerization, depolymerization, and branching are regulated by small G-proteins that serve as molecular switches for controlling and coordinating a variety of cellular processes in response to extracellular stimuli. Small G-proteins such as Rac, Rho, and Ras, among others, have been implicated in the cytoskeletal dynamics that accompany developmental, morphological, and physiological plasticity of excitatory synapses [101, 103]. Historically, the morphological and developmental plasticity of spines and dendrites have received attention as a component of learning and memory, as cellular alterations to the structure of excitatory circuits correlate with diseases that affect these cognitive functions [104]. However, the role of actin filaments is not limited to morphology: they also serve as anchors for cytoplasmic and membrane proteins, coordinate and maintain dynamic cellular compartmentalization, and mediate vesicle trafficking. It is thus not surprising that recent work has implicated small G-proteins in LTP and LTD, although the mechanistic details of these roles are still forthcoming. Progress has been hindered, in part, by the considerable crosstalk that occurs among these signal transduction pathways (*e.g.* Ras can activate Rac), as well as the realization of the diversity of their effects on cellular processes (which often differs according to cell-type). However, research over the past decade, which began in fibroblasts, has highlighted the role of small G-proteins as principal regulators of cytoskeletal organization in all eukaryotic cells, and uncovered their relationship with adhesion molecules such as integrins and cadherins [105], which have been shown to

play a role in both synaptic plasticity and the regulation of MAP Kinase signaling [14, 106].

Genetic mutations or deletions of signaling proteins that regulate dynamic aspects of the actin cytoskeleton have been associated with impairments to learning and memory, abnormal dendritic spine structure, and abnormal LTP and LTD. Interestingly, the brains of patients with human forms of mental retardation commonly show abnormal filamentous actin structures and abnormal spine morphology, such as decreases in the number of "mushroom" shaped spines [104]. In recent years, attention has been turning towards the link between morphological and electrophysiological plasticity, and its apparent relationship with learning and memory. In particular, two proteins that participate in the regulation of actin dynamics upstream of the actin depolymerization factor ADF/Cofilin have recently been highlighted: PAK [107-109] and LIMK [110-112]. LIMK has been implicated in a form of human mental retardation known as Williams Syndrome, which is associated with its deletion, as well as other genes, on chromosome 7 [113, 114]. LIMK is a target of the Rac, Rho, and PKC cascades, which regulate depolymerization of filamentous actin upstream of ADF/Cofilin. PAK, which functions downstream of Rac and Cdc42, has also been shown to regulate synaptic architecture upstream of ADF/Cofilin.

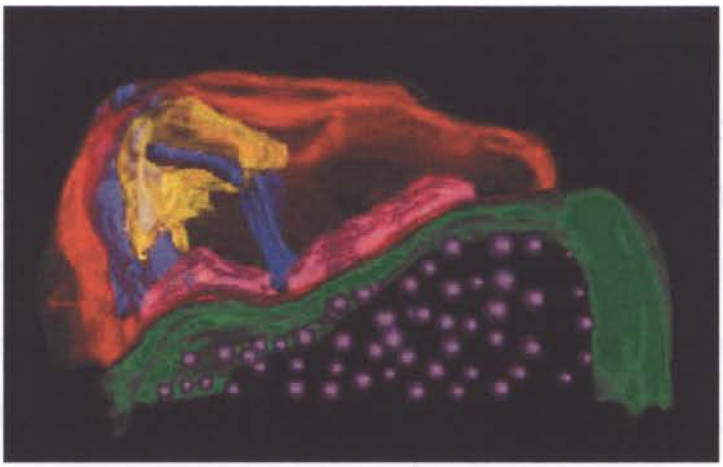
As expected, LIMK homozygous knockout mice, as well as mice deficient in PAK signaling, have alterations to actin structure due to derangements in actin turnover that produce abnormal dendritic spine morphology [111]. Interestingly, LTP magnitude is

increased in both the LIMK knockout and dominant-negative (dn) PAK mice, and LTD magnitude is also reduced in the dnPAK mice. Finally, LIMK knockout and dnPAK mice show decreased learning and memory. In particular the LIMK knockout mice exhibit a decreased capacity for extinction (a type of "unlearning") in the Morris water maze (a hippocampal-dependent learning task), and the dnPAK mice exhibit defects in the consolidation of hippocampal-dependant learning. These and other findings add to the observations that dynamic regulation of the cytoskeleton is critical for proper cognitive function, as well as normal bidirectional synaptic plasticity. Also notable is the observation that larger LTP does not always equate to enhanced learning and memory, which highlights the importance of bidirectional synaptic plasticity in learning and memory (**Chapter 3, Table 3.1**). Thus, it is becoming clear that proteins which regulate the actin cytoskeleton, and hence spine morphology, are potent regulators of synaptic plasticity and learning and memory, but exactly how changes in bidirectional plasticity produce deficits to learning and memory is less clear.

As mentioned above, dendritic spines are rich in filamentous actin, which serves a role in structural dynamics. Additionally, actin interacts with protein complexes such as the PSD and anchors cytoplasmic vesicles and signaling complexes (**Fig. 1.9**). Importantly, actin interacts directly or indirectly with postsynaptic proteins implicated in the trafficking of AMPARs, including GRIP/ABP, protein 4.1N, SAP-97, PSD-95, and others [115]. For this reason, it is reasonable to suspect that dynamic actin plays a role in neurotransmitter receptor trafficking. Indeed, good evidence exists to support this hypothesis [116-119]. Additionally, electrical and chemical stimuli which induce AMPAR trafficking-

dependent LTP and LTD regulate actin polymerization and depolymerization [102, 120, 121], and pharmacological disruption of actin dynamics inhibits LTP [122], perhaps through a disruption of activity- and cytoskeleton-dependent AMPAR trafficking. Consistent with this idea, pharmacological manipulation of actin polymerization with latrunculin A (depolymerizer) and jasplakinolide (stabilizer), results in the stimulation and inhibition of constitutive AMPAR internalization, respectively [119]. This perhaps suggests an additional role of the actin cytoskeleton in homeostatic maintenance of synaptic strength over time as proteins are turned over. When these findings are considered in the context of the role of small G-protein-coupled pathways in regulating AMPAR trafficking during synaptic plasticity, a clear picture begins to emerge regarding the molecular mechanisms that may underlie disorders of learning and memory resulting from their disruption.

Figure 1.9) Actin at the Synapse: Dual Roles in Structure and Vesicular Trafficking



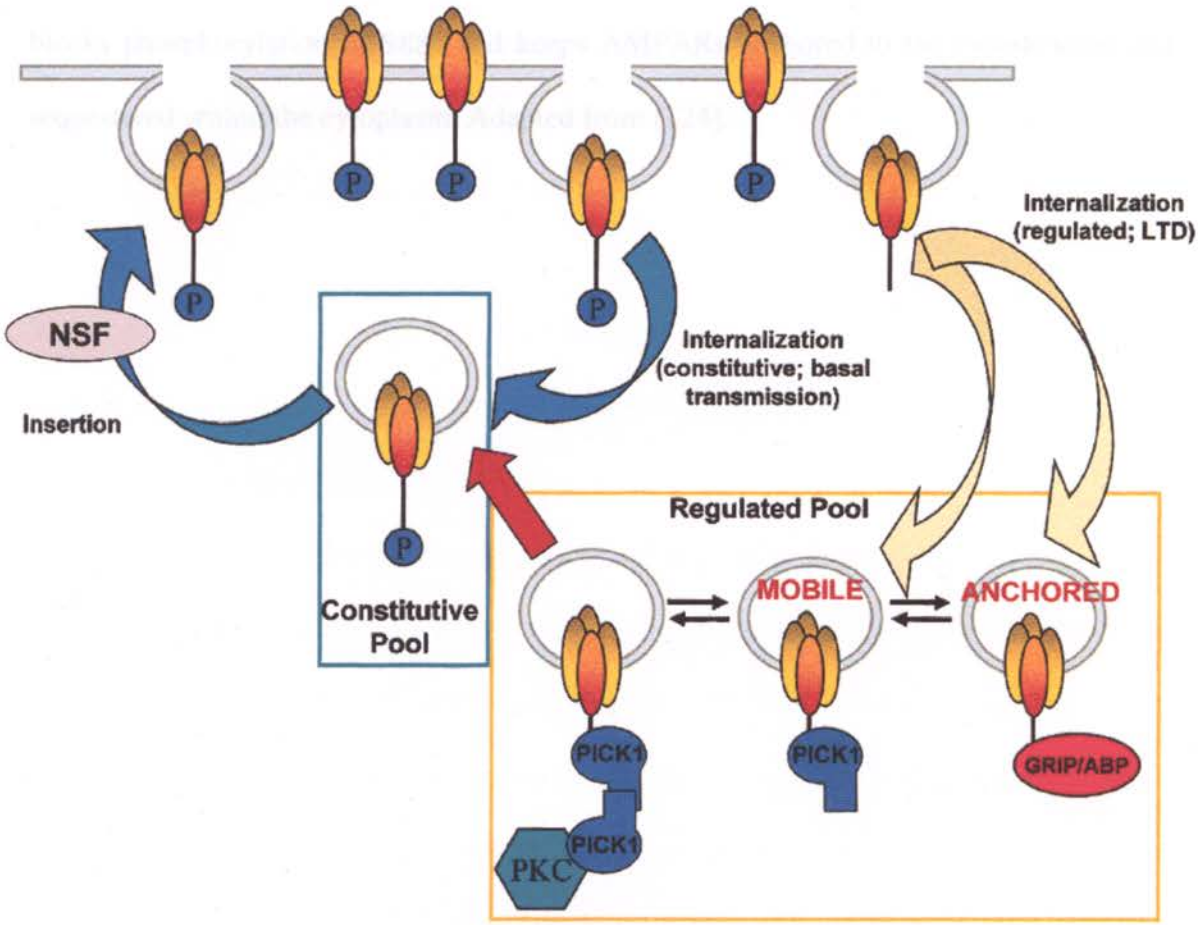


Fig. 1.9) Actin at the synapse: dual roles in structure and vesicular trafficking. Top panel: stereo computer graphic reconstruction from electron micrographs (phaloidin-eosin photo conversion) of actin structure in a hippocampal area CA1 synapse. Actin labeling (blue) is concentrated between the lamellae of the spine apparatus (yellow), the postsynaptic plasma membrane (orange), and the post synaptic density (fuchsia). The presynaptic structure (green, with purple vesicles) is largely devoid of F-actin. Adapted from [123]. Bottom panel: model for constitutive and regulated (activity-dependent) AMPAR trafficking. Shown are GRIP- and PICK-containing protein complexes, which interact with both GluR2-containing vesicles and the actin cytoskeleton (not shown). "P" indicates regulatory phosphorylation of GluR2 at S880 by PKC. Interaction with GRIP blocks phosphorylation of S880 and keeps AMPARs anchored to the cytoskeleton and sequestered within the cytoplasm. Adapted from [124].

1.9 Cellular and Cognitive Functions of WAVE-1

Rac-1 couples to dynamic actin, in part, through its interaction with the WAVE-1 complex [125] (**Fig. 1.10**), but thus far a role for WAVE-1 in synaptic plasticity has not been tested. WAVE-1, or Wiskott-Aldrich syndrome protein (WASP)-family VErprolin homologous protein-1, is technically an A-Kinase Anchoring Protein (AKAP), but has also been shown to function as a molecular scaffold for a variety of signaling molecules other than PKA, including Abl, Abi, HSPC300, CYFIP, actin, Arp 2/3, and WRP [126]. Rac promotes activation of WAVE-1 by causing the release of a multiprotein inhibitory complex [127, 128]. As opposed to LIMK and PAK, which regulate actin depolymerization, WAVE-1 regulates actin *via* a direct association with the ARP 2/3 complex, which catalyzes actin nucleation and branching at the leading edge of cells[129, 130]. Interestingly, Rac signaling is terminated by a WAVE-1 associated GTPase activating protein (GAP) called WRP [128].

The WAVE family of proteins is thought to guide local actin polymerization required for morphological plasticity of neurons, including structures such as the axon growth cone and dendritic spines [131]. WAVE-1 clusters demonstrate a high degree of F-actin-dependent motility and translocate to the leading edge of lamellipodia in response to Rac activity, where it may play a role in spinogenesis and regulation of spine morphological plasticity. In the fruit fly *Drosophila melanogaster,* the WAVE complex has been shown to regulate neuronal function and connectivity, including nerve and synapse formation [132]. Members of the WASP/WAVE family of proteins have a conserved modular structure and share most of their interacting partners, but have distinct physiological

roles. For example, WASP knockout mice display cytoskeletal abnormalities in lymphocytes and platelets (similar to the immunodeficiency seen in Wiskott-Aldrich syndrome patients), whereas N-WASP (neuronal WASP) gene disruption results in embryonic lethality that is associated with neural tube and cardiovascular defects [133]. WAVE has 3 isoforms, WAVE 1-3. WAVE-1 is a brain-specific isoform and, disruption of WAVE-1 signaling by gene knockout reproduces behavioral learning and memory deficits comparable to 3p-syndrome, a form of human mental retardation [134]. Thus, the finding that WRP interacts with WAVE-1 is particularly interesting since WRP has been identified as a gene that is disrupted in 3p-syndrome [135]. In addition to runting and reduced viability, WAVE-1 knockout mice exhibit poor balance, reduced coordination, reduced anxiety, and impaired spatial learning and memory, as demonstrated by a failure to learn the location of a hidden platform in the Morris water maze [134]. WAVE-1 knockout mice also have strong impairment in novel-object recognition, a non-spatial hippocampus-dependent learning and memory task. Thus, WAVE-1 is known to be important for both proper regulation of the actin cytoskeleton by Rac, and for normal hippocampal-dependent brain functions, but the physiological mechanisms underlying the alterations to cognitive function in knockout mice have not been explored. A detailed cellular and electrophysiological characterization of two models of WAVE-1 function in the hippocampus, the WAVE-1 knockout and the Δ-WRP knock-in mouse will be the subject of chapter 3 of this thesis.

Figure 1.10) The WAVE-1 Signaling Complex

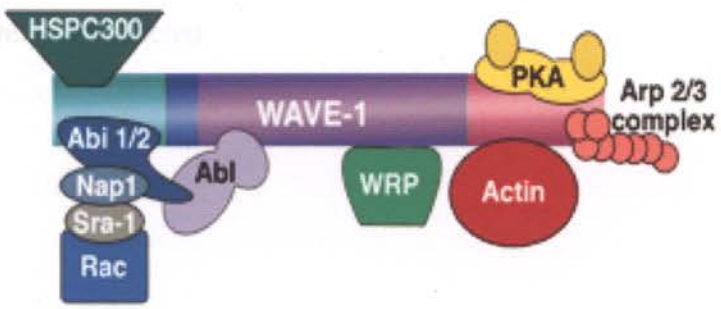


Fig. 1.10) The WAVE-1 Signaling Complex. A schematic diagram of protein components of the WAVE-1 signaling complex. WAVE-1 couples Rac signaling to regulation of the actin cytoskeleton. Rac binds indirectly to WAVE-1 *via* Sra-1, Nap1, and Abi. WAVE-1 directly binds a number of signaling molecules including HSPC300, Abl, WRP (a Rac-GAP), PKA. WAVE-1 also binds actin, and binds and activates the ARP 2/3 complex, which catalyzes acting nucleation and branching. Individual enzymes and effector proteins are labeled.

Chapter 2

CaMKK/CaMKI Activity Gates ERK-dependent Long-term Potentiation

---

*John M. Schmitt, *Eric S. Guire, Takeo Saneyoshi and Thomas R. Soderling

*Co-first authors.

Published Feb. 2, 2005; Journal of Neuroscience; 25(5):1281-90.

My contribution to the findings presented in this chapter included most of the design, acquisition, analysis, and interpretation of the electrophysiology data. I also contributed to the design, acquisition, and interpretation of the biochemical work, and preparation of the manuscript. However, the majority of the biochemistry was masterminded and executed by my colleague Dr. John Schmitt, a post-doctoral fellow in Tom's lab at the time of this collaboration. Some time before this project was initiated in force, while investigating the role of CaMKIV downstream of CaMKK in late-phase LTP, I saw unexpected effects of the novel CaMKK inhibitor STO-609 on LTP. As expected, I had found that the early phase of LTP was unaffected in a CaMKIV knockout mouse, and the late phase of LTP was abolished by STO-609. Unexpected, however, was the partial suppression of the early phase of LTP by STO-609 (**Supplemental Fig. 2.1**). I shared this finding with John, whom it turns out was investigating a potential role for CaMKI (another target of CaMKK) upstream of ERK activation in NG-108 cells. Knowing the established role of ERK in E- LTP, we began our collaboration as soon as John's findings were made conclusive. Thus, the conception, design and execution of this project

reflected an equal contribution on both of our parts. In accordance with this, we determined the order of co-first author listing by coin-toss. Work performed by Takeo, was instrumental to our investigation of the role of Ras-GRF1 in this pathway. Finally, Tom's scientific guidance and wisdom elevated this project to the high standards that are characteristic of the publications that come from his lab.

2.1 Abstract

Intracellular Ca^{2+} and protein phosphorylation play pivotal roles in long-term potentiation (LTP), a cellular model of learning and memory. Ca^{2+} regulates multiple intracellular pathways including the calmodulin-dependent kinases (CaMKs) and the extracellular signal-regulated kinases (ERKs), both of which are required for LTP. However, the mechanism by which Ca^{2+} activates ERK during LTP remains unknown. Here, we describe a requirement for the CaMK-kinase (CaMKK) pathway upstream of ERK in LTP induction. Both the pharmacological inhibitor of CaMKK STO-609 and dominant-negative CaMKI (dnCaMKI), a downstream target of CaMKK, blocked neuronal NMDA receptor-dependent ERK activation. In contrast, an inhibitor of CaMKII and nuclear-localized dnCaMKIV had no effect on ERK activation. NMDA receptor-dependent LTP induction robustly activated CaMKI, the Ca^{2+}-stimulated Ras activator Ras-GRF1 and ERK. STO-609 blocked the activation of all three of these enzymes during LTP without affecting basal synaptic transmission, activation of CaMKII or cAMP-dependent activation of ERK. LTP induction itself was suppressed about 50% by STO-609 in a manner identical to the ERK inhibitor U0126 – either inhibitor occluded the effect of the other, suggesting they are part of the same signaling pathway in LTP induction. STO-609 also suppressed regulatory phosphorylation of two downstream ERK targets during LTP, the general translation factors eIF4E and its binding protein 4E-BP1. Furthermore, suppression of mRNA translation with anisomycin occluded the effects of STO-609 during early-phase LTP. These data indicate an essential role for CaMKK and CaMKI to link NMDA receptor-mediated Ca^{2+} elevation with ERK-dependent LTP.

2.2 Introduction

Protein phosphorylation is one of the most prevalent mechanisms for modulating neuronal functions including long-term potentiation [41, 136], a cellular model of learning and memory [137, 138]. Two protein kinase families highly expressed in brain that have been implicated in molecular mechanisms regulating LTP are the Ca^{2+}/calmodulin-dependent kinases (CaMKs) and the extracellular signal-regulated kinases (ERK 1 & 2) [139, 140]. The CaMK family, which is activated in response to elevations of intracellular Ca^{2+}, includes CaMKII [41, 141, 142] and the CaMKK cascade consisting of CaMKK and its two major downstream targets CaMKI and CaMKIV [143, 144]. CaMKII regulates numerous neuronal functions including phosphorylation of the AMPA-type glutamate receptor, resulting in increased conductance during early-phase LTP (E-LTP) [44, 46]. CaMKIV is largely restricted to the nucleus [64, 145] where it stimulates gene transcription required for late-phase LTP (L-LTP) [146] through phosphorylation of transcription factors such as CREB and CBP [147, 148]. Cytosolic CaMKI modulates cytoskeletal organization [69] and axonal growth cone motility [71], but a potential role for CaMKI in LTP has not been previously examined.

The ERK family of MAP-kinases are activated by growth factors, neurotransmitters, and hormones as well as by Ca^{2+}-permeable N-methyl-D-aspartate receptor (NMDA-R) and voltage-gated channels in neurons [78, 79]. The NMDA-R gates many forms of synaptic plasticity including hippocampal CA1 LTP [149]. Activation of ERK upon neuronal depolarization or NMDA-R stimulation requires elevations of intracellular Ca^{2+}, and it appears to be mediated through the classical Ras/Raf/MEK/ERK cascade [83, 150].

Recent studies suggest that the Ras-guanyl-nucleotide releasing factor, Ras-GRF1 may couple the NMDA-R Ca^{2+} elevation to Ras and ERK activation in neurons [84, 85].

Induction of LTP activates ERK, and inhibitors of the ERK pathway (*e.g.*, SL327 and U0126) partially suppress LTP induction [78, 140]. A role for ERK in the regulation of gene transcription during late-phase LTP (L-LTP) is well established [151, 152], and a role of ERK in mRNA translation has recently been reported [152]. Expression of dominant-negative (dn) MEK1 in region CA1 of the hippocampus suppresses hippocampal-dependent memory tasks in mice, ERK activation, and anisomycin-sensitive LTP in hippocampal slices, as well as mRNA translation in cultured hippocampal neurons.

Because of the key role of ERKs in modulating Ca^{2+}-dependent neuronal plasticity, it was important to determine whether the recently identified cross-talk between CaMKK/CaMKI and ERK [72] regulate LTP. The results of this study provide strong evidence that CaMKK and CaMKI are essential for the full expression of LTP and ERK-dependent translational activation.

2.3 Materials and Methods

Antibodies and plasmids. The following reagents were purchased from the indicated sources: U0126 and Forskolin, Calbiochem (Riverside, CA); STO-609, NMDA, and APV, Tocris Cookson, Inc. (Ellisville, MO); Anisomycin, Alexis Biochemicals (San Diego, CA): Phospho-specific antibodies; ERK1/2 (Thr^{202}, Tyr^{204}), Ras-GRF1 (Ser^{916}), eIF4E (Ser^{209}), 4E-BP1 (Ser^{65}), Akt (Thr^{308}), Cell Signaling (Beverly, MA); CaMKII (Thr^{286}), GluR1 subunit of the AMPA-R (Ser^{831}), Affinity Bioreagents (Golden, CO); CaMKI (Thr^{177}), Dr. Naohito Nozaki [71, 72]. Other antibodies: Akt, Ras-GRF1, eIF4E, and 4E-BP1, Cell Signaling; ERK2 (D-2), Santa Cruz Biotechnology; CaMKII, Transduction Lab.; CaMKI, Dr. Kohji Fukunaga (Tohoku Univ., Sendai, Japan); FLAG (M2), Sigma. The dominant-negative (dn) CaMKK (K71A, T108A, S458A), dnCaMKI (K49E, T177A, 7IHQS286EDDD, F307A), dnCaMKIV nuclear (T196A, K71E, HMDT305DEDD), and CaMKIIN plasmids have been previously described [71, 72]. The Flag-ERK2 plasmid was provided by Dr. Philip Stork (Vollum Institute, Portland, OR). The Ras-GRF1 construct was purchased from Open Biosystems.

Primary hippocampal neuronal culture and treatments. Neurons were isolated from the hippocampus of postnatal day 1-2 Sprague Dawley rats. Neurons were grown 5-6 days *in vitro* (unless otherwise indicated) and placed in serum-free isotonic media consisting of 130mM NaCl, 2.5 mM KCl, 1mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM HEPES-HCl, and 30 mM d-Glucose for 60 minutes at 37 °C with pharmacological inhibitors (5 μM STO-609 for 60 minutes; 10μM U0126 for 20 minutes), as indicated prior to stimulation. Cells

were stimulated with serum- and magnesium-free isotonic media containing NMDA (25 μM) and glycine (1 μM) for the indicated times.

For transfections, 5-day old hippocampal neurons were co-transfected with Flag-ERK2, along with the control vector pcDNA3, or the indicated plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's guidelines. Following transfection, neurons were allowed to recover in complete media for 24 hours, placed in serum-free isotonic media for 60 minutes (see above) treated as indicated and lysed in ice-cold lysis buffer as detailed below. Lysates were then examined for the presence of Flag-ERK2 or myc-Ras-GRF1 (Open Biosystems) expression by western blotting, and Flag-ERK2 or myc-Ras-GRF1 were immunoprecipitated and examined by western blotting for activation.

Western blotting and immunoprecipitation of primary hippocampal neurons. For direct western blotting following primary hippocampal neuron stimulations, media was aspirated and boiling Laemmeli buffer (supplemented with 200 mM EDTA, 20 mM EGTA, 200 mM NaF, 20 mM β-glycerophosphate, 1 mM PMSF, and 5 μM microcystin) was added to each plate of cells. Plates were scraped and cellular lysates were placed in microcentrifuge tubes, rapidly vortexed, boiled for 5 minutes, and centrifuged at 8,000 rpm for 3 minutes to pellet insoluble materials. Equivalent amounts of protein were resolved by SDS-PAGE, blotted onto polyvinylidene difluoride membranes (PVDF), and examined by western blotting with the indicated antibodies.

For immunoprecipitations following primary hippocampal neuron stimulations, media was aspirated and ice-cold lysis buffer (10% glycerol, 1% NP40, 50mM Tris-HCl pH 7.4, 200mM NaCl, 2mM $MgCl_2$) containing freshly added inhibitors (1mM PMSF, 2μg/ml Aprotinin, 1μg/ml Leupeptin, 10μg/ml Trypsin inhibitor, 1mM sodium orthovanadate) was added to each plate of cells on ice. Plates were scraped and cellular proteins placed in ice-cold microcentrifuge tubes. Briefly, cell lysates were spun at 8,000 rpm for 5 minutes at 4 °C to pellet the cytoskeleton and nuclei. Immunoprecipitations were carried out at 4 °C for 4 hours in ice-cold lysis buffer [72]. Precipitated proteins were washed 2 times with lysis buffer, placed in Laemmeli buffer, resolved by SDS-PAGE and western blotted for phosphorylated Flag-ERK2 (pFlag-ERK2, pFlag-ERK2 or myc-Ras-GRF1). Western blotting for total Flag-ERK2 with anti-Flag antibody was performed on lysates to serve as a loading and transfection control. For quantitation of western blots, autoradiographs were scanned and densitized using Kodak ID 3.0.2 system software (New Haven, CT). For each phospho-protein of interest, separate western blots were run for that phospho-protein (*e.g.*, pCaMKI) and for the total protein (CaMKI). For each western blot, band densities were first normalized to untreated controls, and then phospho-protein was normalized to total protein and the value presented as fold-increase in phosphorylation relative to the control.

Mouse hippocampal slice production and treatments. Adult male C57BL6 mice (8-12 weeks old, Charles River) were anesthetized with pentobarbital (60 mg/kg I.P.) and decapitated using procedures reviewed and approved by IACUC at the OHSU Department of Comparative Medicine. Brains were removed within one minute of

decapitation and immediately submerged in ice-cold, sucrose-modified artificial cerebrospinal fluid (S-ACSF) for hippocampal dissection and slicing (in mM): sucrose 110, NaCl 60, KCl 2.5, $NaHCO_3$ 28, NaH_3PO_4 1.25, $CaCl_2$ 0.5, $MgCl_2$ 7, glucose 5, sodium ascorbate 0.6, pH 7.4@4 °C (pre-saturated by bubbling with 95% O_2/5% CO_2 at 21-22°C). Hippocampal slices (400 μm, transverse) were prepared using a vibratome and an agar backing (2%), and each slice was transferred to warm ACSF (in mM: NaCl 125, KCl 2.5, $NaHCO_3$ 22.6, NaH_3PO_4 1.25, $CaCl_2$ 2, $MgCl_2$ 1, glucose 11.1, continuously gas saturated with 95% O_2/5% CO_2, pH 7.4 @ 32°C) for recovery (30 minutes, 37°C). Slices from the dorsal and ventral thirds of the hippocampus were discarded. Following recovery, the chamber was equilibrated at 21-22 °C, and slices were held for 2-8 hours before recording, or 1 hour before beginning NMDA and Forskolin treatments. For NMDA and Forskolin treatments, slices were then placed at 30° C for an additional hour, in the presence or absence of pharmacological inhibitors (5 μM ST0-609 for 60 minutes, 10 μM U0126 for 20 minutes, and 50 μM APV for 20 minutes), prior to chemical stimulations. Hippocampal slices were then stimulated with NMDA plus glycine (25 μM, 1μM, respectively) or Forskolin (50 μM) for the indicated times in ACSF, in the presence or absence of STO-609 or U0126. Following chemical stimulation, and at various time points following the electrical induction of LTP, slices were frozen on a filter paper-covered liquid nitrogen-cooled aluminum block, and placed in microcentrifuge tubes in pairs. Slices were kept submerged at all times until frozen. For LTP experiments, area CA1 was isolated by microdissection following freezing. Ice-cold lysis buffer (10% glycerol, 1% NP40, 50 mM Tris-HCl pH 7.4, 200 mM NaCl, 2 mM $MgCl_2$) plus freshly added inhibitors (1 mM PMSF, 2 μg/ml Aprotinin, 5 μM

microcystin, 1 μg/ml Leupeptin, 10 μg/ml Trypsin inhibitor, 1 mM sodium orthovanadate) was added to each tube containing frozen slices, which were rapidly homogenized as they thawed in lysis buffer. 6X Laemmeli buffer was then added, and tubes were rapidly vortexed and boiled for 5 minutes. Equivalent volumes of lysate were resolved by SDS-PAGE, blotted onto polyvinylidene difluoride membranes (PVDF), and examined by western blotting with the indicated antibodies.

Electrophysiology. For electrophysiological recordings, submerged hippocampal slices were suspended in pairs on a nylon mesh in a small (350-400 μL) recording chamber, and perfused with ACSF at a rate of 2.5-3 mL/minute. The temperature in the recording chamber was raised to 30-32 °C over the course of a few minutes and held for the duration of the experiment. Synaptic responses were evoked by Schaffer collateral stimulation using a bipolar tungsten electrode (FHC, inc., tip spacing 140μm) and a 100 μsec square wave test-pulse (typically 30-40 μA) delivered at 60 second intervals. The stimulation intensity was adjusted to produce a basal response of 1.2-1.4 mV (typically 25% of the maximum fEPSP amplitude). Recordings were made using ACSF filled glass micropipettes (2-4 MΩ) placed in the stratum radiatum area of CA1 (see Fig. 2.2C), and connected *via* head-stages to an A-M Systems model 1800 amplifier. Signals were digitized at 100 kHz using Axon Instrument's digidata 1200 series interface running Clampex 8.0, and the initial slope (linear portion of the first msec) of the fEPSPs were analyzed with Clampfit 8.0 software. Drug treatments began 30 minutes (STO-609, U0126, and anisomycin) or 20 minutes (APV) before, and ended (with the exception of anisomycin) 5 minutes following the induction of LTP using *theta*-bursts. Anisomycin

treatments persisted for the duration of the recordings. E-LTP was induced using an acute *theta*-burst protocol: 4 pulses/burst (100 Hz), 5 bursts/train (5 Hz), 3 trains (beginning 20 seconds apart). L-LTP was induced using a recurrent *theta*-burst protocol: 4 epochs of the acute *theta*-burst protocol delivered 5 minutes apart. With the exception of the first data point following LTP induction (PTP), 3-minute fEPSP averages are shown. The first data point following LTP induction was taken 20 seconds following the final train of *theta*-bursts. All treatment groups were balanced such that the average time of the slices in the recording chamber was equal, and the average age of animals was equal.

Statistics. To determine whether significant differences existed among treatments, an analysis of variance (ANOVA) was performed on the data with significance set at 0.05. To compare whether significant differences existed between two treatments a Student's T-test was performed on the data with significance set at 0.05. Significance levels (p value) are indicated in the Figures: * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$.

2.4 Results

NMDA activation of ERK requires CaMKK and CaMKI. To investigate the potential role of CaMKs in NMDA-dependent ERK activation in hippocampal neurons, we used the membrane-permeable CaMKK inhibitor STO-609 [60] or expression of dominant-negative CaMKs (dnCaMK) or the CaMKII inhibitor protein CaMKIIN [153]. STO-609 appears to be highly specific for CaMKK: 1) it has an *in vitro* IC_{50} of 0.13-0.38 μM for CaMKK and 32 μM for CaMKII with little or no inhibition of CaMKI, CaMKIV, PKA, PKC, ERK or myosin light chair kinase [60], and 2) in cultured hippocampal neurons [71] and NG108 cells [72] subjected to depolarization, STO-609 inhibits CaMKK activation, as assessed by its ability to block phosphorylation of the activation loop Thr^{177} in CaMKI, with no effect on CaMKII activation (*i.e.*, autophosphorylation of Thr^{286}). Furthermore, we used dnCaMKs as an independent confirmation of the involvement of this pathway. CaMKIIN is an endogenous CaMKII inhibitor protein (IC_{50} of 50-100 nM) that exhibits no *in vitro* inhibition of CaMKI, CaMKIV, CaMKK, PKA, PKC [153] or ERK (Nygaard, S., and Soderling, T.R., unpublished result). When transfected into cultured hippocampal neurons, CaMKIIN potently blocks CaMKII activation [71] but not ERK activation [72] upon depolarization.

Stimulation of cultured hippocampal neurons with NMDA activated endogenous ERK within 5 minutes, an effect that was completely blocked by either the MEK inhibitor U0126 or by STO-609 (**Fig. 2.1A**), indicating involvement of the CaMKK cascade. To confirm the specificity of STO-609, we used a mutant (L233F) of CaMKK [60] that is insensitive to inhibition by this compound. Transfection of neurons with $CaMKK_{L233F}$

completely rescued inhibition by STO-609 (**Fig. 2.1A**), establishing that STO-609 was exerting its effect through CaMKK. To further characterize which member of the CaMKK cascade mediated ERK activation, hippocampal neurons were co-transfected with Flag-ERK2 plus various dominant-negative (dn) CaMK constructs. ERK activation was completely inhibited by dnCaMKK as well as by dnCaMKI (**Fig. 2.1B**). Endogenous CaMKIV is predominantly nuclear restricted [145], and nuclear-localized (nuc) dnCaMKIVnuc did not block ERK activation (**Fig. 2.1B**) even though we have previously shown that it suppressed NMDA-stimulated CREB-dependent transcription in neurons [71]. To examine the role of CaMKII, we expressed the specific CaMKII inhibitor protein CaMKIIN [153] that completely blocked neuronal CaMKII activation in response to depolarization [71]. CaMKIIN had no effect on NMDA-dependent ERK activation (**Fig. 2.1B**). These results demonstrate in cultured hippocampal neurons that NMDA-dependent activation of ERK was mediated by the CaMKK and CaMKI pathway but not by CaMKII or nuclear CaMKIV.

Next, we examined the role of CaMKK in ERK activation using acute mouse hippocampal slices. The rapid activation of ERK in response to NMDA treatment was blocked by the NMDA receptor antagonist APV, the MEK inhibitor U0126 and the CaMKK inhibitor STO-609 (**Figs. 2.1C, D**). CaMKK and CaMKI were also rapidly activated by NMDA as assessed by phosphorylation of the activation loop Thr^{177} in CaMKI (a target of CaMKK), and, as expected, these were also inhibited by STO-609 but not U0126 (**Fig. 2.1E**). In agreement with previous studies [154], CaMKII was also activated (*i.e.*, autophosphorylation of Thr^{286}) by NMDA stimulation, and one of its

substrates, Ser^{831} in the GluR1 subunit of the AMPA-R, was phosphorylated [155], but neither of these reactions was inhibited by STO-609 or U0126 (**Fig. 2.1F**). This result confirms our previous observation that STO-609 does not block CaMKII activation in hippocampal neurons [71] or NG108 cells [72]. The specificity of STO-609 for CaMKK was further demonstrated by the fact that activation of ERK in response to Forskolin treatment, which was NMDA-receptor independent (**Fig. 2.1C**), was not blocked by STO-609 but was suppressed by U0126 (**Fig. 2.1G**).

We have previously shown a requirement for the small G protein Ras in the CaMKK/CaMKI activation of ERK upon depolarization [72]. Ras is required for NMDA receptor-dependent activation of ERK in neurons [101], and the dnRas RasN17 blocked ERK activation in response to NMDA treatment of cultured hippocampal neurons in our experiments (data not shown). Furthermore, recent studies demonstrate the involvement of the Ca^{2+}-dependent Ras activator, Ras-GRF1 in ERK activation [84, 85]. Phosphorylation of Ras-GRF1 at Ser^{916} appears to play a pivotal role in its physiological functions and serves as a read-out for activation [87, 88]. Endogenous Ras-GRF1 was rapidly phosphorylated on Ser^{916} in response to NMDA treatment, an effect that was blocked by STO-609 but not U0126 (**Fig. 2.1H**). To confirm the roles for CaMKK and CaMKI in Ras-GRF1 phosphorylation, cultured hippocampal neurons were co-transfected with myc-Ras-GRF1 plus dnCaMKI or CaMKIIN. Ras-GRF1 phosphorylation was completely suppressed by dnCaMKI but not by the CaMKII inhibitor (**Fig. 2.1I**). Taken together, the data of Figure 2.1 support the specificity of

STO-609 for CaMKK and suggest that the requirement for CaMKK/CaMKI in NMDA-dependent ERK activation in hippocampal neurons is upstream of Ras-GRF1.

Induction of LTP requires the CaMKK pathway. The CaMKK cascade can mediate NMDA receptor-dependent ERK activation (**Fig. 2.1**) and ERK plays an important role in LTP induction [78, 140], so we investigated the role of CaMKK in LTP induction. Mouse hippocampal slices treated with STO-609 exhibited a normal input-output relationship over a wide range of stimulus intensities (**Fig. 2.2A**) as well as normal paired-pulse facilitation (**Fig. 2.2B**), indicating that acute blockade of the CaMKK pathway does not effect basal synaptic transmission. Additionally, we integrated the area above the curve of the fEPSPs during stimulation with theta-burst trains (E-LTP protocol, see Methods) and found no significant difference among the dendritic field responses of STO-609 or U0126 treated and untreated slices during E-LTP induction (**Fig. 2.2C**). This finding held true for the first (naïve) *theta*-bust, each individual train, the plasticity within each train (area of burst 5/burst 1) as well as the sum of all trains. These results indicate that STO-609 does not impair normal synaptic transmission nor the dendritic field response to high frequency theta-burst trains, important prerequisites for our study of CaMKK's role in LTP.

To determine if CaMKK and its downstream targets are involved in the induction of early-phase LTP (E-LTP), we treated acute hippocampal slices with 5μM STO-609. Analysis of the initial slope of field excitatory post-synaptic potentials (fEPSPs) taken from area CA1 (**Fig. 2.2C**) before and after *theta*-burst stimulation (Methods) revealed a

significant (~50%) STO-609 sensitivity of E-LTP out to 60 minutes (**Fig. 2.2D**). Our acute *theta*-burst protocol produced activation of CaMKI and ERK within 5 minutes, effects that were completely blocked by the NMDA receptor antagonist APV, as was the induction of E-LTP (**Fig. 2.2E**).

The role of CaMKK in L-LTP lasting 3 hours was examined using a recurrent *theta*-burst protocol spaced over 15 minutes (see Methods). Again, STO-609 treatment gave approximately 50% suppression over the first hour and completely obviated LTP at 3 hours (**Fig. 2.2F**), demonstrating that the requirement for CaMKK in the first hour of LTP was not overcome by a more robust induction protocol. Although CaMKIV is also activated by CaMKK, it does not participate in E-LTP induced with either HFS or recurrent *theta*-bursts in mouse hippocampal region CA1 but is thought to contribute to CREB/CBP-dependent transcription during L-LTP [146, 156]. Based on these observations we conclude that CaMKI mediated the downstream effects of CaMKK during E-LTP whereas CaMKIV may contribute significantly to L-LTP.

CaMKK mediates ERK activation in E-LTP. Induction of E-LTP in hippocampal slices activates ERK, and this activation is required for full expression of LTP [31]. In light of our findings that CaMKI functions upstream of ERK activation, we examined whether the effects of STO-609 on LTP occurred upstream of ERK. Induction of LTP with our acute *theta*-burst protocol (see Methods, **Figs. 2.2D, E**) resulted in 3- to 4-fold activation of CaMKI and ERK within 5 minutes that was sustained for at least 60 minutes (**Fig. 2.3A, B**). Both CaMKI and ERK activation were suppressed by STO-609 at all time

points examined. Another cytoplasmic target for CaMKK is the protein kinase AKT, which is slowly phosphorylated upon depolarization of NG108 cells [72, 157]. A dnAKT [72, 157] did not block ERK activation by NMDA treatment of cultured hippocampal neurons (data not shown) or upon depolarization of NG108 cells [72, 157]. Induction of LTP in hippocampal slices produced a slow phosphorylation of AKT that was blocked by STO-609 (**Fig. 2.3B**). However, ERK was maximally activated within 5 minutes following LTP induction whereas robust AKT activation was not observed until after 15 minutes (**Fig. 2.3B**). Therefore, we propose that CaMKK was not acting through AKT to activate ERK or to mediate LTP induction. However, endogenous Ras-GRF1 was significantly phosphorylated within 5 minutes, and this was obviated by STO-609 treatment (**Fig. 2.3C**). LTP induction also resulted in rapid activation of CaMKII and phosphorylation of its substrate Ser^{831} in the GluR1 subunit of the AMPA-R [155], but these effects were not suppressed by STO-609 (**Fig. 2.3D**). This confirms that STO-609 does not suppress other Ca^{2+}-dependent pathways such as CaMKII. These data support our conclusion that ERK activation during LTP is mediated by CaMKK acting through CaMKI.

CaMKK and ERK mediate E-LTP by the same pathway. As reported previously [31], the MEK inhibitor U0126 produced a partial suppression of E-LTP elicited with *theta*-bursts (**Fig. 2.4A**). The same drug application and acute *theta*-burst protocol was used here as in Figures 2.2D and E. The inhibition of E-LTP by U0126 (**Fig. 2.4A**) was strikingly similar to that produced by STO-609 (**Fig. 2.2D**), consistent with the data in Figure 2.3 demonstrating that ERK activation during LTP is mediated by CaMKK. If this model is

correct and CaMKK's role in LTP induction is upstream of ERK, then the effect of U0126 should be occluded by STO-609 and *visa versa.* As shown in Figure 2.4B, combined bath treatment with U0126 plus STO-609 was no more effective at blocking E-LTP than treatment with STO-609 alone. In these experiments, U0126 completely blocked ERK activation at both 5 and 60 minutes (**Fig. 2.4C**). The specificity of U0126 was confirmed in that it did not inhibit activation of CaMKI, AKT, CaMKII or Ras-GRF1 (**Fig. 2.4D**). This data demonstrates that the role of CaMKK in E-LTP is mediated by an ERK-dependent mechanism.

Role of CaMKK in translational activation during LTP. A recent study [152] shows that protein synthesis-dependent L-LTP induced by four trains of HFS is partially suppressed in mice expressing a dominant-negative MEK1 (the upstream activator of ERK) in region CA1. In fact, the degree and kinetics of L-LTP suppression in the dnMEK1 mice was equivalent to suppression of L-LTP in wild-type mice by the mRNA translation inhibitor anisomycin. These authors did not observe any decrement in E-LTP induced by two trains of HFS in the dnMEK1 mice, but the decrement of L-LTP (induced by four trains of HFS) developed within minutes in both dnMEK1 and anisomycin-treated mice. This is consistent with our results since E-LTP induced by two trains of HFS in mouse is not sensitive to the MEK inhibitors U0126 or SL327 [31]. Therefore, we tested whether E-LTP induced with our acute *theta*-burst protocol was dependent on protein synthesis using anisomycin. Not only did anisomycin produce a partial suppression of this E-LTP, but also both the magnitude and kinetics of E-LTP blockade with anisomycin (**Fig. 2.5A**) were nearly identical to those of U0126 (**Fig. 2.5B**) and STO-609 (**Fig. 2.5C**), indicating

that the CaMKK/ERK pathway in E-LTP may exert its effects largely through a translation-dependent mechanism. Furthermore, anisomycin treatment did not significantly affect the dendritic field responses (area above the curve) during theta-bursts (data not shown), as was the case with STO-609 and U0126 (**Fig. 2.2C**). Our observation that E-LTP generated by *theta*-burst stimulation are equally sensitive to STO-609 (**Fig. 2.2D**) and anisomycin (**Fig. 2.5A**), suggest that protein synthesis during *theta*-burst E-LTP is regulated by CaMKK and is required for concurrent CaMKK-dependent LTP expression. Indeed, treatment of slices with STO-609 was able to occlude further inhibition of E-LTP by anisomycin (**Fig. 2.5D**).

Regulation of general mRNA translation by ERK during LTP may be related to phosphorylation of several translation factors including eIF4E and its inhibitory binding protein 4E-BP1 [152]. Induction of LTP with our acute *theta*-burst protocol produced a rapid (5 minutes) and sustained (60 minutes) phosphorylation of eIF4E and 4E-BP1 (**Fig. 2.6A-D**), both of which required CaMKK activity since STO-609, when present during LTP induction, was inhibitory at all time points examined. U0126 completely blocked phosphorylation of eIF4E and 4E-BP1 (**Fig. 2.6D**), suggesting a role for eIF4E and 4E-BP1 in translation-dependent LTP downstream of ERK. We conclude that regulatory eIF4E and 4E-BP1 phosphorylations are mediated by the CaMKK/ERK pathway in NMDA receptor-dependent *theta*-burst LTP.

Figure 2.1) NMDA Activation of ERK and Ras-GRF1 in Hippocampus Requires CaMKK and CaMKI

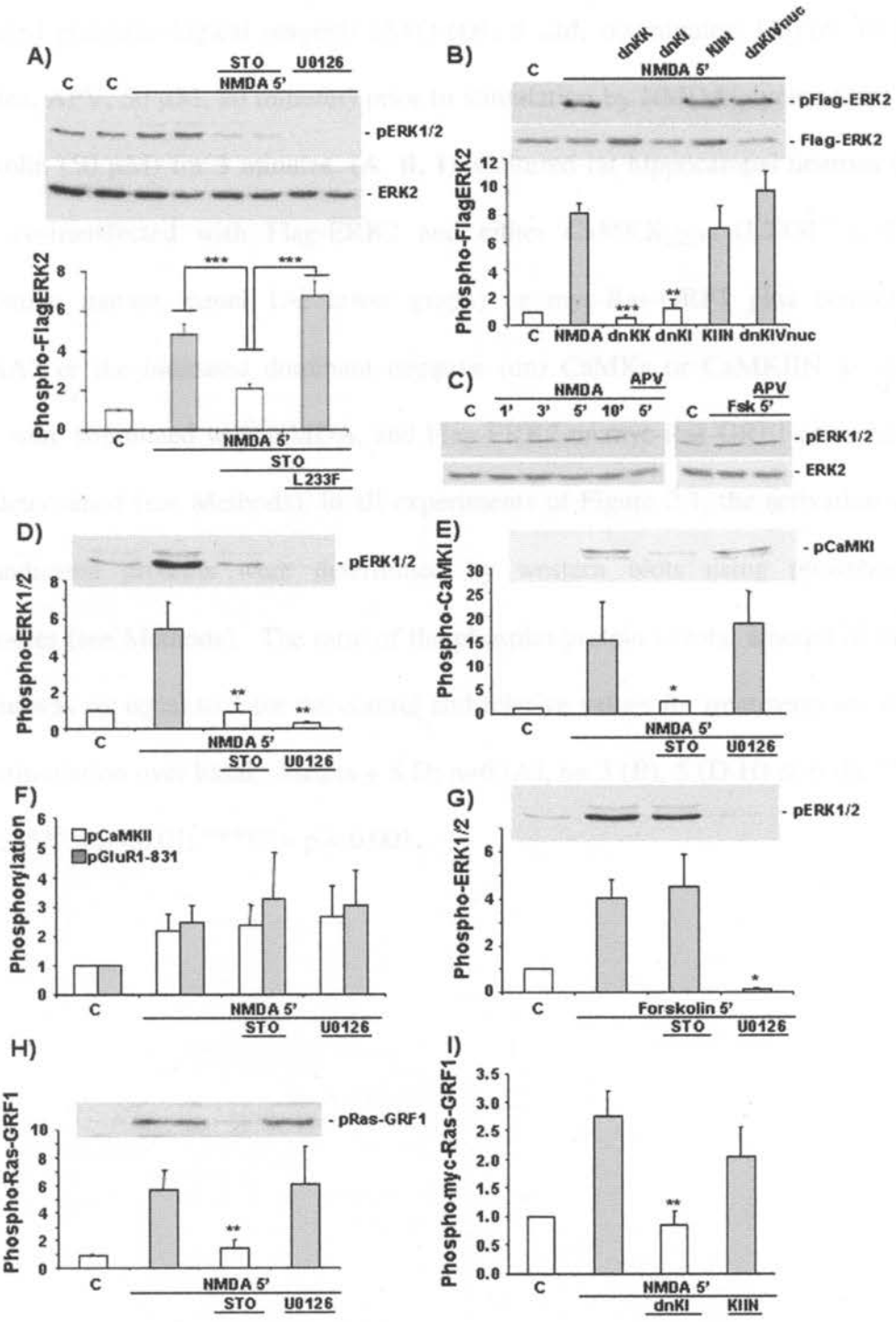


Fig. 2.1) NMDA activation of ERK and Ras-GRF1 in hippocampus requires CaMKK and CaMKI. Primary cultures of rat hippocampal neurons (**A**, **B, I** 6 DIV) or acute mouse hippocampal slices (**C-H**, 8-12 weeks old) were pre-incubated with the indicated pharmacological reagents (STO-609, 5 μM, 60 minutes; U0126, 10 μM, 20 minutes; APV, 50 μM, 20 minutes) prior to stimulation by NMDA/glycine (25/1 μM) or Forskolin (50 μM) for 5 minutes. (**A**, **B, I**). Cultured rat hippocampal neurons (5 DIV) were co-transfected with Flag-ERK2 and either $CaMKK_{L233F}$ (L233F, a STO-609 insensitive mutant, panel 1A, lower graph) or myc-Ras-GRF1 plus control vector pcDNA3 or the indicated dominant negative (dn) CaMKs or CaMKIIN as indicated. Cells were stimulated with NMDA, and Flag-ERK2 or myc-Ras-GRF1 phosphorylation was determined (see Methods). In all experiments of Figure 2.1, the activation states of the indicated proteins were determined by western blots using phospho-specific antibodies (see Methods). The ratio of the phospho-protein to total amount of that same protein was set equal to 1 for the control and relative values for treatments are shown as fold-stimulation over basal. Means $\pm$ S.D; n=6 (A), n= 3 (B), 5 (D-H) or 6 (I). "*" = $p \leq 0.05$; "**" = $p < 0.01$; "***" = $p < 0.001$.

Figure 2.2) Inhibition of CaMKK Markedly Attenuates NMDA Receptor-Dependent LTP

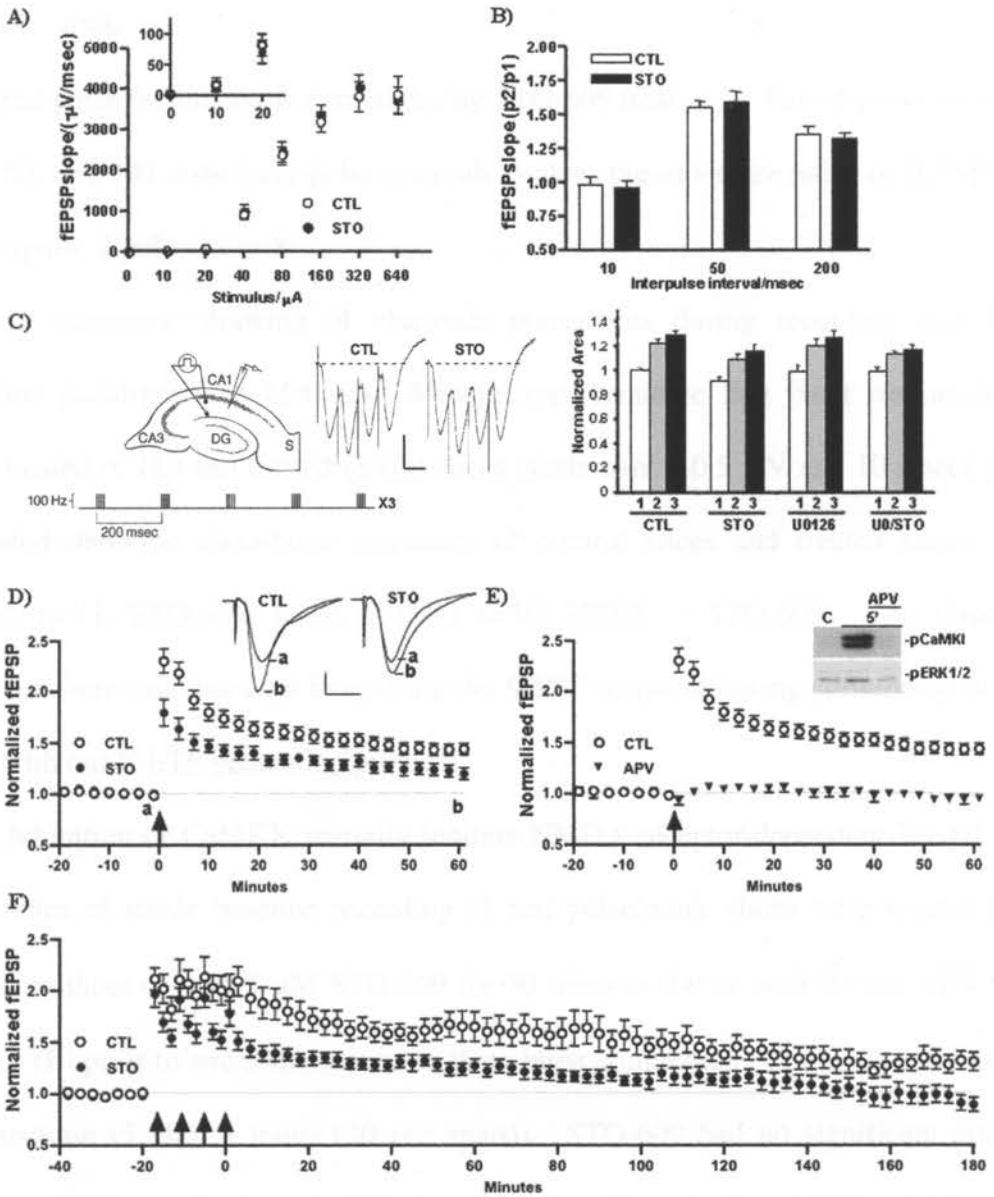


Fig. 2.2) Inhibition of CaMKK markedly attenuates NMDA receptor-dependent LTP.

A. STO-609 does not affect basal synaptic transmission. Input-output relationship for Schaffer collateral stimulation and fEPSP initial slopes recorded from area CA1 of mouse hippocampal slices pre-incubated without or with 5 μM STO-609 for 30 minutes. Inset, 0-20 μA. n=8.

B. Paired-pulse facilitation is normal during STO-609 treatment. Paired-pulse facilitation at 10, 50, and 200 msec inter-pulse intervals. Values presented are ratios of fEPSP initial slopes (pulse 2/pulse 1). n=8.

C. Left: schematic drawing of electrode placements during recording and E-LTP induction paradigm (see Methods). Middle: representative first burst responses from mock-treated (CTL) and treated (STO) slices (scale bars = 0.5 mV and 10 msec). Right: integrated dendritic *theta*-burst responses of control slices and treated slices: n=24, control; n=11, STO-609; n=10, U0126; n=10, U0126 + STO-609. The *theta*-burst responses were calculated by integrating the fEPSP response during stimulation of naive slices with our E-LTP generating protocol.

D, E. Inhibition of CaMKK partially inhibits NMDA receptor-dependent E-LTP. After 20 minutes of stable baseline recording (1 test pulse/min), slices were treated for 30 minutes without or with 5 μM STO-609 for 30 minutes (D) or with 50 μM APV for 20 minutes (E) prior to and 5 minutes after *theta*-burst stimulation (4 pulses/burst (100 Hz), 5 bursts/train (5 Hz), 3 trains (20 sec apart)). STO-609 had no significant effect on baseline fEPSP amplitude or initial slope kinetics. Control slices (LTP = 144 $\pm$ 5.4%, n=24) exhibited approximately 2-fold greater LTP than STO-treated slices (LTP = 120 $\pm$

5.9%, n=11) at 60 minutes whereas APV treatment (n=8) completely blocked LTP. D inset: average of 10 responses, 1-10 minutes before (a) and 50-60 minutes after LTP induction (b). E inset: Western blots from micro-dissected region CA1 showing pCaMKI and pERK 5 minutes after mock-stimulation (Control) or *theta*-burst stimulation without or with APV treatment.

F. Inhibition of CaMKK blocks L-LTP. Slices were treated without or with STO-609 as in panel D prior to stimulation with recurrent *theta*-burst patterned activity (4 pulses/burst (100 Hz), 5 bursts/train (5 Hz), 6 trains (20 sec apart), 4 epochs (5 minutes apart)) to generate late-phase LTP. (Control, n=6; STO-609, n=6)

Figure 2.3) LTP-Activation of CaMKI, Ras-GRF1, and ERK Requires CaMKK

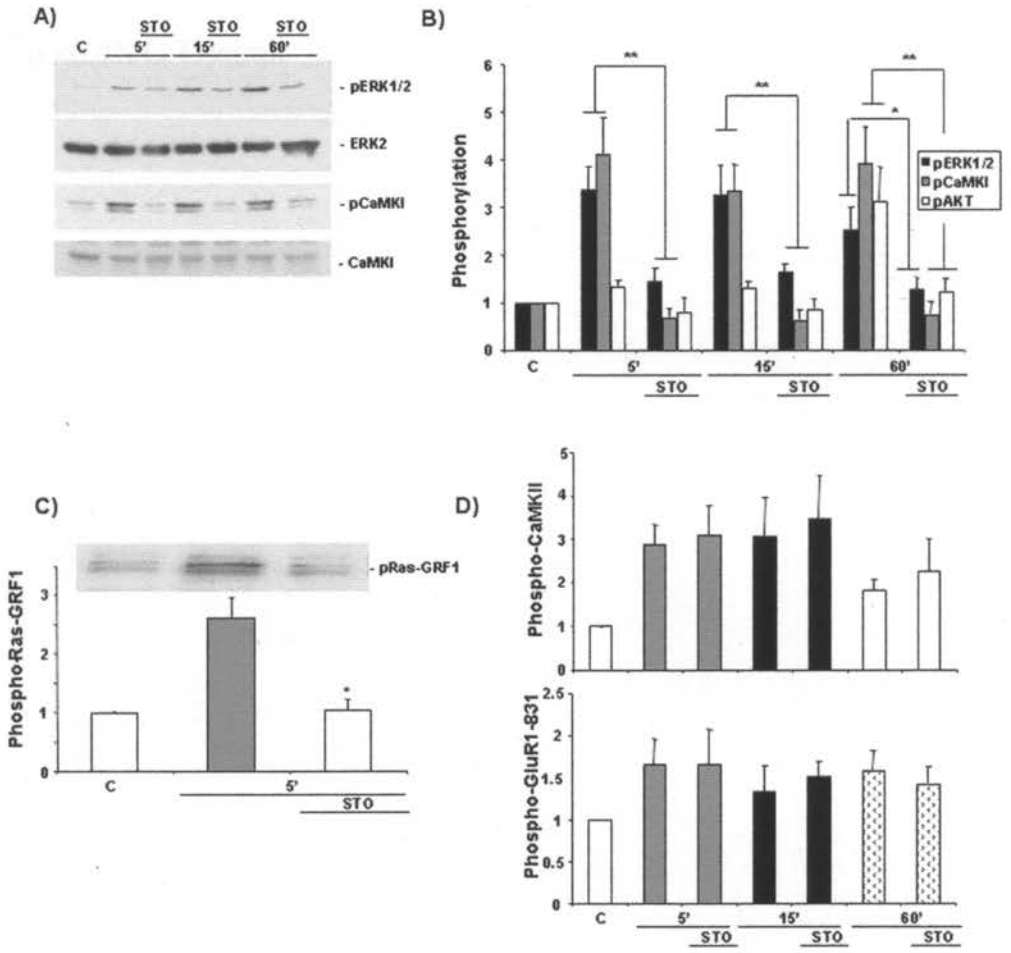


Fig. 2.3) LTP-activation of CaMKI, Ras-GRF1, and ERK requires CaMKK. Mouse hippocampal slices were pre-incubated without or with STO-609 (5 μM, 30 minutes) and subjected to *theta*-burst stimulation as in Figure 2.2D. The activation states, assessed by phospho-specific antibodies (see Figure 2.1), of ERK1/2, CaMKI and AKT (**A, B**), Ras-GRF1 (**C**) CaMKII and the GluR1 subunit of the AMPA-type glutamate receptor (a CaMKII substrate) (**D**) were determined at the indicated times. Mean ± S.E., n=6. $^{*}p$ <0.05; $^{**}p$ <0.01. C, Control.

Figure 2.4) CaMKK and ERK Mediate E-LTP *via* a Common Mechanism

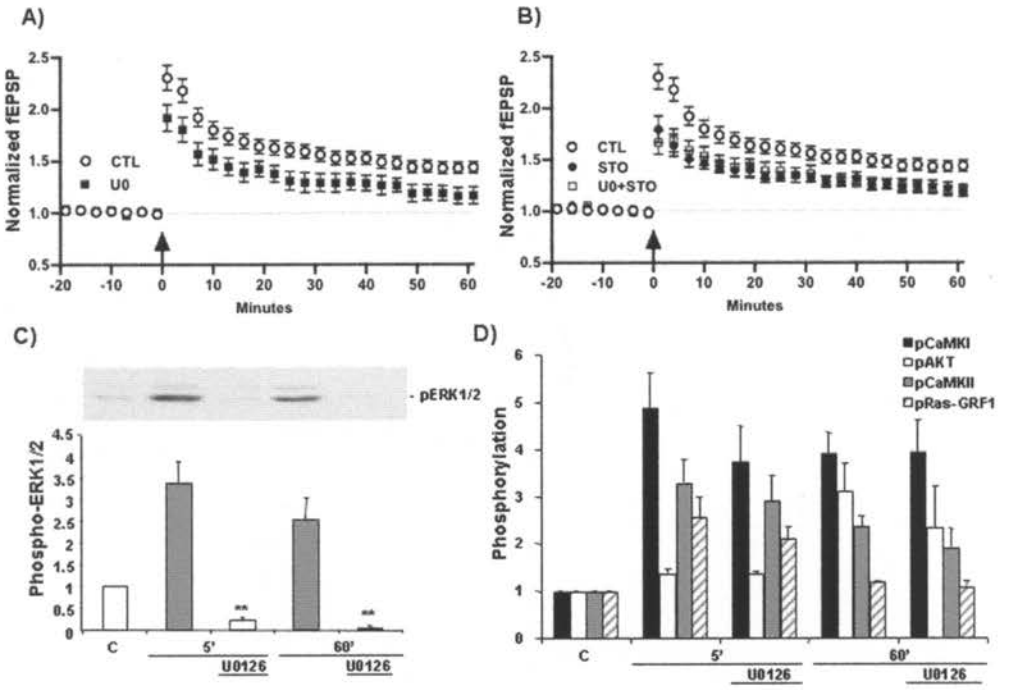


Fig. 2.4) CaMKK and ERK mediate E-LTP *via* a common mechanism.

A, B. Mouse hippocampal slices were treated without or with the MEK inhibitor U0126 (10 μM) and/or the CaMKK inhibitor STO-609 (5 μM) by bath application as in Figure 2.2D for 30 minutes prior to and 5 minutes after E-LTP induction. Control, n=24; U0126, n=10; STO-609, n=11; UO+STO, n=10. **C, D**. At various times after E-LTP induction (as in Figure 2.2D) the activation status of ERK (panel C), CaMKI, AKT, CaMKII and Ras-GRF1 (panel D) were determined at the indicated times. Mean ± S.E., n=5. $^{**}p$ <0.01.

Figure 2.5) Inhibitors of CaMKK and MEK Suppress LTP by a Translation-Dependent Pathway

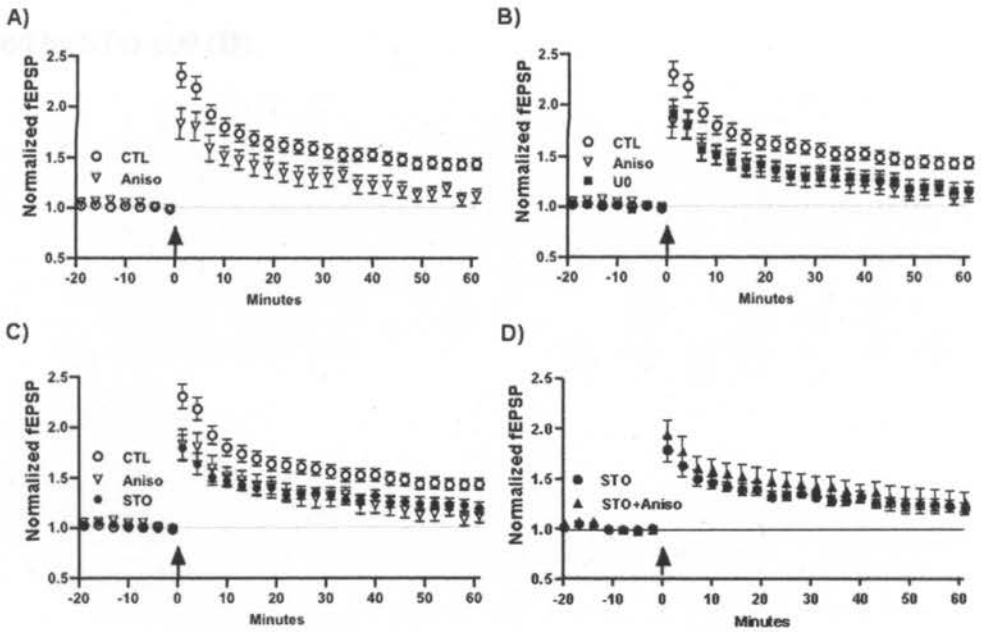


Fig. 2.5) Inhibitors of CaMKK and MEK suppress LTP by a translation-dependent pathway.

A. Mouse hippocampal slices were treated by bath application with the translation inhibitor anisomycin (40 μM) 30 minutes prior to and for the duration of recording after E-LTP (as in Figure 2.2D). n=8. Note that the magnitude and kinetics of anisomycin suppression of LTP are identical to those of U0126 (**B**) and STO-609 (**C**), and are occluded by STO-609 (**D**).

Figure 2.6) Translation Factor Activation in E-LTP Requires CaMKK and ERK

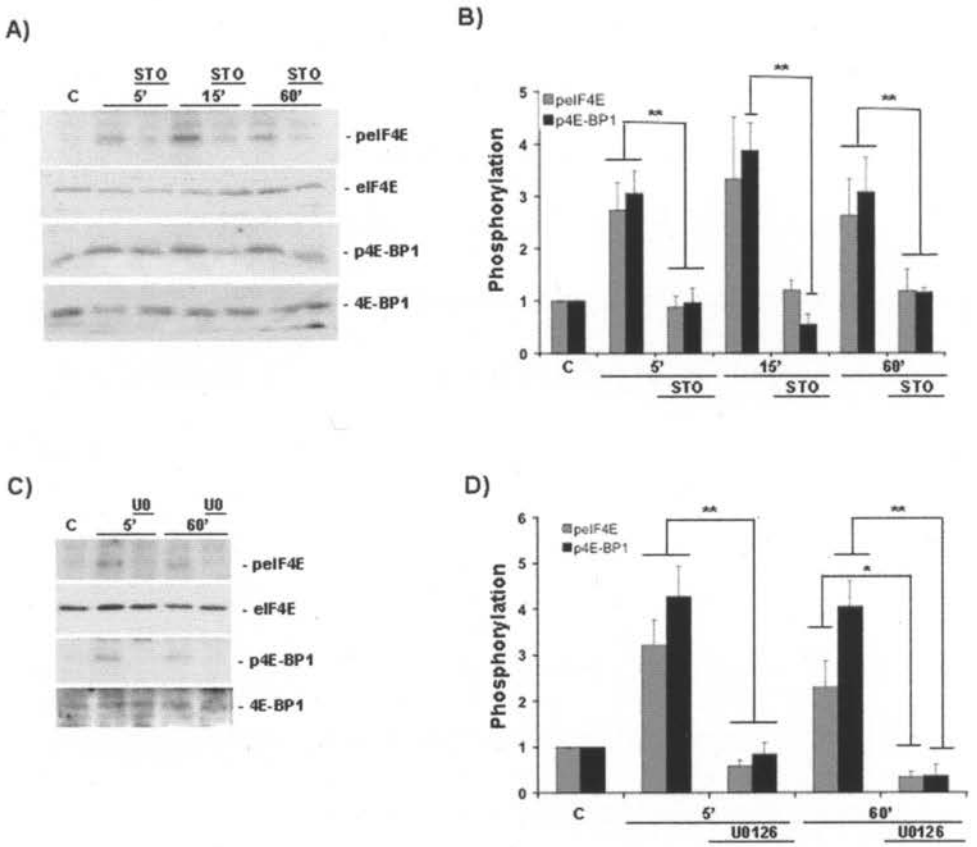


Fig. 2.6) Translation factor activation in E-LTP requires CaMKK and ERK. Mouse hippocampal slices were pre-incubated with the indicated pharmacological reagents (see Figure 2.1) and then subjected to E-LTP induction (as in Figure 2.2D). The activation state of translation factors eIF4E and its inhibitory binding protein 4E-BP1 were determined using phospho-specific antibodies against their respective activation sites. Mean $\pm$ S.E., n=6 (B) or 5 (D). $^{*}p < 0.05$; $^{**}p < 0.01$.

2.5 Discussion

Ca^{2+} signaling and protein phosphorylation play essential roles in regulating the multiple mechanisms that produce hippocampal CA1 LTP, and recent studies have focused on the roles of CaMKII [41, 141], CaMKIV [63, 146], and ERK [31, 97, 152]. Although ERK activation upon LTP induction is Ca^{2+}-dependent, the mechanisms responsible for this are unclear [78]. Utilizing cultured hippocampal neurons and acute slices, we reveal a robust Ca^{2+}-mediated activation of CaMKI by CaMKK that is required for both the activation of ERK and the full expression of NMDA-receptor dependent LTP. Additionally, our study suggests that CaMKK mediates mRNA translation, as measured by regulatory phosphorylation of several translation initiation factors, *via* ERK during LTP. Taken together, our data demonstrate that CaMKK and CaMKI are required for ERK activation, regulatory phosphorylation of two limiting general translation factors and E-LTP at the Schaeffer-collateral CA1 synapse.

A role for CaMKI in E-LTP. Previous studies have examined the role of the CaMKK cascade in LTP, focusing on the role of CaMKIV in CREB/CBP-mediated gene transcription. LTP induction does result in activation of CaMKIV [63], but since CaMKIV is predominantly nuclear it is unlikely to play a major role in cytoplasmic ERK activation. Indeed, nuclear-localized dominant-negative CaMKIV has no effect on ERK activation in NG108 cells [72] or cultured hippocampal neurons (**Fig. 2.1B**). Furthermore, Ca^{2+}-dependent ERK activation in dnCaMKIV-expressing [146] and CaMKIV knockout mice [156] was normal. Moreover, expression of dnCaMKIV in region CA1 of hippocampus does not inhibit E-LTP induced with *theta*-burst [146], nor

does knocking out the CaMKIV gene inhibit E-LTP induced by HFS [156]. These findings support the conclusion that CaMKI, but not CaMKIV or AKT, mediates the CaMKK-dependent portion of LTP induction and E-LTP.

Although LTP induced with *theta*-bursts, was sensitive to U0126 or STO-609, it was not completely inhibited during the first hr. (~50% inhibition) (**Fig. 2.2E,F**). This observation is consistent with the well-established role in LTP of CaMKII to phosphorylate the AMPA receptor subunit GluR1, thereby enhancing its conductance [158]. This mechanism is thought to mediate about 50% of the change in synaptic efficacy that occurs during E-LTP [27, 159]. We found that neither the activation of CaMKII nor the phosphorylation of GluR1 at Ser^{831} were affected by STO-609 during LTP, demonstrating both the specificity of STO-609 and that CaMKK and ERK effects were independent of CaMKII actions. We interpret these findings as evidence that the CaMKK/ERK and CaMKII pathways mediate E-LTP by distinct mechanisms. However, STO-609 did completely suppress L-LTP after about 2 hrs, consistent with a dual role for CaMKK in regulation of CaMKI (E-LTP) and CaMKIV (L-LTP). This is consistent with the report that a mouse lacking the β isoform of CaMKK, which is thought to be predominantly nuclear and to regulate CaMKIV, showed no deficit in E-LTP but lacked L-LTP [68].

CaMKI has previously been shown to promote hippocampal axon outgrowth and growth cone dynamics [71], structural components of plasticity. In this study we find that CaMKI is robustly activated by CaMKK following induction of E-LTP with *theta*-bursts.

This stimulation of the CaMKK/CaMKI pathway was required for ERK activation, providing a compelling mechanism for Ca^{2+}-mediated ERK activation during LTP. Accordingly, the effects of pharmacological inhibition of CaMKK and ERK (with STO-609 and U0126, respectively) on E-LTP are mutually occlusive, indicating that these kinases share the same pathway. We chose *theta*-burst rather than HFS to induce E-LTP as it has been shown in mice that U0126 does not block E-LTP resulting from HFS (2 trains of 100 Hz) but is partially inhibited using *theta*-burst induction [31]. However, we should point out that we used lower stimulation intensity, as well as older animals, than used by Selcher et al. to demonstrate a role for ERK in temporal integration during LTP induction. These differences may help explain why inhibition of the ERK pathway did not significantly reduce the integrated field responses during E-LTP induction in our study. It is interesting that induction of LTP by both of our *theta*-burst protocols was partially suppressed by STO-609 (**Fig. 2.2F**) and induction of L-LTP by HFS (4 trains of 100 Hz, 5 minutes apart) was partially suppressed by anisomycin [152], even during the E-LTP phase (first 60 minutes). These results suggest that the CaMKK, ERK, and protein synthesis-dependent processes can affect LTP within minutes depending on the induction protocol.

In addition to CaMKII-mediated phosphorylation of AMPARs to enhance conductivity [158], recruitment of additional AMPARs to synapses is thought to be a second major postsynaptic mechanism of E-LTP expression at the CA1 synapse [160]. One possible explanation for the rapid deficit of LTP following STO-609, U0126 or anisomycin treatment could be that synaptic delivery of AMPA receptors may be coupled to

translation. Indeed, it has been shown that the Ras/ERK pathway is required for this rapid trafficking of AMPARs [101]. AMPA-R trafficking is also dependent on CaMK activity, with current evidence supporting a role for CaMKII [159]. However, since recent work indicates that CaMKII probably has either an inhibitory effect on ERK [161] or no effect [93], we propose that the requirement for a CaMK in AMPA-R trafficking may be due to CaMKK/CaMKI activation of ERK.

ERK Regulation in Synaptic Plasticity. A role for ERK1/2 in NMDA-receptor-dependent hippocampal LTP is well established [78, 140], and a number of mechanisms for how Ca^{2+} may activate ERK have been proposed including roles for Pyk2 and Src, CalDAG-GEFs, EGF receptor transactivation, and Ras-GRF1/2. The data presented here demonstrate that NMDA receptor-dependent ERK activation during E-LTP requires CaMKK acting through CaMKI. Other components of NMDA receptor-dependent ERK activation include Ras [78] and its activator Ras-GRF1 that directly interacts with the NMDA-receptor and is required for ERK activation [84, 85]. Ras-GRF is regulated through phosphorylation of Ser^{916} by several kinases including PKA and CaMKII, and this phosphorylation is required for full activation of Ras and for neurite outgrowth [86-88]. We find that Ser^{916} is rapidly phosphorylated in response to NMDA treatment and *theta*-burst stimulation in a CaMKK-dependent manner. It should be noted that Ras-GRF1 contains multiple phosphorylation sites [86] and activation appears complex and may be regulated by the binding of co-factor(s) such as CaM and/or by its subcellular localization [162, 163]. In this context, Ras-GRF1 is a very poor *in vitro* substrate for

activated CaMKI relative to PKA (unpublished observation). The precise mechanism for CaMKK-mediated activation of the Ras pathway remains an area of investigative interest.

Regulation of translational activation by CaMKK and ERK. ERK activation has been implicated in regulating multiple aspects of LTP including gene transcription [78], AMPA-R trafficking [101], dendritic excitability [97] and translation factor phosphorylation [152]. The data presented here is consistent with a role of ERK in regulation of mRNA translation *via* phosphorylation of both eIF4E and 4E-BP1 (**Fig. 2.6**). Phosphorylation of 4E-BP1 frees up additional eIF4E, a limiting translation factor, and phosphorylation of eIF4E enhances its functionality [164]. Moreover, the partial inhibition of E-LTP by the protein synthesis inhibitor anisomycin was identical to inhibition by U0126 and STO-609, and occluded by STO-609, implying that CaMKK and ERK regulate E-LTP by a translation-dependent mechanism.

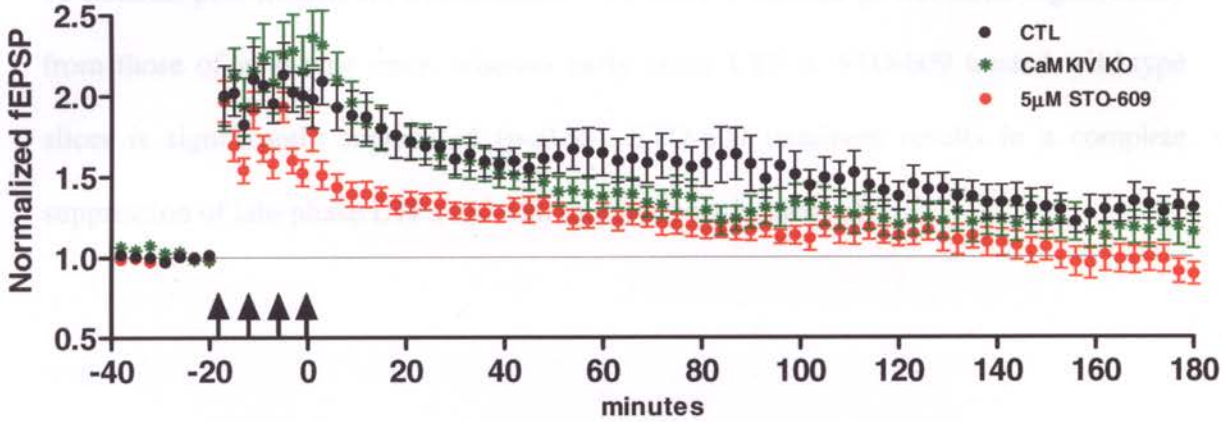
There are several reports documenting inhibition of NMDA receptor-dependent LTP by anisomycin and other translation inhibitors during the early phase in hippocampus and amygdala [37, 152]. The partial inhibition of E-LTP by anisomycin in our acute *theta*-burst protocol was very rapid (**Fig. 2.5**), but it should be noted that translation of a typical 50 kDa protein, at a rate of 7 amino acids per second, would only require about 1 minute. Indeed, a significant increase in dendritic CaMKII, due to localized synthesis, was observed within 5 min of LTP induction [165]. Additionally, anisomycin was present for 30 minutes prior to LTP induction, and, although it had little effect on basal synaptic transmission, it could suppress basal synthesis of some component necessary for the rapid

insertion or modulation of AMPARs upon induction [160]. Alternatively, it is possible that translation and post-synaptic exocytosis are coupled through the secretory pathway, and that inhibiting one process may affect the other. Furthermore, it is now recognized that protein synthesis from mRNAs localized within dendrites and even dendritic spines is essential for synaptic plasticity, so the possibility that protein synthesis may have acute regulatory effects has to be considered. CaMKK, CaMKI [71], ERK [166], and eIF4E [167, 168] are all localized in dendrites as is the mRNA for the GluR1 subunit of the AMPA-R [169]. Thus, it will be important to determine whether the CaMKK/CaMKI pathway regulates a pool of ERK that in turn stimulates AMPAR synthesis and/or trafficking to synapses.

2.6 Acknowledgements

We would like to thank members of the Soderling lab. (Drs. Monika Davare, Victor Derkach and Gary Wayman and Wilmon Grant) for helpful discussions and technical assistance, and Drs. Philip Stork and Soren Impey for critiquing the manuscript. This work was supported by NIH R01 grants NS27037 (T.R.S.) and GM41292 (T.R.S.), N.L. Tartar fellowship (E.G.), training grant DK007680 (J.M.S.), and Human Frontier Science Program fellowship LT00193 (T.S.).

Supplemental Figure 2.1) CaMKK Plays an Important Role in CaMKIV-Independent E-LTP



Supplemental Fig. 2.1) CaMKK plays an important role in CaMKIV-independent E-LTP. Electrophysiological analysis of hippocampal L-LTP in area CA1 of wild-type (Ctl) +- treatment with the CaMKK inhibitor STO-609, and CaMKIV knockout (Ko) mice. **A**) Graph comparing L-LTP induced by 4 epochs of *theta*-burst stimulation, spaced 5 minutes apart (arrows). Data represents mean +/- SEM. In early-phase LTP (30-60 minutes post-induction), the responses of CaMKIVko mice do not differ significantly from those of wild-type mice, whereas early-phase LTP in STO-609 treated wild-type slices is significantly suppressed ($p<0.05$). STO-609 treatment results in a complete suppression of late-phase LTP (150-180 minutes post-induction).

Chapter 3

Regulation of Spine Morphology, Bidirectional Synaptic Plasticity, and Metaplasticity by WAVE-1

My contribution to the findings presented in this chapter included most of the design, acquisition, analysis, and interpretation of data, except for those findings presented in Figures 3.1 and 3.2. This project was initiated by Dr. Scott Soderling, who initially approached me about looking at LTP in the WAVE-1 knockout mouse. Scott has done a tremendous amount of work (including the generation of both mouse models) on this project that will not be presented here for the sake of brevity. Scott, as well as Drs. Victor Derkach and Thomas Soderling were consulted during the design of the electrophysiology and AMPAR trafficking experiments. Scott, Victor, and Tom, and Dr. John Scott, contributed meaningfully to the interpretation of this data. In particular, discussions with Victor greatly facilitated my efforts to build a simple and coherent model which conveys my interpretation of the depotentiation and dedepression findings in the WAVE-1 knockout mouse. Finally, I would like to thank Daniel Silverman for assistance with the data analysis. The findings that I present in this chapter are currently in preparation as two separate manuscripts, as follows:

1) Regulation of Spine Density, Bidirectional Synaptic Plasticity, and Memory by WAVE-1 (working title). Scott H. Soderling, Eric S. Guire, Stefanie Kaech, & John D. Scott

2) WAVE-1 Regulates Synaptic Metaplasticity: Implications for Cellular Models of Learning and Memory (working title). Eric S. Guire, Scott H. Soderling, Victor A. Derkach, John D. Scott and Thomas R. Soderling.

3.1 Abstract

The WAVE family of scaffolding proteins (WAVE-1,2,3) coordinate signaling between the small GTPase Rac and the actin cytoskeleton. Mutations in genes encoding effectors of the Rho-family of GTPases have been linked to mental retardation [104], a family of disorders associated with alterations in dendritic spine morphology and defects in synaptic plasticity. Mice lacking WAVE-1 exhibit impairments in hippocampal-dependent learning and memory tasks [134], but the cellular and electrophysiological defects underlying these impairments are not known. One attractive hypothesis is that WAVE-1 functions in the regulation of excitatory spine density (*i.e.* network connectivity), spine morphology, and use-dependent synaptic plasticity. Accordingly, alterations in WAVE-1 signaling may adversely impact hippocampal Long-Term Potentiation (LTP) and Long-Term Depression (LTD), two complimentary cellular models of learning and memory. Here we report that the WAVE-1 knockout mouse has altered bidirectional synaptic plasticity in area CA1 of adult hippocampal slices, a decrease in the synaptic input-output curve, and a basal redistribution of AMPARs towards the plasma membrane, likely at extrasynaptic sites. This is accompanied by a reduction in density and a decrease in the head-to-neck ratio of hippocampal spines. Genetic deletion of the WRP binding domains of WAVE-1 also results in alterations to LTP, the synaptic input-output curve, and spine morphology, suggesting that the interaction of WRP with WAVE is important for the proper regulation of actin dynamics in spines by the Rac pathway. Furthermore, slices from WAVE-1 knockout mice exhibit pronounced alterations in metaplasticity. In naïve slices, the basal synaptic strength set point is depressed such that LTP is bigger and LTD is smaller in WAVE-1 knockout

mice relative to wild-type mice. However, following the expression of naïve LTP and LTD, the dynamic range of synaptic strengths occurring during Depotentiation and Dedepression becomes dramatically reduced in knockout slices, whereas the dynamic range remains stable or increases in wild-type slices. This use-dependant decrease in the capacity for synaptic plasticity in area CA1 of WAVE-1 knockout mice likely underlies the previously observed spatial learning deficits, and may in general be a better predictor of the capacity for learning and memory than measurements of LTP and LTD alone.

3.2 Introduction

Dynamic changes in the actin cytoskeleton play a role in neuronal development and experience-dependent synaptic plasticity [104, 170]. Members of the Rho family of small GTPases have emerged as key regulators of the actin cytoskeleton. Through their interaction with scaffolding proteins and multiple effectors, they coordinate diverse neuronal processes such as protein synthesis and morphological plasticity [105]. Recent work has also demonstrated a link between actin dynamics and the expression of activity-dependent forms of synaptic plasticity, in particular LTP and LTD: agents which perturb the actin cytoskeleton interfere with LTP and LTD [122], and the electrical or chemical stimuli used to generate these changes in synaptic strength alter the structure of actin in spines and dendrites [102, 120, 121]. Interestingly, dynamic changes in the actin cytoskeleton not only mediate the morphological plasticity of spines [102, 120-122, 171, 172] but also influence the trafficking of postsynaptic neurotransmitter receptors [119, 173, 174], providing one potential mechanism whereby the cytoskeleton regulates LTP and LTD and hence learning and memory. Consistent with these observations, cytoskeletal abnormalities, as well as genes encoding effectors of Rho-family GTPases such as αPIX, PAK3, LIMK, and oligophrenein 1, have been linked to mental retardation [175-177].

Downstream targets for Rac, a Rho-family GTPase, include the WAVE family of Wiskott-Aldrich-syndrome (WASP) scaffolding proteins. WAVE has 3 isoforms (WAVE-1, WAVE-2 and WAVE-3), which assemble multi-protein complexes that appear to function as cytoskeletal signal integrators. Importantly, WAVE is thought to

relay Rac signaling to the Arp 2/3 complex, a group of seven proteins that catalyze actin nucleation and branching. The WAVE-1 isoform is selectively expressed in neurons [134], and removal of this core component is likely to impede assembly of signaling complexes that regulate neuronal actin dynamics. In addition to the Arp 2/3 complex, WAVE-1 binding partners include Abi 1/2 (a Rac activator), Abl, actin, HSPC300, PKA and a recently identified Rac-selective GAP called WRP [125]. The interaction of WAVE-1 with WRP *via* its SH3 domain may serve to terminate Rac signaling following activation of the WAVE-1 complex [128]. This interaction may be important for WAVE-1's role in cognitive function, as WRP has been identified as a gene that is disrupted in a human form of mental retardation known as 3p-syndrome [135].

WAVE-1-dependent neuronal defects may be particularly acute at excitatory synapses, as the vast majority of excitatory synapses located in actin-rich dendritic spines. Accordingly, WAVE-1 knockout mice exhibit pronounced deficits in sensorimotor function and perform poorly in behavioral tests of hippocampal-dependent learning and memory [134], likely due to functional defects in hippocampal network plasticity and neuronal connectivity. Therefore, we have investigated the morphological and electrophysiological properties of excitatory hippocampal synapses in a WAVE-1 knockout mouse, as well as a newly generated model for testing WAVE-1 function, the delta-WRP knock-in mouse (in which WRP binding to WAVE-1 is partially disrupted). Importantly, we find that delta-WRP mice harbor selective deficits to hippocampal-dependent memory, in the absence of potentially confounding alterations to sensorimotor function, strongly implicating WAVE-1 as an essential component of normal

hippocampal function. Through our investigation we reveal that WAVE-1 is an important molecule for regulating hippocampal spine morphology, AMPAR trafficking, and bidirectional excitatory synaptic plasticity. Furthermore, we show that WAVE-1 also plays a pronounced role in the regulation of metaplasticity. Metaplasticity, or the plasticity of synaptic plasticity, is thought to play an important role in maintaining synaptic strengths within a dynamic range that is optimal for learning and memory [7], and facilitate integration of synaptic events over relatively long time periods [178]. Our finding that WAVE-1 regulates synaptic metaplasticity may have significant implications for studies of the relationship between bidirectional synaptic plasticity and learning and memory.

3.3 Methods

Cultured hippocampal neurons and quantitative image analysis.

Primary neuronal cultures were prepared for imaging from hippocampi of E18 rats, as previously described [179]. Dissociated neurons were plated onto poly-L-lysine-treated glass coverslips at a density of 25-50 cells/mm^2 and co-cultured over a monolayer of astrocytes. Cells were maintained in Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with B27 and Glutamax. Plasmid DNA was introduced by electroporation using the AMAXA nucleofection protocol (Amaxa, Gaithersburg, MD). Transfected neurons were plated on coverslips previously plated with 15 cells/mm^2 and imaged on DIV21. Digital images were collected using high-numerical aperture objectives either on a MicroMax 1300YHS CCD camera (Roper Scientific, Tucson, AZ) mounted on a Leica DM-RXA microscope or through a Yokogawa CSU-10 Nipkow Spinning Disk confocal head (Solamere Technologies, UT) on a Cascade 512B CCD camera (Roper Scientific, Tucson, AZ) mounted on a Nikon TE-2000 microscope. Representative images are presented as maximum projections of z-series. Quantitative analysis from 3 independent experiments was performed using Image J software (Research Services Branch, NIH). Spine heads were defined as terminal enlargements that were at least twice the width of the spine shaft. Data obtained were compared by a Students *t* test analysis.

Histological and Morphological analysis.

Brains from three wild-type and three age matched WAVE knockout adult mice were impregnated according to the Golgi procedure *(Ramon-Moliner, E. (1970). The Golgi-Cox technique (New York: Springer)* using Rapid GolgiStain reagents (FD

NeuroTechnologies, Ellicott City, MD), cut at 150 μM with a cryostat and mounted in Permount (Fisher Scientific, Pittsburgh, PA). Brightfield z series images from area CA1 were acquired using a CoolSnap HQ CCD camera (Roper Scientific, Tuscan, AZ) mounted on a Leica AS MDW (Fig. 3.1H-K). Representative images displayed are minimum projections from z series stacks. Spine density was obtained in different lengths of dendrites and maximum spine diameter was calculated from each dendrite imaged. Data obtained were compared by a Students *t* test analysis.

Post-synaptic density (PSD) fractionation and immunoblot detection.

PSD fractions were prepared from frozen rat brains as previously described [180]. Briefly, PSD fractions were separated by SDS-PAGE on a 12% gel, electrotransferred to nitrocellulose membranes (Schleicher & Schuell, Florham, NJ). Antibodies used were WAVE-1 (VO101), Arp3 (Upstate, Charlottesville, VA), ArpC2 (Upstate, Charlottesville, VA), PSD-95 (Upstate, Charlottesville, VA), and SNAP-25 (Clontech/Transduction labs, Palo Alto, CA).

Plasmids.

For all experiments a vector using the chicken β-actin promoter (pBetaActin) was used to drive expression in neurons [181]. Plasmids encoding WAVE-1 and fragments were generated by PCR amplification of DNA and ligation of the restriction digested insert at sites downstream of EGFP (Clontech, Palo Alto, CA). The WAVE-1 444-559($R_{512}K_{513}$/EE) actin binding mutant [126] was produced in an expression vector driven by the pBetaActin promotor. The coding region of γ-actin was ligated into the

pBetaActin vector downstream of EYFP (Clontech, Palo Alto, CA) and used as a marker for dendritic spines. All constructs were verified by sequencing.

Hippocampal slice production.

Adult male mice (2-6 months old) were anesthetized with pentobarbital (60 mg/kg, i.p.) and decapitated using procedures reviewed and approved by Institutional Animal Care and Use Committee at the Oregon Health and Sciences University Department of Comparative Medicine. Brains were removed within 1 min of decapitation and immediately submerged in ice-cold, sucrose-modified artificial CSF (ACSF) for hippocampal dissection and slicing (in mM): 110 sucrose, 60 NaCl, 2.5 KCl, 28 $NaHCO_3$, 1.25 NaH_3PO_4, 0.5 $CaCl_2$, 7 $MgCl_2$, 5 glucose, and 0.6 sodium ascorbate, pH 7.4 at 4°C (presaturated by bubbling with 95% O_2/5% CO_2 at 21-22°C). Hippocampal slices (400 μm, transverse) were prepared using a vibratome and an agar backing (2%), and each slice was transferred to warm ACSF [in mM: 125 NaCl, 2.5 KCl, 22.6 $NaHCO_3$, 1.25 NaH_3PO_4, 2 $CaCl_2$, 1 $MgCl_2$, and 11.1 glucose (continuously gas saturated with 95% O_2/5% CO_2, pH 7.4 at 32°C)] for recovery (30 min, 37°C). Slices from the dorsal and ventral thirds of the hippocampus were discarded. After recovery, the holding chamber was equilibrated at 21-22°C, and slices were held for 2-8 h before recording or 3-4 h before biotinylation.

Slice biotinylation and immunoblotting

For biotinylation, unstimulated hippocampal slices (from animals 8-12 weeks old) were placed in ice-cold HEPES buffered saline, pH 7.4, with 1 mg/mL Sulfo-NHS-SS-biotin

(Pierce Biotechnology, Rockford, IL) for 30 minutes with gentle agitation to selectively label surface proteins. Slices were then rinsed 4 times for 10 minutes each in ice cold TBS, pH 7.4, to quench the biotinylation reaction. Following quenching, slices were rapidly frozen on a filter paper-covered liquid nitrogen-cooled aluminum block and pooled in microcentrifuge tubes in pairs, and stored at -80°C. Frozen hippocampal slices were lysed in ice-cold lysis buffer (TBS supplemented with 1% SDS, 10mM EDTA, 1mM Orthovanadate, 50mM NaF, 25mM NaPPi, protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL) and phosphatase inhibitor cocktail (Calbiochem, San Diego, CA), pH 7.4) and homogenized. Following homogenization, lysates were diluted 1:10 with RIPA buffer (Triton X-100 substituted for SDS in the above recipe) to dilute SDS prior to incubation with avidin beads. A sample was removed for analysis of total proteins, and the remaining lysates were then centrifuged (15 minutes at 20,000g, 4 deg. C). Following centrifugation, the supernatant was incubated with ImmunoPure Immobilized Avidin (Pierce Biotechnology, Rockford, IL) at 4°C overnight. Supernatant from the avidin precipitation was removed (intracellular protein fraction), and the avidin beads (surface protein fraction) were washed 4 times in 10:1 RIPA:lysis buffer, before resuspension in an equal volume of the same. Laemmeli's buffer (6x) was then added to all samples, and tubes were heated for 10 min at 95°C. Lysates were resolved by SDS-PAGE, transferred to Immobilon-FL membranes (Millipore), and examined by immunoblotting. Primary antibodies: GluR2 1:500 (Zymed/ Invitrogen, Carlsbad, CA), tubulin 1:5000 (E7). Secondary antibody: mouse IgG IR800 conjugated 1:2000 (Rockland Immunochemicals, Gilbertsville, PA). Immunoreactivity was imaged and quantified using the Odyssey Li-Cor system (Li-Cor, Lincoln, Nebraska). Total receptor

levels were calculated from combined measurements of intracellular and surface levels and all data collected were compared by a Students *t*-test analysis.

Electrophysiology.

For electrophysiological recordings, submerged hippocampal slices were suspended in pairs on a nylon mesh in a small (350-400 µl) recording chamber and perfused with ACSF (see above) at a rate of 2.5-3 ml/min. The temperature in the recording chamber was raised to 30-32°C over the course of a few minutes and held for the duration of the experiment. Synaptic responses were evoked by Schaffer collateral stimulation using a bipolar tungsten electrode (tip spacing, 140 µm; Frederick Haer Company, Bowdoinham, ME) and a 100 µs square wave test pulse (typically 30-40 µA) delivered at 60 s intervals. The stimulation intensity was adjusted to produce a basal response of approximately1.2 mV for E-LTP/LTD, and approximately 1.5 mV for L-LTP/LTD experiments. Recordings were made using ACSF-filled borosilicate micropipettes (2-4 MΩ) placed in the stratum radiatum area of CA1 and connected *via* head stages to an A-M Systems (Carlsborg, WA) model 1800 amplifier. Signals were digitized at 100 kHz using Axon Instruments' Digidata 1200 series interface running Clampex 8.0, and the initial slope of the fEPSPs were analyzed with Clampfit 8.0 software. LTP was induced using *theta*-bursts: four pulses per burst (100 Hz), five bursts per train (5 Hz), three trains (at 20 second intervals) per epoch. For E-LTP experiments one epoch was used, for L-LTP experiments 4 epochs were used (at 5 minute intervals). E-LTD was induced with low frequency paired-pulse stimulation [900 paired-pulses, 50 msec isi (20 Hz), delivered at 1 Hz]. L-LTD was induced with 900 triplet-bursts [50 msec isi (20 Hz), delivered at 1 Hz].

3 minute fEPSP averages are shown. The first data points after LTP/LTD induction were taken 20 seconds after the final stimuli. WAVE-1 knockout (animals 8-12 weeks old) and Δ-WRP knock-in (animals 3-6 months old) slices were balanced with wild-type littermate slices across experiments such that the average time *ex vivo* was equal and the average age of animals used to prepare slices were matched.

3.4 Results

Subcellular localization of WAVE-1 in hippocampal neurons. Immunocytochemistry revealed that WAVE-1 was prevalent in actin-rich spines of mature cultured hippocampal neurons and overlapped with PSD95, a postsynaptic marker (data not shown). ArpC2 and Arp 3, subunits of the Arp2/3 complex were also enriched within the dendritic spine (data not shown). Postsynaptic localization of WAVE-1, ArpC2 and Arp3 was corroborated biochemically, as each protein was present in postsynaptic density fractions isolated from rat brain (**Fig. 3.1A-E**). Imaging experiments confirmed that GFP-tagged WAVE-1 was concentrated within spines of hippocampal neurons (**Fig. 3.1F & G**). Expression of an amino-terminal fragment, WAVE 1-225-GFP, was targeted to dendritic spines suggesting that this region of the scaffolding protein contains determinants for subcellular localization (**Fig. 3.1H**).

WAVE-1 regulation of dendritic spine architecture. Since WAVE-1 appears to be concentrated at synapses, we investigated its importance for spine morphology by examining spine architecture in knockout mice. Hippocampal neurons from WAVE-1 knockout mice had altered spine morphology as revealed by Golgi impregnation (**Fig. 3.2A & B**). Quantitative image analysis revealed a 19% reduction in spine number (spines/μm: +/+ = 1.04 ± 0.05; -/- = 0.83 ± 0.04, $p<0.01$, **Fig. 3.2C**) and an 18% reduction in the maximum diameter of the spine heads when compared to wild-type (μm: +/+ = 1.25 ± 0.07; -/- = 1.03 ± 0.06, $p<0.01$, **Fig. 3.2C**). One explanation for these findings is that signaling through WAVE-1 is required for the maintenance of proper spine density and morphology. Previous studies have shown that WAVE-1 binds the

Arp2/3 complex, and that coordinated actin binding is required for stimulation of actin polymerization and branching [126, 182-184]. Thus WAVE-1 domains that bind Arp2/3, but not actin, act as functional inhibitors of Arp2/3-mediated actin reorganization [184]. To test this hypothesis a dominant interfering mutant (WAVE-1 444-559, $R_{512}K_{513}$/EE) that competes for recruitment of Arp2/3 was expressed in hippocampal neurons. This resulted in a 29% reduction in spine density (spines/μm: control = 0.4460 ± 0.03; WAVE-1 444-559EE = 0.3218 ± 0.03, $p<0.01$); the remaining spines adopted a more filopodial morphology (1.5 fold reduction in head/filopodial ratio, control = 2.52 ± 0.3; WAVE-1 444-559EE = 1.72 ± 0.2, $p<0.01$; **Fig. 3.2E-H**). Similar effects on spine density and morphology were obtained using the WAVE-1 ΔWRP (Δ320-332, Δ420-433) construct, both by transfection of neuronal cultures and by genetic knock-in (data not shown). Collectively, the data in Figures 3.1 and 3.2 demonstrate that WAVE-1 accumulates in dendritic spines where it controls their shape and density.

Regulation of basal synaptic properties and AMPAR distribution by WAVE-1 signaling. Electrophysiological analysis of hippocampal slices (area CA1) isolated from WAVE-1 knockout and Δ-WRP mice revealed basal alterations of synaptic properties. Loss of WAVE-1 resulted in a 29% reduction, and interference of WRP binding to WAVE-1 resulted in a 14% reduction, in the maximal evoked field excitatory postsynaptic potential (fEPSPs; $p<0.01$ using repeated measures ANOVA, **Fig. 3.3A & B**). For the Δ-WRP knock-in mouse, significant alterations to the input-output curve were limited to higher stimulation intensities (> 100 μA). Paired-pulse facilitation, however, was unaltered in both genotypes, suggesting that neurotransmitter release probability is not regulated by WAVE-1 (**Fig. 3.3C & D**). Furthermore, the observation that the synaptic input-output

curves in our two mouse models were shifted downwards, but not to the left or right, is evidence that WAVE-1 signaling likely does not regulate presynaptic excitability (**Fig. 3.3A & B**).

Interestingly, the absence of WAVE-1 was found to significantly alter the basal subcellular distribution of AMPARs. We covalently labeled surface proteins using a membrane-impermeable form of biotin, and then quantified the distribution of AMPARs between the surface and cytosolic fractions in adult hippocampal slices by Western blotting. Equal protein loading in the samples, and the specificity of the biotinylation reagent towards surface proteins, was confirmed in Western blots for tubulin, which was protected from surface biotinylation by its cytoplasmic localization (**Fig. 3.4A**). WAVE-1 knockout mice (-/-) exhibited 42% higher basal levels of surface GluR2 subunit compared to wild-type controls (+/+) (**Fig. 3.4B**, surface/total ratio: +/+ = 0.4 ± 0.03; -/- = 0.57 ± 0.05, $p<0.01$). Similar results, although less robust, were obtained using an anti-GluR1 subunit antibody (data not shown). GluR2 was chosen as the preferred subunit for quantitation because most AMPARs in hippocampal neurons contain the GluR2 subunit, and its expression is largely restricted to the neuronal population [185]. The finding that basal subcellular AMPAR distribution is altered in the WAVE-1 knockout mouse reveals a previously unknown relationship between WAVE-1 regulation of the actin cytoskeleton, dendritic spine morphology, and the basal trafficking of AMPARs.

The role of WAVE-1 signaling in bidirectional synaptic plasticity. Recent evidence suggests that actin remodeling in dendritic spines correlates with changes in synaptic

transmission [102, 121, 122]. Given that WAVE-1 functions as a signal integrator for regulation of the actin cytoskeleton, and that the WAVE-1 knockout mouse was shown previously to harbor deficits in hippocampal-dependent learning and memory tasks [134], we investigated activity-dependent synaptic plasticity in area CA1 of hippocampal slices to better understand the physiological roles of WAVE-1. The magnitude of E-LTP in response to *theta*-burst stimulation (TBS) was increased approximately 2-fold in the WAVE-1 knockout mouse (**Fig. 3.5A**, $p<0.01$ using repeated measures ANOVA for responses from 0-60 min.). Additionally, the magnitude of E-LTD in response to paired-pulse low-frequency stimulation (PP-LFS, 1 Hz) was reduced in the knockout by approximately 50% (**Fig. 3.5B**, $p<0.05$ using repeated measures ANOVA for responses 0-60 min.). Finally, L-LTP using 4 epochs of theta burst trains spaced at 5 minute intervals revealed that the increased LTP magnitude in WAVE knockout mice persisted during strong, repetitive stimulation (data not shown). This finding suggests that the knockout mice have a higher capacity for LTP, as opposed to more efficient induction. The altered LTP and LTD recorded in area CA1 of the WAVE-1 knockout mice likely reflects the hippocampal-dependent behavioral abnormalities previously noted in these animals.

In contrast, the Δ-WRP knock-in mice showed an initial tendency towards increased post-tetanic potentiation (PTP) and short-term potentiation (STP), but E-LTP was not significantly altered 60 minutes following its induction (**Fig. 3.5C**). Additionally, the course of E-LTD was unaffected (**Fig. 3.5D**). The Δ-WRP mouse exhibits about a 50% reduction in the coimmunoprecipitation of WRP, a Rac-selective GAP [128], by WAVE-

1 (Scott Soderling, unpublished). This interference of WAVE-1-WRP interaction, although partial, is thought to lead to an impaired termination of Rac activity subsequent to its interaction with the WAVE-1 complex. This defect in WAVE-1 signaling produces similar alterations to spine density as in the WAVE-1 knockout mouse, but produces more subtle alterations to hippocampal-dependent learning and memory (Scott Soderling, unpublished results). In contrast to the WAVE-1 knockout mice, Δ-WRP knock-in mice display relatively normal acquisition in the Morris water maze, but subsequently performed poorly during delayed probe trials, findings suggestive of a selective deficit in long-term memory. We therefore evaluated late-phase LTP (L-LTP) and L-LTD [186] in the Δ-WRP mouse. L-LTP was significantly altered, approximately 2-fold higher than in wild-type mice (**Fig. 3.6A**, $p<0.01$). This finding held true for both *theta*-burst LTP (**Fig. 3.6A**) and 100 Hz HFS LTP (data not shown). Furthermore, slices from Δ-WRP mice displayed an increased capacity for further potentiation during repetitive stimulation with spaced epochs of theta-burst trains during L-LTP induction (**Fig. 3.6A, inset**), and the evolution of L-LTP expression was significantly different between genotypes (genotype x time period interaction using repeated measures ANOVA, $p < 0.05$). This effect likely explains why L-LTP was altered but E-LTP was not. As with E-LTD, no significant impairment existed in the knock-in slices during the course of L-LTD in response to our induction protocol (**Fig 3.6B**). We should also note, that since the Δ-WRP knock-in mice did not display significant alterations to sensory motor function, they represent stronger evidence for a specific role of WAVE-1 in hippocampal-dependent learning and memory tasks than do the WAVE-1 knockout mice. Collectively, these findings indicate an important role of WAVE-1 signaling in the regulation of hippocampal synaptic plasticity.

Abnormal Reversal of synaptic plasticity in the WAVE-1 knockout mouse: Depotentiation and Dedepression. The capacity for bidirectional synaptic plasticity is a hallmark of modern theories of neural network processing [3-5]. However, it is becoming increasingly clear that alterations to LTP and LTD resulting from genetic or pharmacological manipulations in mammals are not, on their own, accurate predictors of performance on learning and memory tasks (see **table 3.1**). We therefore examined the *reversibility* of LTP and LTD in a subset of experiments from the WAVE-1 knockout mice. The reversal of previously established LTP and LTD, known respectively as depotentiation and dedepression, are phenomena which more likely reflect the neural processes underlying learning and memory (see Chapter 4). Following the establishment of LTP, the resulting change in synaptic strength, as expected, was reversible. However, in contrast to LTD, (**Fig. 3.5B**), normalized reversal of LTP with low frequency paired-pulse stimulation in the knockout was comparable to wild-type slices (**Fig. 3.7A**), indicating that the mechanism leading to impaired synaptic depression had been relieved by prior LTP induction. This finding is consistent with a model in which synapses in slices taken from knockout mice are basally depressed (**Fig. 3.10**). Following the establishment of LTD, the resulting change in synaptic strength was also reversible with high frequency theta-burst stimulation, but less so in the knockout mouse (**Fig. 3.7B**). This is in striking contrast to the relatively large degree of synaptic potentiation expressed in naïve knockout slices in response to high frequency theta-burst stimulation during LTP (**Fig. 3.5A, Ko**). Thus, prior induction of LTD in knockout slices reverses the tendency for WAVE-1 lacking synapses to hyperpotentiate following theta-burst

stimulation. This finding suggests that the alterations to bidirectional plasticity in the knockout mouse likely do not result from an innate hyperexcitability of CA1 pyramidal neurons. In summary, our measurements of synaptic depotentiation and dedepression in the WAVE-1 knockout mouse reveal that plastic responses to patterned activity can depend strongly on prior synaptic events, in sharp contrast to depotentiation and dedepression in wild-type mice.

Effects of prior synaptic experience on the response to pattered stimuli in the WAVE-1 knockout mouse. Prior induction of LTP bestowed knockout slices with an increased capacity for synaptic depression in response to paired-pulse low frequency stimulation. Furthermore, prior induction of LTD blunted the response to theta-burst stimulation in knockout slices. In contrast, wild-type slices were relatively insensitive to the effects of prior synaptic plasticity, as has been reported previously [11]. To better visualize the effects of prior experience on subsequent plasticity, we first grouped, normalized and overlayed the traces from all theta-burst responses (**Fig. 3.8A**) and all paired-pulse low frequency responses (**Fig. 3.8B**). We next defined and calculated the "synaptic history quotient" to measure the effect of prior plasticity on the responses to patterned stimuli (**Insets**). Comparison of these values revealed a significantly heightened dependency of responses in the WAVE-1 knockout mouse to prior plastic experience, revealing a significant alteration of metaplasticity in these mice.

Loss of WAVE-1 results in a use-dependent reduction in the capacity for plasticity. The most extensively examined form of metaplasticity, termed B.C.M. or sliding modification

(**Fig. 1.3**), deals with activity-dependent changes in the thresholds for LTP and LTD *induction*. On theoretical grounds however, the fidelity and density of information storage in a neuronal network may be also be a function of the magnitudes of LTP and LTD. Thus, another form of metaplasticity that remains relatively unexplored, is use-dependent changes in the dynamic range of bidirectional synaptic plasticity – plasticity of the *capacity* for LTP and LTD. We term this form of metaplasticity *"capacitative metaplasticity"* to distinguish it from other forms of metaplasticity that relate to the thresholds for LTP and LTD induction (*i.e. "inductive metaplasticity"*). To quantify capacitative metaplasticity in the wild-type and WAVE-1 knockout mice, we summed the numerical averages of LTP and LTD responses 30 minutes following induction, and compared those to the corresponding sums of depotentiation and dedepression for each genotype (**Fig. 3.9**). The initial state of naïve knockout slices shows an overall increased dynamic range of plasticity and a striking bias towards LTP. In contrast, naïve wild-type slices have a basal set point closer to the middle of the dynamic range. Following use-dependent synaptic plasticity, wild-type slices retain or even increase their dynamic range while maintaining a balance in the strengths of potentiated and depressed states. However, knockout slices display a marked, use-dependant reduction in dynamic range, indicating an important role of WAVE-1 in the regulation of metaplasticity. This use-dependent decrease in the capacity for plasticity likely underlies the spatial learning impairments previously observed in the WAVE-1 knockout mouse [134].

Figure 3.1) WAVE-1 and Arp2/3 are Enriched in Hippocampal Spines

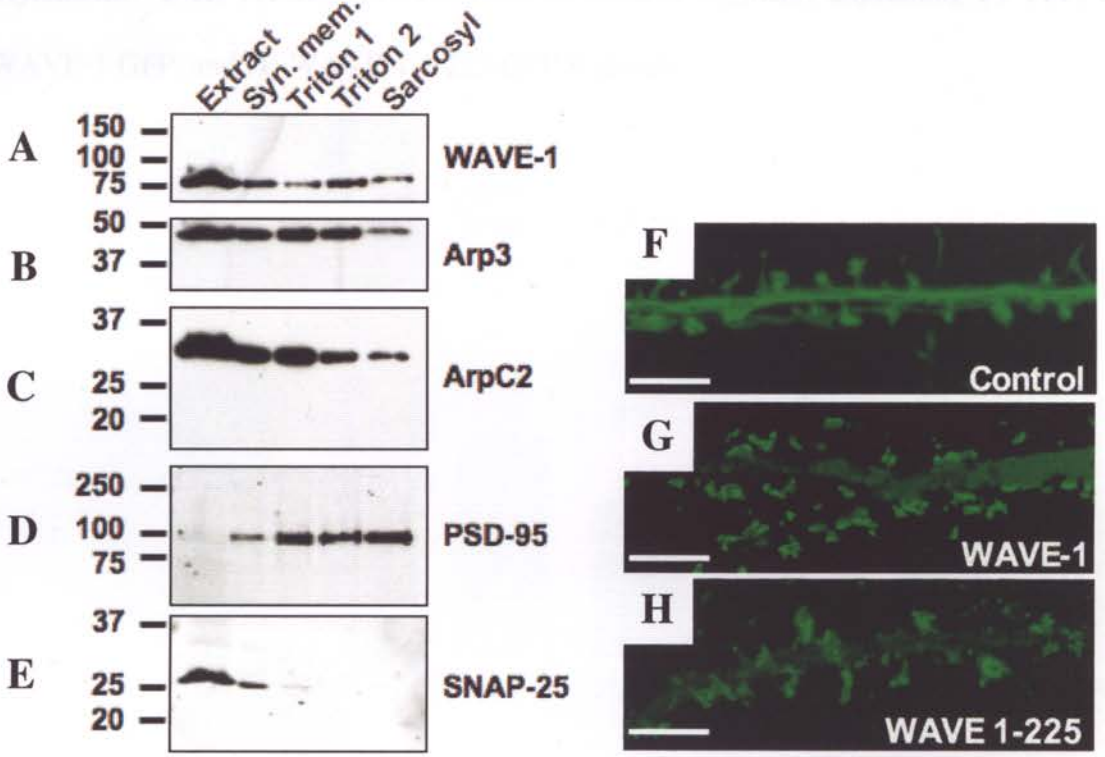


Fig. 3.1) WAVE-1 and ARP2/3 are enriched in hippocampal spines. A-E) Immunodetection of **A)** WAVE-1, **B)** Arp3, **C)** ArpC2, **D)** PSD-95, **E)** SNAP-25 in extracts and insoluble PSD fractions (10μg each). WAVE-1, the Arp2/3 complex subunits, Arp3 and ArpC2, and PSD-95 are present within the core PSD (Sarcosyl). The presynaptic protein, SNAP-25 is only present the synaptic membrane fraction (Syn.mem). **F-H)** Fluorescence detection of dendrite segments expressing **F)** YFP, **G)** WAVE-1 GFP, and **H)** WAVE-1 1-225 GFP fragment.

Figure 3.2) WAVE-1 Regulates Hippocampal Spine Density and Morphology

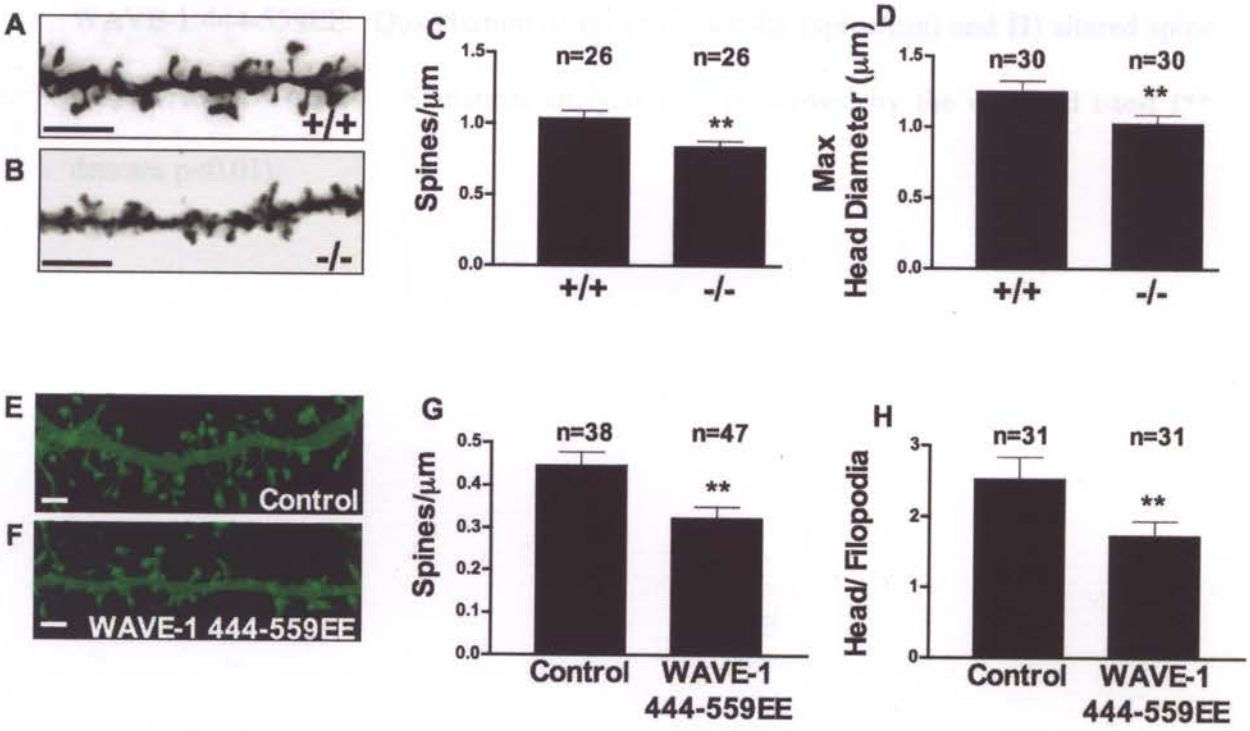


Fig. 3.2) WAVE-1 regulates hippocampal spine density and morphology. A-D) Golgi-impregnated dendritic segments of CA1 pyramidal neurons from **A)** wild-type (+/+), **B)** WAVE-1 knockout (-/-) mice, **C)** quantitation of spine density (spine/μm), and **D)** measurement of maximal spine head diameter (μm). **E-H)** Images of dendrite segments of hippocampal neurons in culture expressing **E)** YFP-actin, **F)** YFP-actin, and WAVE-1 444-559EE. Quantitation of **G)** spine density (spine/μm) and **H)** altered spine shape (Head/filopodia). Statistical analysis was performed by the unpaired t-test (** denotes $p<0.01$).

Figure 3.3) WAVE-1 Signaling Influences Synaptic Field Strength But Not Paired-Pulse Facilitation

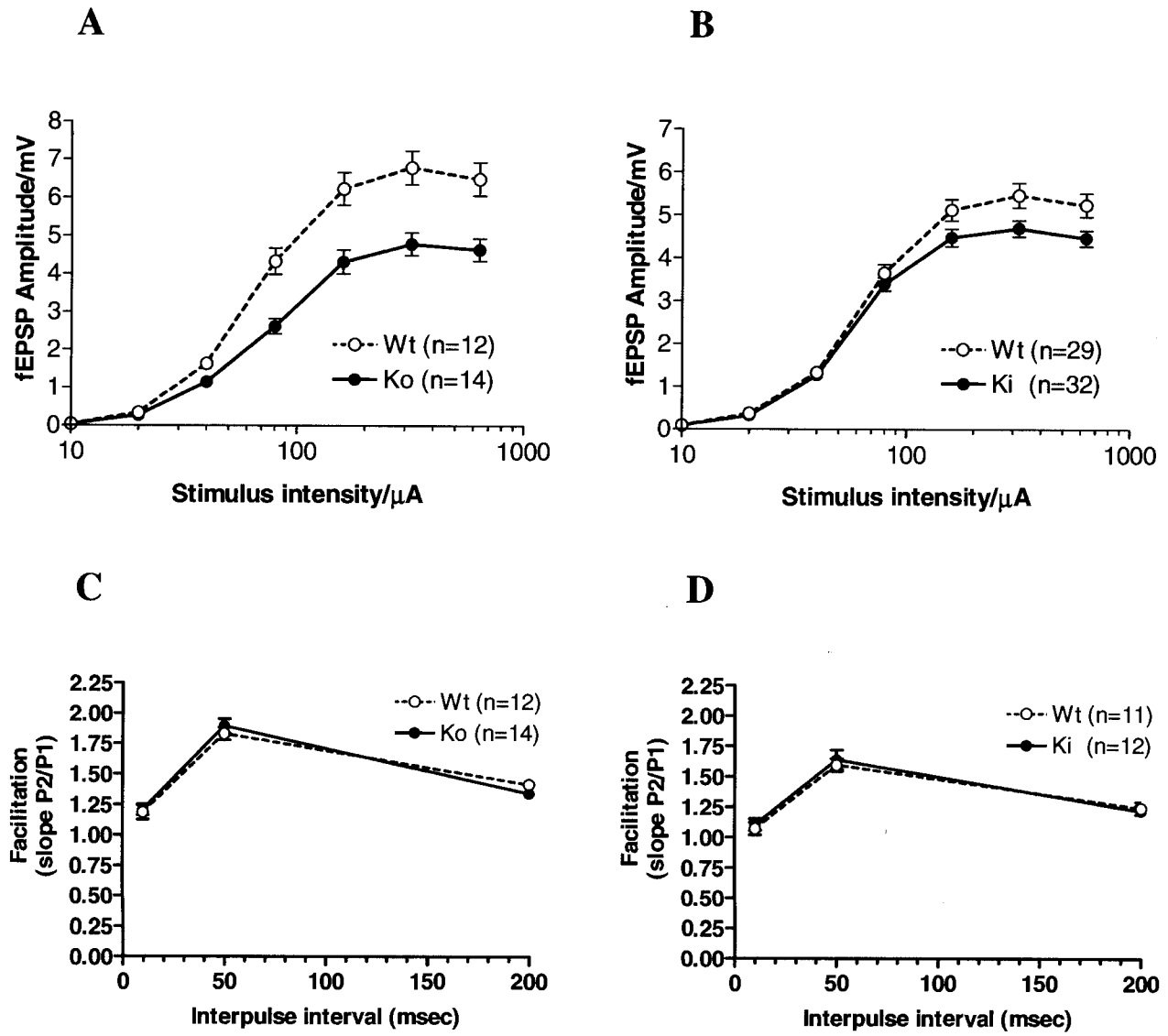


Fig. 3.3) Wave-1 signaling influences synaptic field strength but not paired-pulse facilitation. Measurement of the synaptic input/output relationship in WAVE-1 knockout **A)** and Δ-WRP (knock-in) **B)** mice reveals significantly decreased synaptic field responsiveness for both genotypes ($p<0.01$, $p<0.05$, respectively). Paired-pulse facilitation was normal at 10, 50, and 200 msec inter-pulse intervals in the WAVE-1 knockout **C)** and Δ-WRP (knock-in) **D)** mice, suggesting normal presynaptic release probability. Successive measurements were made at least 20 seconds apart (<0.05Hz).

Figure 3.4) WAVE-1 Regulates Basal AMPAR Distribution

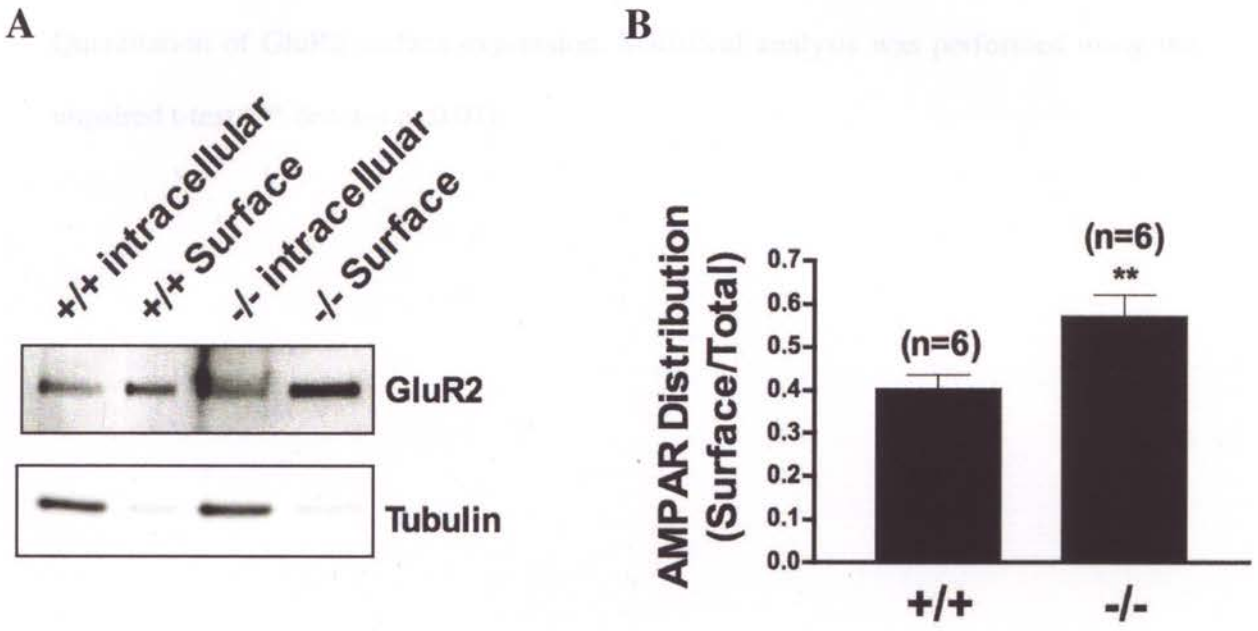


Fig. 3.4) WAVE-1 regulates basal AMPAR distribution. (**A**) Immunodetection of surface and intracellular GluR2 subunit (top) and tubulin loading control (bottom) from a surface biotinylation experiment, showing specificity of the biotinylation reagent for surface protein. Representative immunoblot from 6 independent experiments. (**B**) Quantitation of GluR2 surface expression. Statistical analysis was performed using the unpaired t-test (** denotes $p<0.01$).

Figure 3.5) An Important Role of WAVE-1 Signaling in Bidirectional Synaptic Plasticity

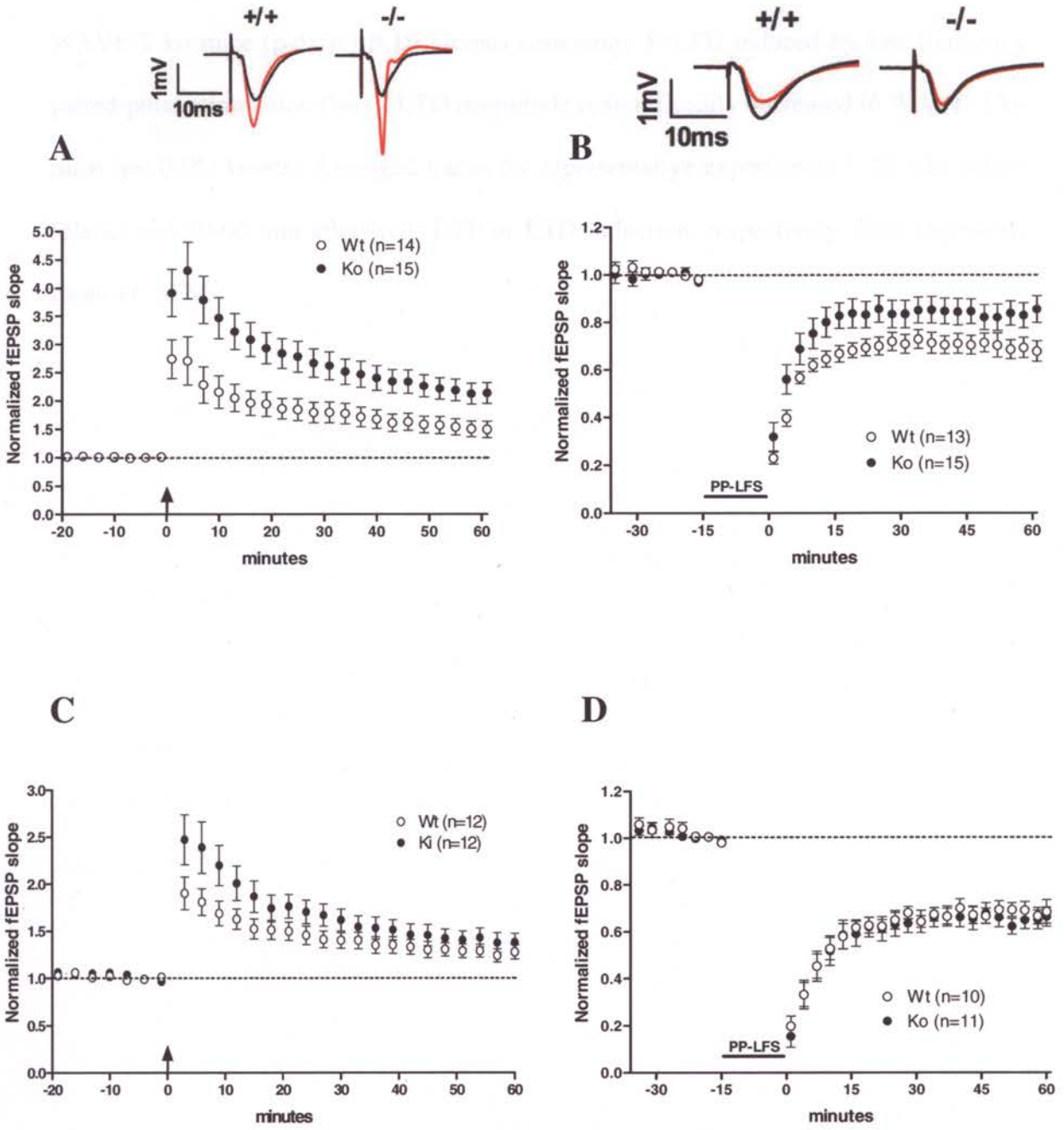


Fig. 3.5) An important role of WAVE-1 signaling in bidirectional synaptic plasticity. Electrophysiological analysis of hippocampal LTP and LTD in area CA1 of wild-type (Wt), WAVE-1 knockout (Ko), and Δ-WRP (Ki) mice. **A,C**) Graphs comparing E-LTP induced by *theta*-burst stimulation (arrows). LTP magnitude is significantly increased in WAVE-1 ko mice ($p<0.01$) **B,D**) Graphs comparing E-LTD induced by low frequency paired-pulse stimulation (bars). LTD magnitude is significantly decreased in WAVE-1 ko mice ($p< 0.05$) **Insets)** Averaged traces for representative experiments 1-10 min before (black) and 50-60 min after (red) LTP or LTD induction, respectively. Data represents mean +/- SEM.

Figure 3.6) WRP Interaction with WAVE-1 Impacts Late-Phase LTP

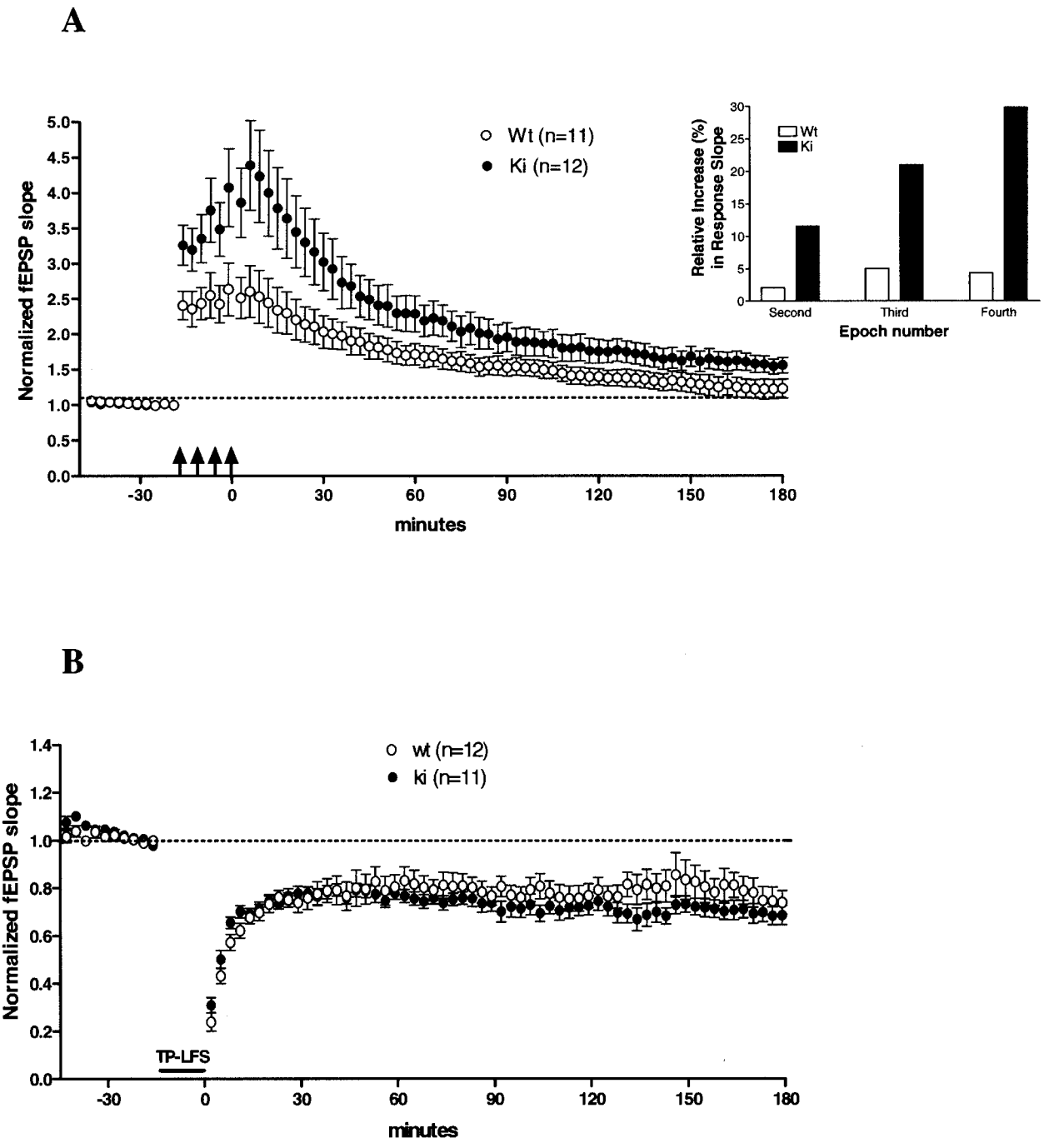


Fig. 3.6) WRP interaction with WAVE-1 impacts late-phase LTP. Electrophysiological analysis of hippocampal L-LTP and L-LTD in area CA1 of wild-type (Wt) and Δ-WRP (Ki) mice. **A**) Graph comparing L-LTP induced by 4 epochs of *theta*-burst stimulation, spaced 5 minutes apart (arrows). The magnitude of late-phase LTP is significantly larger in knock-in mice ($p<0.01$). **Inset**) Quantitation of the relative peak increase in fEPSP slope (%) for wild-type (Wt) and Δ-WRP (Ki) mice per epoch, versus the first epoch. Knock-in mice displayed a trend towards increased capacity for further potentiation during repetitive stimulation with spaced epochs of theta-burst trains during L-LTP induction, and the evolution of L-LTP expression was significantly different between genotypes (genotype x time period interaction using repeated measures ANOVA, $p < 0.05$). **B**) Graphs comparing L-LTD induced by low frequency triplet pulse stimulation (bars). Late-phase LTD did not differ significantly by genotype. Data represents mean +/- SEM.

Figure 3.7) Normal Depotentiation and Impaired Dedepression in the WAVE-1 Knockout Mouse

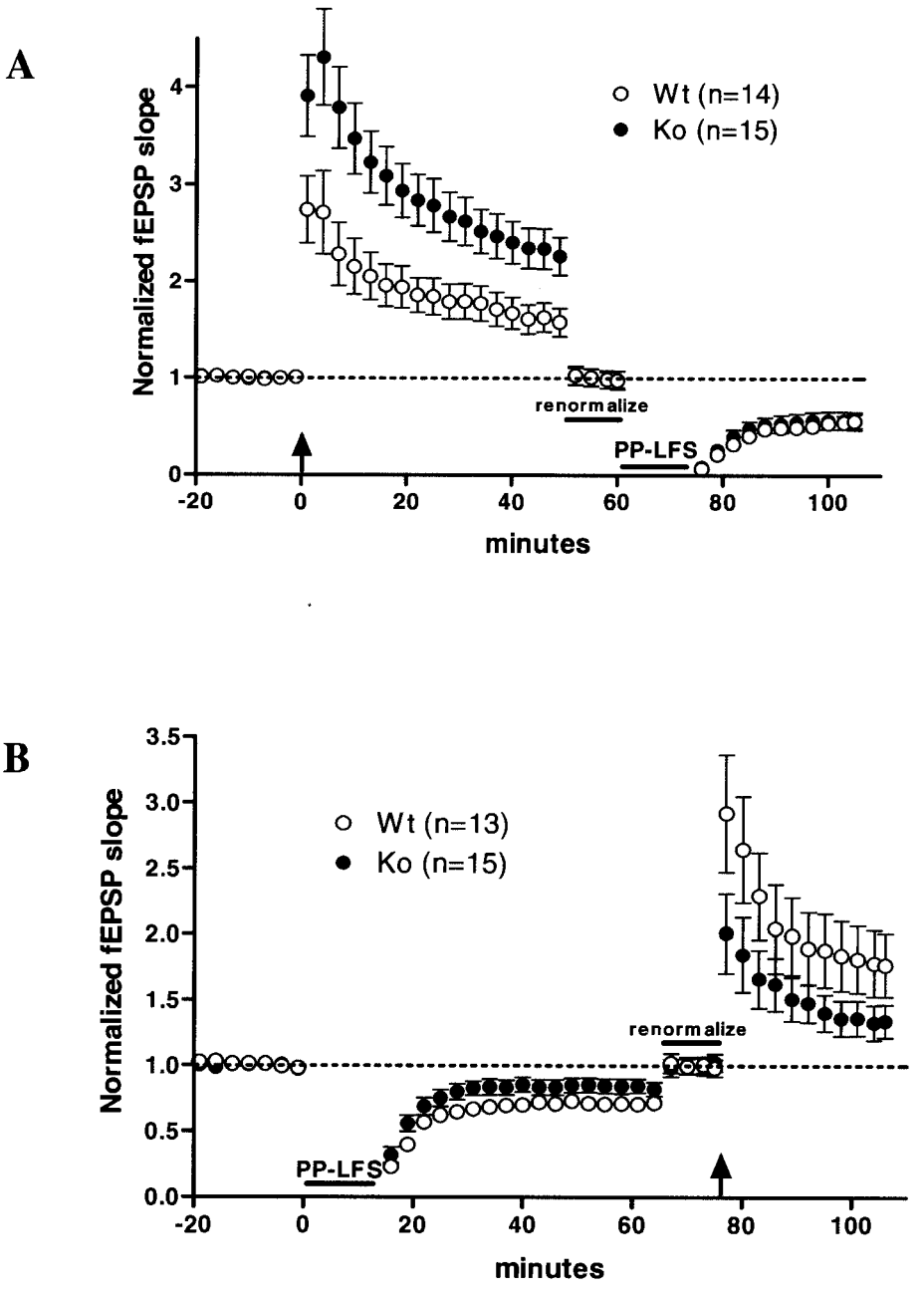


Fig. 3.7) Normal depotentiation and impaired dedepression in the WAVE-1 knockout mouse. Electrophysiological analysis of hippocampal LTP and LTD reversal, 60 minutes following their induction in area CA1 of wild-type (Wt) and WAVE-1 knockout (Ko) mice. **A**) Graph comparing depotentiation (low frequency paired-pulse stimulation: PP-LFS, bar, n=7,8) of previously established LTP (theta-bursts, arrow). **B**) Graph comparing dedepression (theta-bursts, arrow, n=6,6) of previously established LTD (PP-LFS, bar). For graphical purposes, responses were renormalized to the 50-60 minute average following LTP or LTD induction. Data represents mean +/- SEM. The magnitude of relative depotentiation does not differ significantly by genotype. The magnitude of relative dedepression is significantly reduced for ko mice ($p<0.05$) compared to wild-type mice.

Figure 3.8) Analysis of the Effect of Prior Synaptic Experience on Responses to Patterned Stimulation

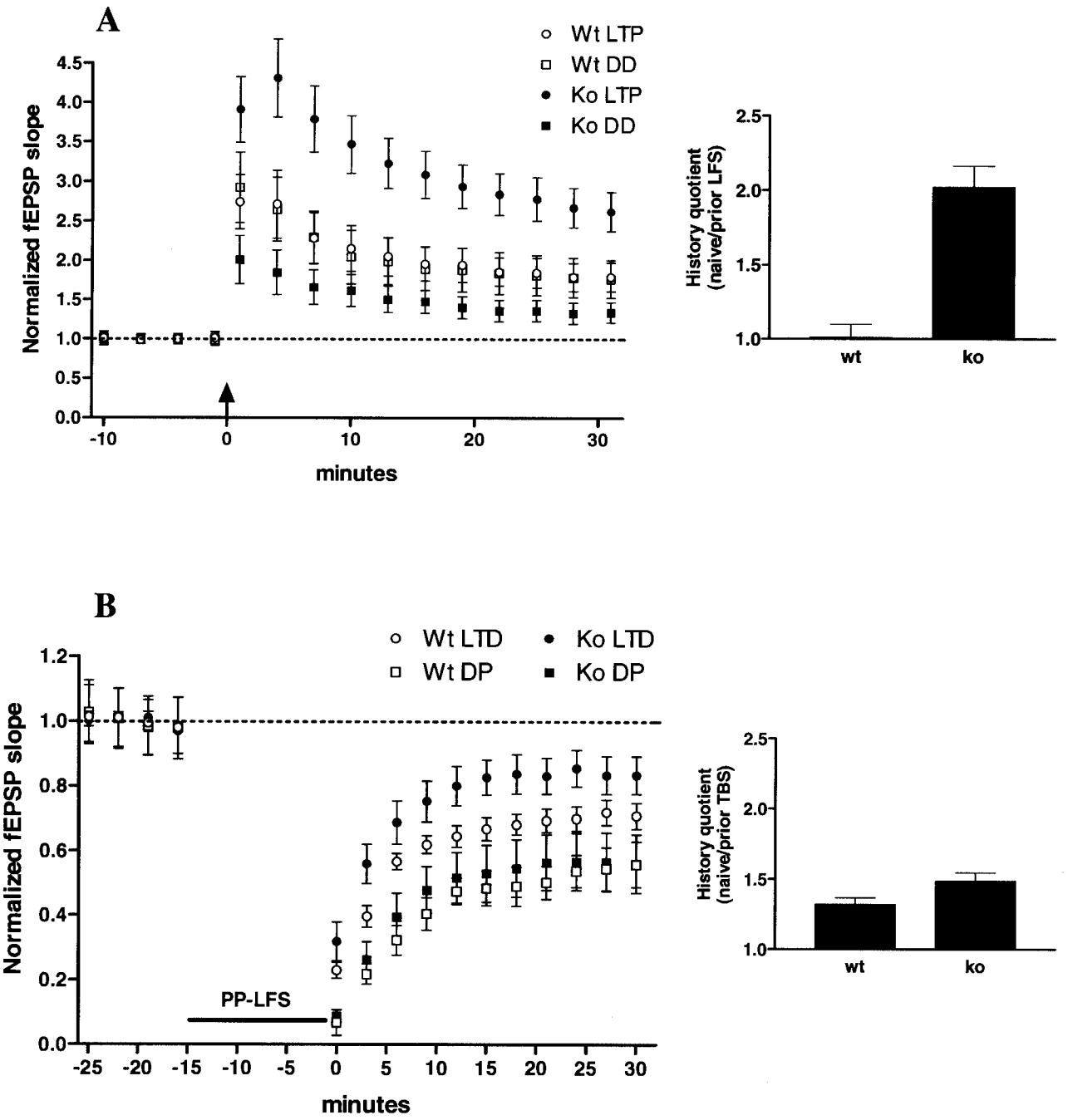


Fig. 3.8) Analysis of the effect of prior synaptic experience on responses to patterned stimulation. Comparison graphs for responses to patterned activity in naïve (circles) and "experienced" (squares) slices. "Experienced" slices are wild-type (open symbols) or WAVE-1 knockout (closed symbols) mice that have experienced prior LTP or LTD. **A**) Overlayed traces from both genotypes comparing responses to theta-bursts in naïve slices (LTP) with slices that have experienced prior LTD (DD). **B**) Overlayed traces from both genotypes comparing responses to paired pulse low-frequency stimulation (PP-LFS) in naïve slices (LTD) with slices that have experienced prior LTP (DP). **Insets)** Synaptic history quotients by genotype. The synaptic history quotient is the ratio of normalized responses resulting from the same stimulus in naïve versus experienced slices, 30 minutes following induction. Data represents mean +/- SEM, established rules for the propagation of uncertainty were applied. The responses to *theta*-bursts and PP-LFS were significantly altered in knockout slices by prior induction of LTD or LTP ($p<0.01$).

Figure 3.9) Loss of WAVE-1 Results in a Use-Dependent Decrease in the Dynamic Range of Synaptic Plasticity

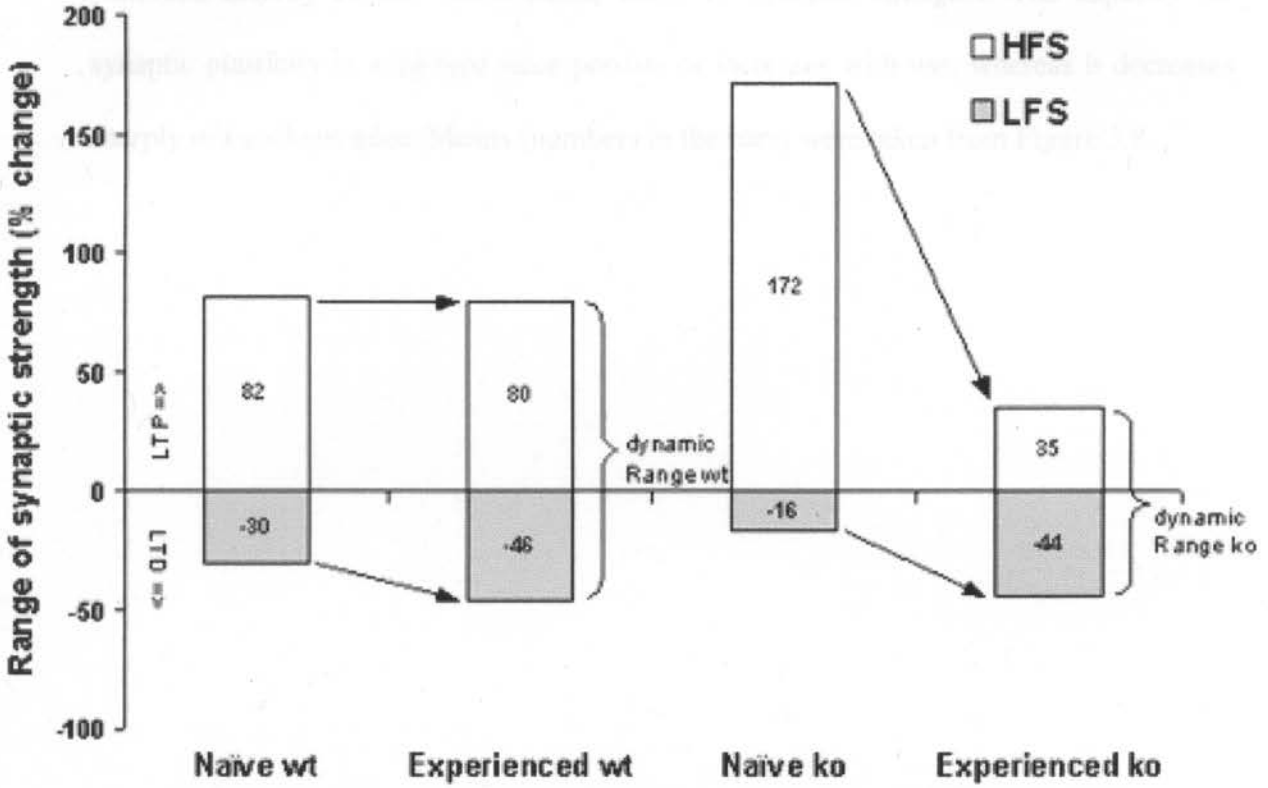


Fig. 3.9) Loss of WAVE-1 results in a use-dependent decrease in the dynamic range of synaptic plasticity. Graphical display of changes in the mean dynamic range of synaptic strengths in area CA1 due to synaptic experience for wild-type (wt) and WAVE-1 knockout (ko) slices. Stacking (summation) of the mean changes (%) in synaptic efficacy 30 minutes following high frequency (white bars) or low frequency (gray bars) patterned activity reveals the dynamic range of synaptic strengths. The capacity for synaptic plasticity in wild-type mice persists or increases with use, whereas it decreases sharply in knockout mice. Means (numbers in the bars) were taken from Figure 3.8.

Figure 3.10) State Diagram Model for the WAVE-1 Knockout Mouse

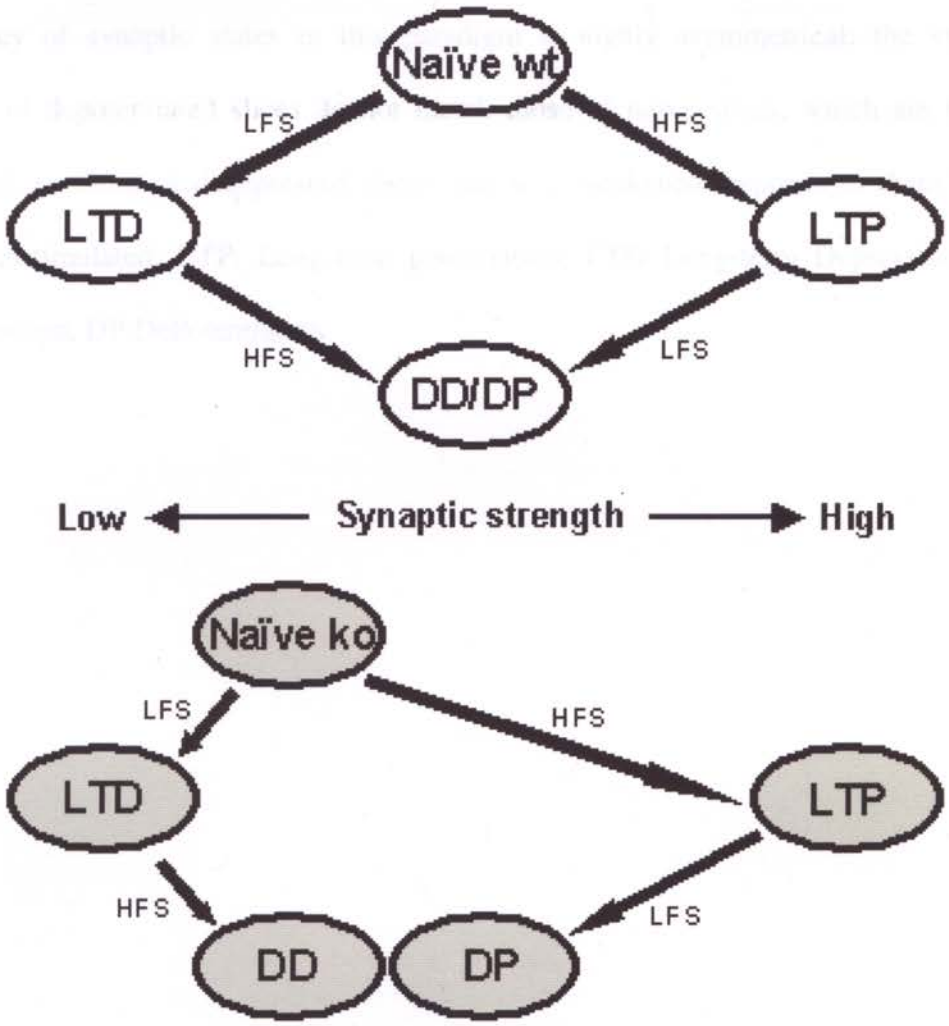


Fig. 3.10) State diagram model for the WAVE-1 knockout mouse. Schematic depiction of the synaptic states occupied during reversible bidirectional plasticity. The reversibility of bidirectional synaptic plasticity in the wild-type mouse (white ovals) displays a high degree of symmetry: following depotentiation or dedepression, synaptic strength is similar to that of naïve slices. In WAVE-1 knockout mice (gray ovals), the occupancy of synaptic states in this paradigm is highly asymmetrical: the synaptic strength of depotentiated slices do not match those of naïve slices, which are basally depressed, or those of dedepressed slices, due to a weakened response to *theta*-bursts. Naïve: unstimulated, LTP: Long-term potentiation, LTD Long-term Depression, DD: DeDepression, DP DePotentiation.

Figure 3.11) AMPAR Trafficking Model for Skewing of Bidirectional Synaptic Plasticity

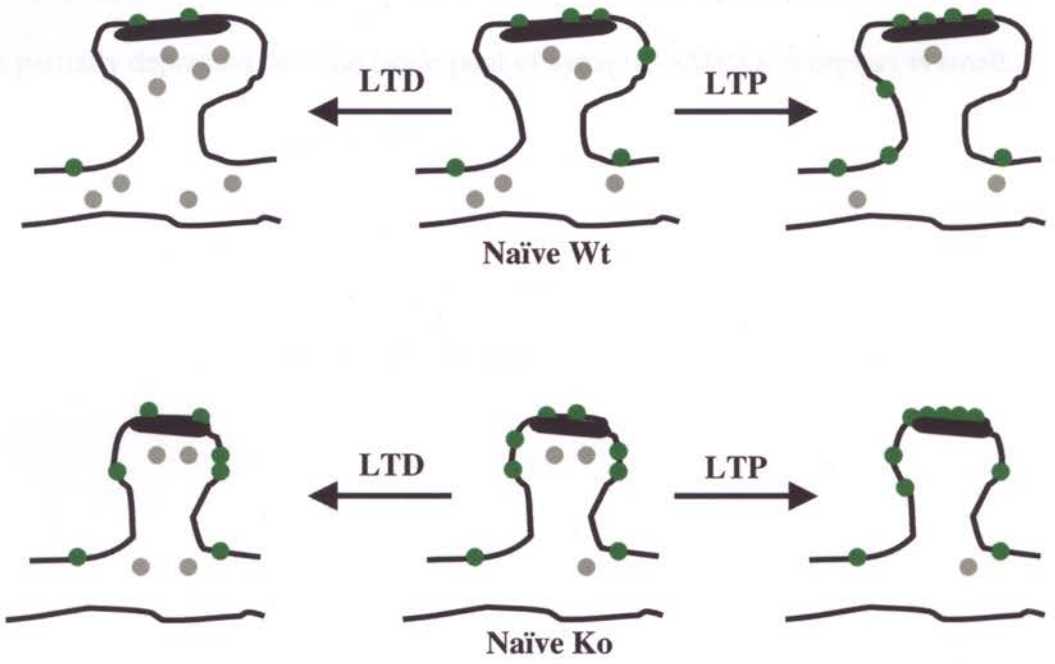


Fig. 3.11) AMPAR trafficking model for skewing of bidirectional synaptic plasticity. Hypothetical model to account for skewing of bidirectional synaptic plasticity in the WAVE-1 knockout mice. In naïve knockout slices, basal levels of surface AMPARs (green circles) are increased at extrasynaptic sites and depleted in the cytoplasm (gray circles) relative to wild-type mice. This results in the facilitation of LTP due to "priming" (*c.f.* two-step synaptic AMPAR delivery model [30]). LTD is impaired because synaptic strength is partially depressed, and the labile pool of synaptic AMPAR receptors is small.

3.5 Discussion and Conclusions

WAVE-1 regulation of spine morphology and density. The synaptic function of WAVE-1 may be to assemble signaling scaffolds in the highly motile regions of actin-rich spines that influence synaptic plasticity. Actin reorganization within dendritic spines is a highly dynamic process. Fluorescence recovery after photobleaching (FRAP) measurements reveal that approximately 85% of the actin within dendritic spines is highly dynamic with a turnover rate of 44 seconds [102]. Actin dynamics are thought to underlie the morphological plasticity associated with LTP and LTD [121, 122, 187, 188], as well as contribute to the trafficking of neurotransmitter receptors [20]. Therefore, the molecules that control actin dynamics have the capacity to profoundly influence synaptic efficacy. In this report we demonstrated that WAVE-1 signaling influences both spine morphology and synaptic physiology.

Comparison of LTP and LTD in the WAVE-1 knockout and Δ-WRP mice. Generally speaking, the loss of WAVE-1 (knockout) resulted in more profound alterations of synaptic physiology than did mutations to WAVE-1 that interfere with the interaction between WAVE-1 and WRP (Δ-WRP knock-in). Perhaps this was because the interaction of the WAVE-1 signaling complex with WRP was only partially disrupted, or perhaps it was due to one of the other functions of WAVE-1. Notable was the lack of effects on LTD in the knock-in mice, despite our use of a late-phase LTD protocol that was shown previously to require *de novo* protein synthesis [189], a hallmark of late-phase LTP and LTD [186]. These findings are consistent with behavioral studies, which demonstrate more profound deficits to learning and memory in the knockout mouse [134] than the

knock-in (Scott Soderling, unpublished). Indeed, the alterations to synaptic plasticity in knock-in mice were largely limited to the late-phase LTP experiments, in good agreement with the selective performance deficit observed during delayed probe trials in the Morris water maze (Scott Soderling, unpublished). Similarly, the profound alterations of E-LTP and E-LTD in knockout mice corresponded nicely with the slower learning acquisition rates of those animals. Typically, transgenic mice that show normal learning but harbor late-memory impairments exhibit selective alterations to the late-phase of LTP, yet have normal L-LTP induction. This is then followed by an accelerated decay of LTP, 1-2 hours following its induction, similar to the decay resulting from pharmacological inhibition of gene transcription [62]. In addition to their regulation of the cytoskeleton, Rho-family members are capable of regulating gene transcription [105]. However, since we observed a rapid alteration to L-LTP in the Δ-WRP knock-in mice, and since L-LTP was increased rather than decreased, we see no reason to suspect abnormal regulation of gene transcription in these mice. Indeed, inhibition of gene transcription does not alter the early phase of LTP (LTP during the first 1-2 hours). However, it is possible that *de novo* protein synthesis is yet altered due to abnormal regulation of mRNA translation, since pharmacological inhibition of translation has been shown to have effects on during the early-phase of LTP [34-38, 62, 152], and to impair the rapid synaptic trafficking of GluR1-containing AMPARs [190]. In any case, the alteration to L-LTP in the knock-in may be a consequence of constitutive Rac activation in cytoskeletal signaling complexes. While it is not immediately clear how larger L-LTP in the knock-in mouse leads to impaired behavior, it is possible that these mice, as with the knockout mice, harbor a

defect in metaplasticity that could be revealed through studies of depotentiation, dedepression, and AMPAR trafficking.

AMPAR distribution and trafficking. WAVE-1 knockout mice had nearly 50% more AMPAR on the plasma membrane surface (normalized to total AMPAR), as assessed by biotinylation and GluR2 immunoreactivity, than did wild-type littermates. The increase in surface GluR2 in the WAVE-1 knockout mouse (**Fig. 3.4**) is inconsistent with a cellular increase in synaptic AMPARs, since an increase of such magnitude would be expected to offset the downwards shift in the synaptic input-output curve that characterizes WAVE-1 knockout mice. We therefore conclude that loss of WAVE-1 most likely increased the surface expression of extrasynaptic AMPARs, and that the downwards shift in the synaptic input-output curve most likely resulted from the observed decrease in spine density. Whether the defect in basal AMPAR trafficking resulting from the loss of WAVE-1 involves alterations to the rates of endocytosis and exocytosis of AMPARs, alterations in the phosphorylation of sites on AMPAR subunits that are associated with its trafficking (*e.g.* GluR2 S880 and GluR1 S845), or the stabilization and destabilization of AMPARs at the plasma membrane and cytoplasmic sites respectively, could prove to be an interesting area for future investigations.

Bidirectional synaptic plasticity. Accumulating evidence suggests that an efficient neural network relies on bi-directional changes in synaptic strength [3-5, 191]. Our finding that LTP is enhanced while LTD is diminished demonstrates that WAVE-1 knockout mice exhibit fundamental alterations in bi-directional synaptic plasticity. This is also consistent with a basal depression of synaptic strength in naïve slices from the knockout (**Figs.**

3.3A, 3.5B, 3.10), which foreshadowed defects in metaplasticity. What mechanism may account for such alterations? A dynamic pool of extrasynaptic AMPA receptors are available for rapid delivery into the synapse upon induction of LTP [160]. Conversely, endocytotic removal of perisynaptic AMPA receptors precedes the loss of synaptic AMPA receptors that occur upon LTD induction [192]. Therefore, basal changes in AMPA receptor distribution between cytoplasmic sites and the extrasynaptic plasma membrane could explain the aberrant electrophysiological profile of WAVE-1 knockout mice (**Fig. 3.11**). Indeed, the alterations in bidirectional synaptic plasticity we observed in the knockout mouse are consistent with our contention that AMPARs are basally enriched in the extrasynaptic plasma membrane, as evidence suggests that enrichment of extrasynaptic surface AMPARs "prime" LTP [30, 193], and may similarly impair LTD. Furthermore, prior induction of LTD in knockout slices reversed the tendency for WAVE-1-lacking synapses to hyperpotentiate following theta-burst stimulation. One possible explanation for this is perhaps LTD induction in the knockout mouse led to the efficient removal of excess extrasynaptic AMPARs, which are believed to facilitate LTP induction (**Figs. 3.3A, B, 3.11).** Furthermore, activation of PKC in neurons has been shown to regulate Rho/Rac, produce alterations to the actin cytoskeleton, regulate the trafficking of AMPARs, and prime LTP [194]. We therefore believe our findings support the exciting possibility that cytoskeleton-dependent AMPAR trafficking may be a mediator of synaptic metaplasticity. Unraveling the molecular mechanisms whereby such cytoskeleton-dependent changes in spine morphology and AMPAR trafficking skew bidirectional plasticity will be of keen interest to the field.

Measurement of capacitative metaplasticity may accurately predict outcomes on learning and memory. Metaplasticity typically refers to changes in the thresholds for induction of LTP and LTD (*e.g.* sliding modification or B.C.M. theory). This form of metaplasticity may therefore be described as *inductive metaplasticity*. However, use-dependent changes in the capacity for bidirectional plasticity represents an additional form of metaplasticity, which may prove to be useful for studies of the cellular and biochemical correlates of learning and memory. It is clear that an analysis of LTP or LTD magnitudes in naïve brain slices is of limited utility in consistently predicting the effects of biochemical manipulations on learning and memory. While we agree with the central dogma that LTP- and LTD-like phenomena ultimately underlie learning and memory, there is generally poor correlation between the extent and direction (*i.e.* increased or decreased) of alterations to plasticity due genetic and pharmacological manipulations, and the resulting effects on learning and memory (**Table 3.1**). For example, mice overexpressing the NR2B subunit of the NMDA receptor have enhanced LTP, as do FMRP2 (Fragile-X Mental Retardation Proetin 2) knockout mice [195, 196] and the WAVE-1 knockout mice examined in this study. However, behavioral testing has revealed that NR2B overexpressing mice have measurable improvements to learning and memory while the FMRP2 and WAVE-1 knockout mice have measurable deficits. Indeed, there are also conflicting results obtained among different labs regarding the degree and direction that a given biochemical manipulation has on LTP and LTD, possibly resulting from subtle differences in methodology. We propose here a simple method for quantitation of use-dependent changes in the capacity for synaptic plasticity (**Fig. 3.9**), which we have termed *capacitative metaplasticity*. In addition to the measurement of bidirectional

synaptic plasticity, capacitative metaplasticity represents an aspect of synaptic plasticity that may be a valuable tool for future studies of learning and memory. Further testing of this important hypothesis likely will be of broad interest to the field.

For wild-type mice, *theta*-burst stimulation produces the same amount of synaptic potentiation regardless of the initial state of the synapses (**Fig. 3.8A**, [11]). In contrast, for mice lacking WAVE-1, *theta*-burst stimulation produced widely varying responses depending on the synaptic history of the slice: *theta*-burst stimulation results in an abnormally large synaptic efficacy increase for naïve slices, yet produces a very weak increase in synaptic efficacy subsequent to LTD expression (dedepression). This phenomenon results in a decreased dynamic range following a round of bidirectional synaptic plasticity, theoretically lowering the information processing capacity of the CA1 region (**Fig. 3.9**). When measuring the responses to patterned activity in conditioned slices whose synapses have undergone prior LTP and LTD, WAVE-1 knockout mice exhibit normal "LTD" and impaired "LTP" – quite a different conclusion than would have been reached through the analysis of naïve slices alone. Furthermore, the observation that WAVE-1 knockout mice respond to such experience by sharply decreasing their capacity for synaptic plasticity, while wild-type mice respond by maintaining or increasing in this capacity, has important implications for the relationship between synaptic plasticity and learning and memory. Finally, the mechanisms underlying such metaplastic phenomena have remained poorly understood. Some potential regulators of metaplasticity include modifications to the NMDAR that regulate its function, mGluR activation, PKC activity, regulation of voltage-gated ion channels, and AMPAR surface priming. The results presented here for the WAVE-1 knockout

mouse are direct evidence implicating cytoskeletal machinery in the regulation of metaplasticity.

Conclusions

1. WAVE-1 regulates hippocampal dendritic spine density and morphology in a manner consistent with the structural alterations observed in human forms of mental retardation.

2. WAVE-1 regulates bidirectional synaptic plasticity and basal AMPAR distribution.

3. Loss of WAVE-1 results in a use-dependent incapacitation of bidirectional synaptic plasticity.

Table 3.1) Relationship Between LTP and Memory*

Genetic model	LTP	Memory	References
RyR3 knockout	Increased	Increased	[197, 198]
NR2B overexpression	Increased	Increased	[199, 200]
PP2B dominant-negative	Increased	Increased	[201]
HB-GAM overexpression	**Decreased**	**Increased**	[202]
CaMKII, various mutants	Decreased	Decreased	[53, 55, 203]
mGluR1 knockout	Decreased	Decreased	[204]
Ras GRF-1 knockout	Decreased	Decreased	[205]
CREB interfering mutant	Decreased	Decreased	[206]
CaMKIV dominant negative	Decreased	Decreased	[62]
PAK dominant negative	**Increased**	**Decreased**	[107, 109]
LIMK knockout	**Increased**	**Decreased**	[111]
FMRP2 knockout	**Increased**	**Decreased**	[195]
PSD-95 mutant (PDZ-3 stop)	**Increased**	**Decreased**	[3]
WAVE-1 knockout, interfering mutant	**Increased**	**Decreased**	[134]

*A more extensive list can be found in *Mechanisms of Memory, 2003 Academic press, David Sweatt, p269.*

Chapter 4

Summary and Significance

The molecular mechanisms underlying synaptic plasticity

can be likened to an intricate clock:

the removal of a single cog may cause time

to be lost.

4.1 Recapitulation

The work presented in chapters 2 and 3 of this thesis enhances our understanding of the cellular and molecular mechanisms of LTP and LTD, and provides new insight into how LTP and LTD *ex vivo* may relate to learning and memory *in vivo*. In chapter 2, CaMKK, operating upstream of CaMKI, was shown to play an important role in the activation of the Ras-ERK cascade during NMDAR-dependent neuronal signaling. Both the NMDAR and the ERK-MAPK cascade are central players in most forms of LTP induction in hippocampal area CA1. Since prior studies had failed to demonstrate how calcium influx through the NMDAR during LTP induction activates the Ras-ERK cascade, further progress in our understanding of the factors important for, and the mechanisms of, LTP induction had been limited. Our finding that the CaMKK/CaMKI cascade regulates E-LTP induction, and does so through the activation of ERK, has provided a missing link between the NMDAR and the Ras-ERK cascade. We also showed that the CaMKK/CaMKI pathway modulates the regulatory phosphorylation of known substrates downstream of ERK in LTP, namely eIF4e and 4EBP1. These two factors are believed to participate in the regulation of activity-dependent initiation of local mRNA translation in dendrites during LTP induction [152], and interestingly, the Ras-ERK pathway is also believed to regulate synaptic AMPAR trafficking during LTP [101]. Furthermore, CaMKI signaling events may regulate the actin cytoskeleton, given the role of CaMKI upstream of Ras in the regulation of axon extension and growth cone motility [71], and given the role of CaMKI in the regulatory phosphorylation of myosin II regulatory light chain and Numb [69, 73]. Thus, the finding that the CaMKK inhibitor STO-609, the mRNA translation inhibitor anisomycin, and the MEK (the upstream activator of ERK)

inhibitor U0126 all inhibit E-LTP similarly, and with out additive effects, raises the exciting possibility that CaMKI regulates activity-dependent AMPAR trafficking, and that this process is somehow coupled with translational initiation and the dynamic regulation of the actin cytoskeleton.

The brain-specific cytoskeletal regulatory scaffolding protein WAVE-1 couples Rac activity to actin nucleation and branching [127]. WAVE-1 was shown previously to be essential for normal hippocampal-dependent learning and memory. Furthermore, its function as a scaffold for WRP binding may have implications for human forms of mental retardation such as 3p-syndrome. In chapter 3, WAVE-1 was shown to play an important role in the regulation of hippocampal spine morphology, bidirectional synaptic plasticity, metaplasticity, and the basal subcellular distribution of AMPARs between the cytoplasm and the plasma membrane. The increase of surface AMPAR resulting from deletion of WAVE-1 appeared to be selectively extrasynaptic. This finding potentially provides a mechanistic understanding of how WAVE-1 and the actin cytoskeleton may contribute to alterations in activity-dependent synaptic plasticity. The work presented in chapter 3 also furthers our understanding of how LTP and LTD relate to learning and memory *in vivo,* and provides a new molecular mechanism for the regulation of metaplasticity. I suspect the observation that WAVE-1 regulates *use-dependent changes* in the capacity for bidirectional synaptic plasticity, may represent a general mechanism whereby cytoskeletal dynamics, possibly through the regulation of AMPAR trafficking, influence the extent to which neuronal networks can process and store new information. Importantly, the routine measurement of this form of metaplasticity, which I call

capacitative metaplasticity (to distinguish it from other forms of *inductive metaplasticity,* such as B.C.M. or sliding modification, that deal explicitly with the thresholds for LTP and LTD induction), may improve our ability to predict how changes in activity-dependent synaptic plasticity resulting from alterations of cellular and biochemical processes may impact behavior. Thus, the regulation of the neuronal cytoskeleton by signaling cascades that impact LTP and LTD, and the resulting effects on AMPAR trafficking and metaplasticity, may represent an essential component of a more unified theme for future studies of the cellular basis for learning and memory.

In the following sections I will reexamine some of the key findings presented in this thesis, my interpretation of them, and my thoughts for future work to further test and extend these hypotheses.

4.2 Caveats and Hypotheses for Chapter 2

While we identified a role for CaMKK and CaMKI in ERK-dependent E-LTP, our efforts to identify a direct substrate for CaMKI upstream of Ras were unsuccessful. Recent reports have identified a key role for RasGRF1 in the regulation of ERK activation by calcium influx through the NMDAR [84, 85], as well as during LTP [205, 207] and hippocampal-dependent learning [207]. Inhibition of CaMKK/CaMKI signaling with STO-609 was able to suppress phosphorylation of RasGRF-1 at S916 during LTP induction, a regulatory site believed to be important for the activation of Ras and ERK in response to NMDAR-dependent elevations of intracellular calcium in neurons. However, S916 was very weakly phosphorylated by CaMKI *in vitro*, compared to PKA or CaMKII, although transfections of heterologous cells with active CaMKI did produce pronounced elevations is S916 phosphorylation (Saneyoshi, unpublished). Thus, phosphorylation of S916 by CaMKI may require a cofactor, or perhaps more likely, another molecule or signaling pathway dependent on CaMKK/CaMKI activity is responsible for the activation of RasGRF1. To further examine the role of Ras-GRF in CaMKK/CaMKI-dependent E-LTP, it would be interesting to test whether STO-609 produces behavioral deficits on hippocampal-dependent learning and memory tasks similar to those observed in the RasGRF knockout mouse, and if so, to examine whether STO-609 is able to further suppress LTP induction in these mice. If STO-609 produced a further inhibition of E-LTP in the RasGRF knockout, this would be an indication that CaMKK has effects on E-LTP that lie outside of RasGRF signaling. It would also be interesting to test whether inhibition of CaMKK signaling negatively impacts PKA activity, since PKA is able to robustly phosphorylate S916 *in vitro*, and since cross-talk is known to occur between the

two signaling pathways: PKA activity negatively impacts CaMKK function in hippocampal neurons [208].

One possible interpretation of our data is that CaMKI regulates LTP through the phosphorylation of AMPARs at sites that regulate their channel properties. We cannot exclude this possibility completely, but we consider it unlikely since neither ERK nor CaMKI phosphorylated the C-terminus of GluR1 *in vitro* (Guire and Oh, unpublished), which contains the two known phospho-regulatory sites for AMPAR channel properties: S831 and S845. Additionally, elevations of S831 phosphorylation during LTP were not blocked by STO-609, and preliminary evidence suggested that phosphorylation of S845 was not blocked by STO-609 during NMDA-stimulation of hippocampal slices (Guire, unpublished).

A considerable body of evidence suggests that AMPARs are a dynamic component of the active synapse. AMPARs are mobile on the surface of dendrites, and NMDAR-dependent processes regulate their trafficking between intracellular pools and the surface of dendrites, as well as their delivery to spines. Genetic and pharmacological studies have further elucidated roles for CaMKs in the regulation of postsynaptic AMPARs through trafficking [50, 209, 210]. As mentioned above, the Ras-ERK pathway was shown previously to play a role in activity- and GluR1-dependent AMPAR trafficking. AMPAR trafficking appears to be a mechanism that operates rapidly during E-LTP, and likely involves redistribution of surface AMPARs to synaptic sites (*e.g.* clustering) as well as AMPAR exocytosis from intracellular stores [211]. The cycling of AMPARs between the

cytoplasm and the plasma membrane has been well documented [47, 212-215], as has regulated synaptic AMPAR translocation [50, 173, 216], although it remains unclear whether AMPAR exocytosis can occur directly at synaptic sites. Accordingly, current models of regulated AMPAR trafficking favor a so-called two-step process [30, 217]. Perhaps the most significant and straightforward hypothesis generated from the results presented in Chapter 2 is that CaMKI, acting upstream of the Ras-ERK pathway, regulates some component of AMPAR trafficking that impacts LTP. Indeed, preliminary evidence suggests that CaMKI activity is sufficient to rapidly induce the synaptic potentiation of miniature EPSCs, consistent with the synaptic accumulation of AMPARs (Guire, unpublished). Further research will be required to fully test this hypothesis, and also to examine the potential roles of local translation and cytoskeletal dynamics in this process.

Intriguingly, there are several reports that document an inhibition of NMDA receptor-dependent E-LTP that develops rapidly following its induction in hippocampus and amygdala, using a variety of pharmacological inhibitors of translation including anisomycin, emetine, and cyclohexamide [34-38]. Furthermore, a role for MEK, the upstream activator of ERK, in activity-dependent mRNA translation was recently reported [152]. Expression of dnMEK1in region CA1 of the hippocampus suppressed hippocampal-dependent memory tasks, ERK activation, anisomycin-sensitive LTP, and mRNA translation in that study. In fact, the degree and kinetics of LTP suppression in slices taken from dnMEK1 mice was equivalent to wild-type slices treated with the mRNA translation inhibitor anisomycin. The authors did not observe any reduction in LTP induced by two trains of HFS in the dnMEK1 mice, but the deficit in LTP induced

by four trains of HFS developed within a few minutes in both dnMEK1 and anisomycin-treated mice. Regulation of mRNA translation by ERK during LTP may be related to the phosphorylation of translation factors, including the key molecules eIF4E and its inhibitory binding protein 4E-BP1. In dnMEK1 mice, phosphorylation of eIF4E and 4E-BP1 was blocked, suggesting that translational activation was impaired during LTP.

The Ras/ERK pathway appears to be required for both the trafficking of AMPARs [101] and translational activation during LTP [152], but few targets of ERK in E-LTP have been identified. One possible explanation for the rapid deficits in LTP seen after STO-609, U0126, and anisomycin pretreatment could be that synaptic delivery of AMPA receptors is somehow coupled to translation. Previous reports indicate that some, but not all, LTP induction protocols are sensitive to inhibition of translation during induction of LTP [34-38], and inhibition of translation within minutes following LTP induction has no effect on synaptic strength [178, 218], indicating that the role of protein synthesis in E-LTP occurs prior to or during a brief window surrounding its induction. Mechanistically, how could AMPAR trafficking be coupled to mRNA translation? It should be noted that, the translation of a 50 kDa protein, at a rate of seven amino acids per second, would only require ~1 min – perhaps fast enough to account for rapid AMPAR trafficking in the event that synaptically inserted receptors, or some factor required for their trafficking, are synthesized on demand. Indeed, a significant increase in dendritic CaMKII, attributable to localized synthesis, has been observed within 5 minutes of LTP induction [165], and glutamate receptors can also be locally synthesized in dendrites [219, 220]. In a recent study, activation of dopaminergic D1/D5 receptors with DHX stimulated local protein synthesis in dendrites and produced a rapid increase in the frequency of AMPAR minis,

which was blocked by either postsynaptic application of a peptide inhibitor of PKA or by brief pretreatment with anisomycin [190]. However, since pharmacological inhibitors of translation are typically present 30 minutes or more before LTP induction, they could suppress basal synthesis of some component necessary for the rapid insertion or modulation of AMPARs during induction [160], if that component has a sufficiently high rate of turnover. Indeed, this possibility has experimental support in the case of AMPAR trafficking during cerebellar LTD [221]. Another possibility that might explain how rapid deficits in LTP often result from inhibitors of translation is that translation and postsynaptic exocytosis could be biomechanically coupled through the secretory pathway, and that inhibiting one process may affect the other. This possibility is consistent with the effects of Brefeldin A on synaptic plasticity in hippocampus: Brefeldin A induces fusion of the ER and Golgi membranes, thus blocking exocytosis of newly synthesized membrane proteins, including GluR1 [222-225]. Furthermore, it is now recognized that protein synthesis from mRNAs localized within dendrites and even dendritic spines is essential for synaptic plasticity, so the possibility that protein synthesis may have acute regulatory effects has to be considered. CaMKK, CaMKI [71], ERK [226], and eIF4E [167, 168] are all localized in dendrites, as is the mRNA for the GluR1 subunit of the AMPAR [227]. Thus, it will be important to determine whether the CaMKK/CaMKI pathway regulates synthesis of AMPARs or some other protein critical for synaptic trafficking. The stargazin family of proteins may be a candidate for this potential mechanism as their levels have been shown to be limiting for the surface expression of AMPARs, as well as being required for the activity-dependent synaptic incorporation of GluR1-containing AMPARs [193].

4.3 Caveats and Hypotheses for Chapter 3

An important unresolved piece of the puzzle addressed in chapter 3 is how, at the molecular level, loss of WAVE-1 leads to alterations in AMPAR distribution. AMPAR subunits are known to interact with several proteins believed to be complexed with the actin cytoskeleton, such as ABP/GRIP, TARPs, and protein 4.1N (**chapter 1.3**). These AMPAR interacting proteins have been hypothesized to regulate the synaptic trafficking of AMPARs. Since the trafficking of AMPARs involves regulated endocytosis and exocytosis, molecules that participate in these processes (such as NSF, Clathrin, and likely TARPS) are also good candidates for mediating WAVE-1-dependent effects on basal AMPAR trafficking and activity-dependent synaptic plasticity. Furthermore, the potential role of WAVE-1 in *activity-dependent trafficking* of AMPAR *per se* was not evaluated. Currently, experiments are underway to test whether chemical forms of LTP and LTD (GISP/DHPG) result in altered rates of endocytosis and exocytosis of AMPARs. These experiments will also attempt to differentiate between AMPARs in dendrites and spines, and between SDS- and Triton-soluble surface fractions in lysates. Finally, it would be straightforward and perhaps enlightening to test whether the basal phosphorylation of regulatory sites on AMPAR subunits that are associated with their trafficking are altered in the WAVE-1 knockout mice, such as phospho-S845 of GluR1 and phospho-S880 of GluR2. Alterations in the basal phosphorylation of either of these two sites could potentially help explain why naïve LTP and LTD is altered in these mice, as well as shed light on the signaling mechanisms downstream of WAVE-1 that mediate its effects on AMPAR trafficking. Along these lines, another useful experiment would be to determine whether AMPAR trafficking is altered in the Δ-WRP knock in mouse, in

order to assess the potential role of WRP in the regulation of AMPAR trafficking. For both mouse models, the direct visualization of fluorescently tagged AMPARs at the level of spines, and the quantitation of miniature EPSCs *versus* responses to extrasynaptic application of AMPA, would be instrumental in conclusively testing the hypothesis that the increased surface expression of GluR2 due to disruption of WAVE-1 function is extrasynaptic. Indeed, one intriguing hypothesis consistent with the physiological and biochemical findings in the WAVE-1 knockout mouse is that there are a relatively large number of AMPA-silent synapses present prior to the induction of LTP in these mice.

Perhaps the most pressing hypothesis to be further tested is the relationship between capacitative metaplasticity and learning and memory. Simply speaking, the best way to do this is would be to reexamine bidirectional synaptic plasticity, including depotentiation and dedepression, in other situations where learning and memory is impaired, such as in any number of genetic mouse models (see Table 3.1). This relationship could also be evaluated in pharmacological models of metaplasticity, such as treatment of acute brain slices with activators of PKA signaling (*e.g.* Forskolin/Rolipram) or PKC signaling (phorbol ester or mGluR1/5 activation), transient removal of extracellular magnesium, or low concentrations of NMDA blockers such as APV. These manipulations have been reported to alter the responses of a hippocampal slice to LTP- and LTD- inducing stimuli independently of any effect on baseline synaptic strength. Furthermore, it would be quite interesting to test the capacitative metaplasticity model using the application of drugs that are under investigation for their purported nootropic effects, such as ampakines or selective PDE inhibitors. Finally, much of the early work

on metaplasticity was performed using conditioning stimuli, such as weak tetani, which on their own fail to induce LTP. Thus, it would also be informative to test such forms of inductive metaplasticity in the WAVE-1 knockout mouse and other mouse models of mental retardation. Clearly, much work remains to be done.

The reversal of LTP and LTD maintain the capacity for network plasticity and are essential components in models of learning and memory[228]. Indeed, long-term synaptic depotentiation (DP) and dedepression (DD) have been reported in area CA1, and satisfy the hypothesized requirement for reversibility in saturation-resistant neuronal networks *in vivo* [229]. The *ex vivo* hippocampal slice differs from the *in vivo* hippocampus in important respects, however, including the pronounced attenuation of intrinsic firing rates and the severing of neuromodulatory inputs. Furthermore, a recent investigation demonstrated that about half of the synaptic spines in acute hippocampal slices prepared from rodents are truly naïve, in the sense that they were not physically present in the intact hippocampus prior to dissection and recovery [230, 231]. However, the neural pathways used in the acquisition, storage, and retrieval of behavioral modifications during a particular learning event in an adult animal are almost certainly not naïve. Newly formed synapses in acute slices are likely to react differently to patterned activity than mature, experienced, synapses *in vivo*. Because synapses in the hippocampus participate in multimodal sensory information processing, they likely undergo repetitive, reversible transitions between different states of synaptic strength. Accordingly, depotentiation and dedepression are more likely to reflect the nature of synaptic plasticity

occurring *in vivo* during learning, since these forms of plasticity reflect the responses of "experienced" synapses to patterned activity.

Would *in vivo* LTP/LTD then be a better predictor of learning and memory than LTP/LTD in naïve slices? The answer to that question is unclear. Mature hippocampus is quite efficient at synaptogenesis and therefore might be expected to contain a reservoir of naïve synapses. Since the electrical stimuli used to induce synaptic plasticity (*e.g.* patterned stimulation of the Schaffer collateral pathway) during *in vivo* LTP and LTD results in an artificial correlation of synaptic activities, it may activate a significant proportion of naïve or pseudonaïve synapses that would not have participated in a given learning task. Two means of probing this issue come to mind. First, it would be informative to test *in vivo* LTP and LTD in the WAVE-1 knockout mouse, as well as other genetic modes of mental retardation, such as the dominant-negative PAK mouse. Indeed, LTP magnitude may be decreased, rather than increased, relative to wild-type mice when measured in this way. Furthermore, the behavioral properties of the LIMK knockout mouse make it an excellent candidate for further testing this model, since the learning deficits observed in these mice relate to impaired behavioral extinction or "unlearning" [109], a process which may represent the reversal of LTP and LTD *in vivo*. Second, it has been demonstrated *in vivo* that CA1 LTP, and *ex vivo* that LTP in cortical and amygdalar slices, is decreased when measured in recently trained animals [232-235]. Thus, a different result may have been obtained in the WAVE-1 knockout mice during "naïve" LTP and LTD, if the animals had been trained prior to harvesting their hippopcampi. In any event, I believe our analysis of capacitative metaplasticity in the WAVE-1 knockout mouse opens up new and exciting questions pertaining to the

relationship between synaptic plasticity and learning and memory *in vivo*, and may provide a valuable tool for cellular and biochemical studies of the molecular basis of synaptic plasticity *in vitro*.

4.4 Central Themes

One central theme underlying this thesis, are implications for small G-proteins, such as Ras (downstream of CaMKK/CaMKI) and Rac (*via* WAVE-1-dependent signaling), in the coordinated regulation of cellular processes important for activity-dependent changes in synaptic strength. Small G-proteins have received much attention for their ability to reorganize the actin cytoskeleton as well as regulate *de novo* protein synthesis through the regulation of DNA transcription and mRNA translation. Indeed, such processes are integral to adaptive cellular reorganization, such as the morphological plasticity of dendritic spines that accompanies synaptic plasticity, and the activity-dependent trafficking of AMPARs. When viewed in this light, the role of the actin cytoskeleton in localizing AMPARs, and the potential coupling of AMPAR trafficking to activity-dependent regulation of mRNA translation, may be an important mechanism for controlling synaptic metaplasticity.

Several examples of these coordinated relationships have been identified, and I will briefly cover two of them. First, signal transduction *via* the PKC pathway has been shown to regulate AMPAR trafficking through the phosphorylation of GluR2 at S880, and evidence suggests this event releases GluR2-containing AMPARs from cytoplasmic sequestration [236, 237], making them available for exocytosis and synaptic delivery [236, 237]. Furthermore, manipulation of PKC signaling in rats and mice has been demonstrated to alter dendritic spine morphology through Rac-, Rho-, and actin-dependent mechanisms [194]. Interestingly, PKC signaling also has been shown to mediate a form of synaptic metaplasticity [238-240]. Second, recent work on FMRP

alludes to a potentially general relationship between mRNA translation and actin dynamics in spines and dendrites. FMRP is associated with dendritic polyribosomes and has been demonstrated to regulate local dendritic protein synthesis [241], which occurs in response to LTP and LTD inducing stimuli. Loss of FMRP, such as occurs in human fragile-X mental retardation syndrome, results in dendritic spine abnormalities that strikingly resemble those obtained in mutants affecting the Rac-1 signaling pathways. In a recent paper, the *drosophila* ortholog of the FMRP interacting protein CYFIP was shown to interact with both FMRP and Rac-1, and its functional disruption reproduced synaptic alterations characteristic of manipulations to FMRP signaling [241, 242]. Furthermore, the *in vivo* interaction of CYFIP and Rac-1 was activity-dependent, suggesting that Rac-1 and FMRP regulate neuronal connectivity by a common pathway. Thus, it appears likely that the cellular events occurring during synaptic plasticity, such as AMPAR trafficking, cytoskeleton-based morphological plasticity, and translational activation, are coordinated in a highly interdependent fashion by small G-proteins of the Ras superfamily.

"The bottom line of all this is that if you are thinking of a single phosphorylation event or the synthesis of a new protein or the insertion of a membrane receptor or ion channel or even the formation of a new synapse as being capable of storing memory for any appreciable period of time, you must adjust your thinking. As a first approximation, the entirety of the functional components of your whole CNS have been broken down and resynthesized over a 2-month time span. This should scare you. Your apparent [physical] stability as an individual is a perceptual illusion."

-David Sweatt

References

1. Sweatt, J.D., *Mechanisms of Memory.* Academic Press, 2003.
2. Bliss, T.V. and T. Lomo, *Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path.* J Physiol, 1973. 232(2): p. 331-56.
3. Migaud, M., et al., *Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein.* Nature, 1998. 396(6710): p. 433-9.
4. Morris, R.G. and D.J. Willshaw, *Memory. Must what goes up come down?* Nature, 1989. 339(6221): p. 175-6.
5. Zeng, H., et al., *Forebrain-specific calcineurin knockout selectively impairs bidirectional synaptic plasticity and working/episodic-like memory.* Cell, 2001. 107(5): p. 617-29.
6. Abraham, W.C., *Metaplasticity: Key Element in Memory and Learning?* News Physiol Sci, 1999. 14: p. 85.
7. Abraham, W.C. and M.F. Bear, *Metaplasticity: the plasticity of synaptic plasticity.* Trends Neurosci, 1996. 19(4): p. 126-30.
8. Abraham, W.C. and W.P. Tate, *Metaplasticity: a new vista across the field of synaptic plasticity.* Prog Neurobiol, 1997. 52(4): p. 303-23.
9. Deisseroth, K., et al., *Synaptic plasticity: A molecular mechanism for metaplasticity.* Curr Biol, 1995. 5(12): p. 1334-8.
10. Bienenstock, E.L., L.N. Cooper, and P.W. Munro, *Theory for the development of neuron selectivity: orientation specificity and binocular interaction in visual cortex.* J Neurosci, 1982. 2(1): p. 32-48.
11. Lee, H.K., et al., *Regulation of distinct AMPA receptor phosphorylation sites during bidirectional synaptic plasticity.* Nature, 2000. 405(6789): p. 955-9.
12. Johnston, D., Wu, S., *Foundations of Cellular Neurophysiology.* MIT Press, 1999.
13. Fazeli, M.S., *Synaptic plasticity: on the trail of the retrograde messenger.* Trends Neurosci, 1992. 15(4): p. 115-7.
14. Chan, C.S., et al., *Integrin requirement for hippocampal synaptic plasticity and spatial memory.* J Neurosci, 2003. 23(18): p. 7107-16.
15. Rohrbough, J., et al., *Integrin-mediated regulation of synaptic morphology, transmission, and plasticity.* J Neurosci, 2000. 20(18): p. 6868-78.
16. Nagy, V., et al., *Matrix metalloproteinase-9 is required for hippocampal late-phase long-term potentiation and memory.* J Neurosci, 2006. 26(7): p. 1923-34.
17. Xiao, P., et al., *Evidence that matrix recognition contributes to stabilization but not induction of LTP.* Neuroreport, 1991. 2(8): p. 461-4.
18. Diamond, J.S. and C.E. Jahr, *Synaptically released glutamate does not overwhelm transporters on hippocampal astrocytes during high-frequency stimulation.* J Neurophysiol, 2000. 83(5): p. 2835-43.
19. Frerking, M., *When astrocytes signal, kainate receptors respond.* Proc Natl Acad Sci U S A, 2004. 101(9): p. 2649-50.
20. Bredt, D.S. and R.A. Nicoll, *AMPA receptor trafficking at excitatory synapses.* Neuron, 2003. 40(2): p. 361-79.

21. Collingridge, G.L., J.T. Isaac, and Y.T. Wang, *Receptor trafficking and synaptic plasticity.* Nat Rev Neurosci, 2004. 5(12): p. 952-62.
22. Luscher, C. and M. Frerking, *Restless AMPA receptors: implications for synaptic transmission and plasticity.* Trends Neurosci, 2001. 24(11): p. 665-70.
23. Triller, A. and D. Choquet, *Surface trafficking of receptors between synaptic and extrasynaptic membranes: and yet they do move!* Trends Neurosci, 2005. 28(3): p. 133-9.
24. Derkach, V., A. Barria, and T.R. Soderling, *Ca2+/calmodulin-kinase II enhances channel conductance of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors.* Proc Natl Acad Sci U S A, 1999. 96(6): p. 3269-74.
25. Thiagarajan, T.C., M. Lindskog, and R.W. Tsien, *Adaptation to synaptic inactivity in hippocampal neurons.* Neuron, 2005. 47(5): p. 725-37.
26. Oh, M.C. and V.A. Derkach, *Dominant role of the GluR2 subunit in regulation of AMPA receptors by CaMKII.* Nat Neurosci, 2005. 8(7): p. 853-4.
27. Benke, T.A., et al., *Modulation of AMPA receptor unitary conductance by synaptic activity.* Nature, 1998. 393(6687): p. 793-7.
28. Luthi, A., et al., *Bi-directional modulation of AMPA receptor unitary conductance by synaptic activity.* BMC Neurosci, 2004. 5(1): p. 44.
29. Banke, T.G., et al., *Control of GluR1 AMPA receptor function by cAMP-dependent protein kinase.* J Neurosci, 2000. 20(1): p. 89-102.
30. Oh, M.C., et al., *Extrasynaptic membrane trafficking regulated by GluR1 serine 845 phosphorylation primes AMPA receptors for long-term potentiation.* J Biol Chem, 2006. 281(2): p. 752-8.
31. Selcher, J.C., et al., *A role for ERK MAP kinase in physiologic temporal integration in hippocampal area CA1.* Learn Mem, 2003. 10(1): p. 26-39.
32. Adams, J.P., et al., *MAPK regulation of gene expression in the central nervous system.* Acta Neurobiol Exp (Wars), 2000. 60(3): p. 377-94.
33. Pittenger, C. and E. Kandel, *A genetic switch for long-term memory.* C R Acad Sci III, 1998. 321(2-3): p. 91-6.
34. Okulski, P., G. Hess, and L. Kaczmarek, *Anisomycin treatment paradigm affects duration of long-term potentiation in slices of the amygdala.* Neuroscience, 2002. 114(1): p. 1-5.
35. Scharf, M.T., et al., *Protein synthesis is required for the enhancement of long-term potentiation and long-term memory by spaced training.* J Neurophysiol, 2002. 87(6): p. 2770-7.
36. Barea-Rodriguez, E.J., et al., *Protein synthesis inhibition blocks the induction of mossy fiber long-term potentiation in vivo.* J Neurosci, 2000. 20(22): p. 8528-32.
37. Huang, Y.Y., K.C. Martin, and E.R. Kandel, *Both protein kinase A and mitogen-activated protein kinase are required in the amygdala for the macromolecular synthesis-dependent late phase of long-term potentiation.* J Neurosci, 2000. 20(17): p. 6317-25.
38. Osten, P., et al., *Protein synthesis-dependent formation of protein kinase Mzeta in long-term potentiation.* J Neurosci, 1996. 16(8): p. 2444-51.
39. Bliss, T.V. and G.L. Collingridge, *A synaptic model of memory: long-term potentiation in the hippocampus.* Nature, 1993. 361(6407): p. 31-9.

40. Bear, M.F. and R.C. Malenka, *Synaptic plasticity: LTP and LTD.* Curr Opin Neurobiol, 1994. 4(3): p. 389-99.
41. Lisman, J., H. Schulman, and H. Cline, *The molecular basis of CaMKII function in synaptic and behavioural memory.* Nat Rev Neurosci, 2002. 3(3): p. 175-90.
42. Lisman, J.E., *A mechanism for memory storage insensitive to molecular turnover: a bistable autophosphorylating kinase.* Proc Natl Acad Sci U S A, 1985. 82(9): p. 3055-7.
43. Isaac, J., *Protein phosphatase 1 and LTD: synapses are the architects of depression.* Neuron, 2001. 32(6): p. 963-6.
44. Soderling, T.R. and V.A. Derkach, *Postsynaptic protein phosphorylation and LTP.* Trends Neurosci, 2000. 23(2): p. 75-80.
45. Mayer, M.L., G.L. Westbrook, and P.B. Guthrie, *Voltage-dependent block by Mg2+ of NMDA responses in spinal cord neurones.* Nature, 1984. 309(5965): p. 261-3.
46. Song, I. and R.L. Huganir, *Regulation of AMPA receptors during synaptic plasticity.* Trends Neurosci, 2002. 25(11): p. 578-88.
47. Shi, S.H., et al., *Rapid spine delivery and redistribution of AMPA receptors after synaptic NMDA receptor activation.* Science, 1999. 284(5421): p. 1811-6.
48. Heynen, A.J., et al., *Bidirectional, activity-dependent regulation of glutamate receptors in the adult hippocampus in vivo.* Neuron, 2000. 28(2): p. 527-36.
49. Lu, W., et al., *Activation of synaptic NMDA receptors induces membrane insertion of new AMPA receptors and LTP in cultured hippocampal neurons.* Neuron, 2001. 29(1): p. 243-54.
50. Hayashi, Y., et al., *Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction.* Science, 2000. 287(5461): p. 2262-7.
51. Yasuda, H., et al., *A developmental switch in the signaling cascades for LTP induction.* Nat Neurosci, 2003. 6(1): p. 15-6.
52. Ito, I., H. Hidaka, and H. Sugiyama, *Effects of KN-62, a specific inhibitor of calcium/calmodulin-dependent protein kinase II, on long-term potentiation in the rat hippocampus.* Neurosci Lett, 1991. 121(1-2): p. 119-21.
53. Hinds, H.L., S. Tonegawa, and R. Malinow, *CA1 long-term potentiation is diminished but present in hippocampal slices from alpha-CaMKII mutant mice.* Learn Mem, 1998. 5(4-5): p. 344-54.
54. Kirkwood, A., A. Silva, and M.F. Bear, *Age-dependent decrease of synaptic plasticity in the neocortex of alphaCaMKII mutant mice.* Proc Natl Acad Sci U S A, 1997. 94(7): p. 3380-3.
55. Silva, A.J., et al., *Deficient hippocampal long-term potentiation in alpha-calcium-calmodulin kinase II mutant mice.* Science, 1992. 257(5067): p. 201-6.
56. Hvalby, O., et al., *Specificity of protein kinase inhibitor peptides and induction of long-term potentiation.* Proc Natl Acad Sci U S A, 1994. 91(11): p. 4761-5.
57. Smith, M.K., R.J. Colbran, and T.R. Soderling, *Specificities of autoinhibitory domain peptides for four protein kinases. Implications for intact cell studies of protein kinase function.* J Biol Chem, 1990. 265(4): p. 1837-40.
58. Bayer, K.U., et al., *Interaction with the NMDA receptor locks CaMKII in an active conformation.* Nature, 2001. 411(6839): p. 801-5.

59. Omkumar, R.V., et al., *Identification of a phosphorylation site for calcium/calmodulindependent protein kinase II in the NR2B subunit of the N-methyl-D-aspartate receptor.* J Biol Chem, 1996. 271(49): p. 31670-8.
60. Tokumitsu, H., et al., *STO-609, a specific inhibitor of the Ca(2+)/calmodulin-dependent protein kinase kinase.* J Biol Chem, 2002. 277(18): p. 15813-8.
61. Ho, N., et al., *Impaired synaptic plasticity and cAMP response element-binding protein activation in Ca2+/calmodulin-dependent protein kinase type IV/Gr-deficient mice.* J Neurosci, 2000. 20(17): p. 6459-72.
62. Kang, H., et al., *An important role of neural activity-dependent CaMKIV signaling in the consolidation of long-term memory.* Cell, 2001. 106(6): p. 771-83.
63. Kasahara, J., K. Fukunaga, and E. Miyamoto, *Activation of calcium/calmodulin-dependent protein kinase IV in long term potentiation in the rat hippocampal CA1 region.* J Biol Chem, 2001. 276(26): p. 24044-50.
64. Jensen, K.F., et al., *Nuclear and axonal localization of Ca2+/calmodulin-dependent protein kinase type Gr in rat cerebellar cortex.* Proc Natl Acad Sci U S A, 1991. 88(7): p. 2850-3.
65. Lemrow, S.M., et al., *Catalytic activity is required for calcium/calmodulin-dependent protein kinase IV to enter the nucleus.* J Biol Chem, 2004. 279(12): p. 11664-71.
66. Enslen, H., H. Tokumitsu, and T.R. Soderling, *Phosphorylation of CREB by CaM-kinase IV activated by CaM-kinase IV kinase.* Biochem Biophys Res Commun, 1995. 207(3): p. 1038-43.
67. Impey, S., et al., *Phosphorylation of CBP mediates transcriptional activation by neural activity and CaM kinase IV.* Neuron, 2002. 34(2): p. 235-44.
68. Peters, M., et al., *Loss of Ca2+/calmodulin kinase kinase beta affects the formation of some, but not all, types of hippocampus-dependent long-term memory.* J Neurosci, 2003. 23(30): p. 9752-60.
69. Suizu, F., et al., *Characterization of Ca2+/calmodulin-dependent protein kinase I as a myosin II regulatory light chain kinase in vitro and in vivo.* Biochem J, 2002. 367(Pt 2): p. 335-45.
70. Saneyoshi, T., S. Kume, and K. Mikoshiba, *Calcium/calmodulin-dependent protein kinase I in Xenopus laevis.* Comp Biochem Physiol B Biochem Mol Biol, 2003. 134(3): p. 499-507.
71. Wayman, G.A., et al., *Regulation of axonal extension and growth cone motility by calmodulin-dependent protein kinase I.* J Neurosci, 2004. 24(15): p. 3786-94.
72. Schmitt, J.M., et al., *Calcium activation of ERK mediated by calmodulin kinase I.* J Biol Chem, 2004. 279(23): p. 24064-72.
73. Tokumitsu, H., et al., *Phosphorylation of Numb family proteins. Possible involvement of Ca2+/calmodulin-dependent protein kinases.* J Biol Chem, 2005. 280(42): p. 35108-18.
74. Qin, H., et al., *Phosphorylation screening identifies translational initiation factor 4GII as an intracellular target of Ca(2+)/calmodulin-dependent protein kinase I.* J Biol Chem, 2003. 278(49): p. 48570-9.
75. Matsushita, M. and A.C. Nairn, *Characterization of the mechanism of regulation of Ca2+/ calmodulin-dependent protein kinase I by calmodulin and by*

Ca2+/calmodulin-dependent protein kinase kinase. J Biol Chem, 1998. 273(34): p. 21473-81.
76. Tokumitsu, H., H. Enslen, and T.R. Soderling, *Characterization of a Ca2+/calmodulin-dependent protein kinase cascade. Molecular cloning and expression of calcium/calmodulin-dependent protein kinase kinase.* J Biol Chem, 1995. 270(33): p. 19320-4.
77. Selcher, J.C., et al., *Protein kinase signal transduction cascades in mammalian associative conditioning.* Neuroscientist, 2002. 8(2): p. 122-31.
78. Thomas, G.M. and R.L. Huganir, *MAPK cascade signalling and synaptic plasticity.* Nat Rev Neurosci, 2004. 5(3): p. 173-83.
79. Pearson, G., et al., *Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions.* Endocr Rev, 2001. 22(2): p. 153-83.
80. Gallagher, S.M., et al., *Extracellular signal-regulated protein kinase activation is required for metabotropic glutamate receptor-dependent long-term depression in hippocampal area CA1.* J Neurosci, 2004. 24(20): p. 4859-64.
81. Tian, X. and L.A. Feig, *Age-dependent participation of Ras-GRF proteins in coupling calcium-permeable AMPA glutamate receptors to Ras/Erk signaling in cortical neurons.* J Biol Chem, 2006. 281(11): p. 7578-82.
82. Wu, G.Y., K. Deisseroth, and R.W. Tsien, *Activity-dependent CREB phosphorylation: convergence of a fast, sensitive calmodulin kinase pathway and a slow, less sensitive mitogen-activated protein kinase pathway.* Proc Natl Acad Sci U S A, 2001. 98(5): p. 2808-13.
83. Agell, N., et al., *Modulation of the Ras/Raf/MEK/ERK pathway by Ca(2+), and calmodulin.* Cell Signal, 2002. 14(8): p. 649-54.
84. Tian, X., et al., *Developmentally regulated role for Ras-GRFs in coupling NMDA glutamate receptors to Ras, Erk and CREB.* Embo J, 2004. 23(7): p. 1567-75.
85. Krapivinsky, G., et al., *The NMDA receptor is coupled to the ERK pathway by a direct interaction between NR2B and RasGRF1.* Neuron, 2003. 40(4): p. 775-84.
86. Baouz, S., et al., *Sites of phosphorylation by protein kinase A in CDC25Mm/GRF1, a guanine nucleotide exchange factor for Ras.* J Biol Chem, 2001. 276(3): p. 1742-9.
87. Mattingly, R.R., *Phosphorylation of serine 916 of Ras-GRF1 contributes to the activation of exchange factor activity by muscarinic receptors.* J Biol Chem, 1999. 274(52): p. 37379-84.
88. Yang, H., et al., *Phosphorylation of the Ras-GRF1 exchange factor at Ser916/898 reveals activation of Ras signaling in the cerebral cortex.* J Biol Chem, 2003. 278(15): p. 13278-85.
89. English, J.D. and J.D. Sweatt, *A requirement for the mitogen-activated protein kinase cascade in hippocampal long term potentiation.* J Biol Chem, 1997. 272(31): p. 19103-6.
90. Chen, H.J., et al., *A synaptic Ras-GTPase activating protein (p135 SynGAP) inhibited by CaM kinase II.* Neuron, 1998. 20(5): p. 895-904.
91. Oh, J.S., P. Manzerra, and M.B. Kennedy, *Regulation of the neuron-specific Ras GTPase-activating protein, synGAP, by Ca2+/calmodulin-dependent protein kinase II.* J Biol Chem, 2004. 279(17): p. 17980-8.
92. *Erratum.* Neuron, 2002. 33(1):151.

93. Krapivinsky, G., et al., *SynGAP-MUPP1-CaMKII synaptic complexes regulate p38 MAP kinase activity and NMDA receptor-dependent synaptic AMPA receptor potentiation.* Neuron, 2004. 43(4): p. 563-74.
94. Komiyama, N.H., et al., *SynGAP regulates ERK/MAPK signaling, synaptic plasticity, and learning in the complex with postsynaptic density 95 and NMDA receptor.* J Neurosci, 2002. 22(22): p. 9721-32.
95. Sanna, P.P., et al., *Phosphatidylinositol 3-kinase is required for the expression but not for the induction or the maintenance of long-term potentiation in the hippocampal CA1 region.* J Neurosci, 2002. 22(9): p. 3359-65.
96. Zhu, D., R.H. Lipsky, and A.M. Marini, *Co-activation of the phosphatidylinositol-3-kinase/Akt signaling pathway by N-methyl-D-aspartate and TrkB receptors in cerebellar granule cell neurons.* Amino Acids, 2002. 23(1-3): p. 11-7.
97. Morozov, A., et al., *Rap1 couples cAMP signaling to a distinct pool of p42/44MAPK regulating excitability, synaptic plasticity, learning, and memory.* Neuron, 2003. 39(2): p. 309-25.
98. Roberson, E.D., et al., *The mitogen-activated protein kinase cascade couples PKA and PKC to cAMP response element binding protein phosphorylation in area CA1 of hippocampus.* J Neurosci, 1999. 19(11): p. 4337-48.
99. Muller, D., et al., *Protein kinase C activity is not responsible for the expression of long-term potentiation in hippocampus.* Proc Natl Acad Sci U S A, 1990. 87(11): p. 4073-7.
100. Zhang, H.T., et al., *Effects of the novel PDE4 inhibitors MEM1018 and MEM1091 on memory in the radial-arm maze and inhibitory avoidance tests in rats.* Psychopharmacology (Berl), 2005.
101. Zhu, J.J., et al., *Ras and Rap control AMPA receptor trafficking during synaptic plasticity.* Cell, 2002. 110(4): p. 443-55.
102. Star, E.N., D.J. Kwiatkowski, and V.N. Murthy, *Rapid turnover of actin in dendritic spines and its regulation by activity.* Nat Neurosci, 2002. 5(3): p. 239-46.
103. Van Aelst, L. and H.T. Cline, *Rho GTPases and activity-dependent dendrite development.* Curr Opin Neurobiol, 2004. 14(3): p. 297-304.
104. van Galen, E.J. and G.J. Ramakers, *Rho proteins, mental retardation and the neurobiological basis of intelligence.* Prog Brain Res, 2005. 147: p. 295-317.
105. Hall, A., *Rho GTPases and the actin cytoskeleton.* Science, 1998. 279(5350): p. 509-14.
106. Stork, P.J., *Does Rap1 deserve a bad Rap?* Trends Biochem Sci, 2003. 28(5): p. 267-75.
107. Hayashi, M.L., et al., *Altered cortical synaptic morphology and impaired memory consolidation in forebrain- specific dominant-negative PAK transgenic mice.* Neuron, 2004. 42(5): p. 773-87.
108. Boda, B., et al., *The mental retardation protein PAK3 contributes to synapse formation and plasticity in hippocampus.* J Neurosci, 2004. 24(48): p. 10816-25.
109. Meng, J., et al., *Abnormal long-lasting synaptic plasticity and cognition in mice lacking the mental retardation gene Pak3.* J Neurosci, 2005. 25(28): p. 6641-50.

110. Meng, Y., et al., *Regulation of spine morphology and synaptic function by LIMK and the actin cytoskeleton.* Rev Neurosci, 2003. 14(3): p. 233-40.
111. Meng, Y., et al., *Abnormal spine morphology and enhanced LTP in LIMK-1 knockout mice.* Neuron, 2002. 35(1): p. 121-33.
112. Sarmiere, P.D. and J.R. Bamburg, *Head, neck, and spines: a role for LIMK-1 in the hippocampus.* Neuron, 2002. 35(1): p. 3-5.
113. Donnai, D. and A. Karmiloff-Smith, *Williams syndrome: from genotype through to the cognitive phenotype.* Am J Med Genet, 2000. 97(2): p. 164-71.
114. Korenberg, J.R., et al., *VI. Genome structure and cognitive map of Williams syndrome.* J Cogn Neurosci, 2000. 12 Suppl 1: p. 89-107.
115. Lisman, J.E. and A.M. Zhabotinsky, *A model of synaptic memory: a CaMKII/PP1 switch that potentiates transmission by organizing an AMPA receptor anchoring assembly.* Neuron, 2001. 31(2): p. 191-201.
116. Ko, J., et al., *Interaction between liprin-alpha and GIT1 is required for AMPA receptor targeting.* J Neurosci, 2003. 23(5): p. 1667-77.
117. Schulz, T.W., et al., *Actin/alpha-actinin-dependent transport of AMPA receptors in dendritic spines: role of the PDZ-LIM protein RIL.* J Neurosci, 2004. 24(39): p. 8584-94.
118. Wu, H., et al., *Interaction of SAP97 with minus-end-directed actin motor myosin VI. Implications for AMPA receptor trafficking.* J Biol Chem, 2002. 277(34): p. 30928-34.
119. Zhou, Q., M. Xiao, and R.A. Nicoll, *Contribution of cytoskeleton to the internalization of AMPA receptors.* Proc Natl Acad Sci U S A, 2001. 98(3): p. 1261-6.
120. Lin, B., et al., *Theta stimulation polymerizes actin in dendritic spines of hippocampus.* J Neurosci, 2005. 25(8): p. 2062-9.
121. Okamoto, K., et al., *Rapid and persistent modulation of actin dynamics regulates postsynaptic reorganization underlying bidirectional plasticity.* Nat Neurosci, 2004. 7(10): p. 1104-12.
122. Fukazawa, Y., et al., *Hippocampal LTP is accompanied by enhanced F-actin content within the dendritic spine that is essential for late LTP maintenance in vivo.* Neuron, 2003. 38(3): p. 447-60.
123. Lisman, J., *Actin's actions in LTP-induced synapse growth.* Neuron, 2003. 38(3): p. 361-2.
124. Daw, M.I., et al., *PDZ proteins interacting with C-terminal GluR2/3 are involved in a PKC-dependent regulation of AMPA receptors at hippocampal synapses.* Neuron, 2000. 28(3): p. 873-86.
125. Soderling, S.H. and J.D. Scott, *WAVE signalling: from biochemistry to biology.* Biochem Soc Trans, 2006. 34(Pt 1): p. 73-6.
126. Westphal, R.S., et al., *Scar/WAVE-1, a Wiskott-Aldrich syndrome protein, assembles an actin-associated multi-kinase scaffold.* Embo J, 2000. 19(17): p. 4589-600.
127. Eden, S., et al., *Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck.* Nature, 2002. 418(6899): p. 790-3.
128. Soderling, S.H., et al., *The WRP component of the WAVE-1 complex attenuates Rac-mediated signalling.* Nat Cell Biol, 2002. 4(12): p. 970-5.

129. Higgs, H.N. and T.D. Pollard, *Regulation of actin filament network formation through ARP2/3 complex: activation by a diverse array of proteins.* Annu Rev Biochem, 2001. 70: p. 649-76.
130. Welch, M.D., *The world according to Arp: regulation of actin nucleation by the Arp2/3 complex.* Trends Cell Biol, 1999. 9(11): p. 423-7.
131. Pilpel, Y. and M. Segal, *Rapid WAVE dynamics in dendritic spines of cultured hippocampal neurons is mediated by actin polymerization.* J Neurochem, 2005. 95(5): p. 1401-10.
132. Schenck, A., et al., *WAVE/SCAR, a multifunctional complex coordinating different aspects of neuronal connectivity.* Dev Biol, 2004. 274(2): p. 260-70.
133. Miki, H. and T. Takenawa, *Regulation of actin dynamics by WASP family proteins.* J Biochem (Tokyo), 2003. 134(3): p. 309-13.
134. Soderling, S.H., et al., *Loss of WAVE-1 causes sensorimotor retardation and reduced learning and memory in mice.* Proc Natl Acad Sci U S A, 2003. 100(4): p. 1723-8.
135. Endris, V., et al., *The novel Rho-GTPase activating gene MEGAP/ srGAP3 has a putative role in severe mental retardation.* Proc Natl Acad Sci U S A, 2002. 99(18): p. 11754-9.
136. Kandel, E.R., *The molecular biology of memory storage: a dialogue between genes and synapses.* Science, 2001. 294(5544): p. 1030-8.
137. Malenka, R.C., *The long-term potential of LTP.* Nat Rev Neurosci, 2003. 4(11): p. 923-6.
138. Lynch, M.A., *Long-term potentiation and memory.* Physiol Rev, 2004. 84(1): p. 87-136.
139. Soderling, T.R., *CaM-kinases: modulators of synaptic plasticity.* Curr Opin Neurobiol, 2000. 10(3): p. 375-80.
140. Sweatt, J.D., *Mitogen-activated protein kinases in synaptic plasticity and memory.* Curr Opin Neurobiol, 2004. 14(3): p. 311-7.
141. Soderling, T.R., B. Chang, and D. Brickey, *Cellular signaling through multifunctional Ca2+/calmodulin-dependent protein kinase II.* J Biol Chem, 2001. 276(6): p. 3719-22.
142. Colbran, R.J. and A.M. Brown, *Calcium/calmodulin-dependent protein kinase II and synaptic plasticity.* Curr Opin Neurobiol, 2004. 14(3): p. 318-27.
143. Soderling, T.R., *The Ca2+-calmodulin-dependent protein kinase cascade.* Trends in Biochem, 1999. 24: p. 232-235.
144. Means, A.R., *Regulatory cascades involving calmodulin-dependent protein kinases.* Mol Endocrinol, 2000. 14(1): p. 4-13.
145. Lemrow, S.M., Anderson, K.A., Joseph, J.D., Ribar, T.J., Noeldner, P.K., and Means, A.R., *Catalytic activity is required for calcium/calmodullin-dependent protein kinase IV to enter the nucleus.* J. Biol. Chem., 2004. 279(12): p. 11664-71.
146. Kang, H., et al., *An important role of neural activity-dependent CaM-KIV signaling in the consolidation of long-term memory.* Cell, 2001. 106(6): p. 771-83.
147. Enslen, H., et al., *Characterization of Ca2+/calmodulin-dependent protein kinase IV. Role in transcriptional regulation.* J Biol Chem, 1994. 269(22): p. 15520-7.

148. Impey, S., et al., *Phosphorylation of CBP mediates transcriptional activation by neural activity and CaM kinase IV.* Neuron, 2002. 34(2): p. 235-44.
149. Malenka, R.C. and R.A. Nicoll, *Long-term potentiation--a decade of progress?* Science, 1999. 285(5435): p. 1870-4.
150. Wu, G.Y., K. Deisseroth, and R.W. Tsien, *Spaced stimuli stabilize MAPK pathway activation and its effects on dendritic morphology.* Nat Neurosci, 2001. 4(2): p. 151-158.
151. Treisman, R., *Regulation of transcription by MAP kinase cascades.* Curr Opin Cell Biol, 1996. 8(2): p. 205-15.
152. Kelleher, R.J., 3rd, et al., *Translational control by MAPK signaling in long-term synaptic plasticity and memory.* Cell, 2004. 116(3): p. 467-79.
153. Chang, B.H., S. Mukherji, and T.R. Soderling, *Characterization of a calmodulin kinase II inhibitor protein in brain.* Proc. Natl. Acad. Sci. USA, 1998. 95: p. 10890-10895.
154. Fukunaga, K., T.R. Soderling, and E. Miyamoto, *Activation of Ca2+/calmodulin-dependent protein kinase II and protein kinase C by glutamate in cultured rat hippocampal neurons.* Journal of Biological Chemistry, 1992. 267(31): p. 22527-33.
155. Barria, A., et al., *Regulatory phosphorylation of AMPA-type glutamate receptors by CaM-KII during long-term potentiation [see comments].* Science, 1997. 276(5321): p. 2042-5.
156. Ho, N., et al., *Impaired synaptic plasticity and cAMP response element-binding protein activation in Ca2+/calmodulin-dependent protein kinase type IV/Gr-deficient mice.* J Neurosci, 2000. 20(17): p. 6459-72.
157. Yano, S., H. Tokumitsu, and T.R. Soderling, *Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway.* Nature, 1998. 396(6711): p. 584-7.
158. Derkach, V., A. Barria, and T.R. Soderling, *Ca2+/calmodulin-kinase II enhances channel conductance of alpha-amino-3- hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors.* Proc Natl Acad Sci U S A, 1999. 96(6): p. 3269-74.
159. Poncer, J.C., J.A. Esteban, and R. Malinow, *Multiple mechanisms for the potentiation of AMPA receptor-mediated transmission by alpha-Ca2+/calmodulin-dependent protein kinase II.* J Neurosci, 2002. 22(11): p. 4406-11.
160. Malinow, R. and R.C. Malenka, *AMPA receptor trafficking and synaptic plasticity.* Annu Rev Neurosci, 2002. 25: p. 103-26.
161. Oh, J.S., P. Manzerra, and M.B. Kennedy, *Regulation of the neuron-specific Ras GTPase activating protein, synGAP, by Ca2+/calmodulin-dependent protein kinase II.* J Biol Chem, 2004.
162. Arozarena, I., et al., *Activation of H-Ras in the endoplasmic reticulum by the RasGRF family guanine nucleotide exchange factors.* Mol Cell Biol, 2004. 24(4): p. 1516-30.
163. Buchsbaum, R., et al., *The N-terminal pleckstrin, coiled-coil, and IQ domains of the exchange factor Ras-GRF act cooperatively to facilitate activation by calcium.* Mol Cell Biol, 1996. 16(9): p. 4888-96.

164. Raught, B., Gingras, A.C. and Sonenberg, N., *Regulation of ribosomal recruitment in eukaryotes*. Translational Control of Gene Expression, ed. J.W.B.H. M.Sonenberg, and M.B. Mathews. 2000, Cold Spring Harbor, NY: Cold Spring Harbor Press. pp. 245-293.
165. Ouyang, Y., et al., *Tetanic stimulation leads to increased accumulation of Ca(2+)/calmodulin-dependent protein kinase II via dendritic protein synthesis in hippocampal neurons.* J Neurosci, 1999. 19(18): p. 7823-33.
166. Wu, G.Y., K. Deisseroth, and R.W. Tsien, *Activity-dependent CREB phosphorylation: Convergence of a fast, sensitive calmodulin kinase pathway and a slow, less sensitive mitogen- activated protein kinase pathway.* Proc Natl Acad Sci U S A, 2001. 98(5): p. 2808-2813.
167. Asaki, C., et al., *Localization of translational components at the ultramicroscopic level at postsynaptic sites of the rat brain.* Brain Res, 2003. 972(1-2): p. 168-76.
168. Smart, F.M., G.M. Edelman, and P.W. Vanderklish, *BDNF induces translocation of initiation factor 4E to mRNA granules: evidence for a role of synaptic microfilaments and integrins.* Proc Natl Acad Sci U S A, 2003. 100(24): p. 14403-8.
169. Job, C. and J. Eberwine, *Localization and translation of mRNA in dendrites and axons.* Nat Rev Neurosci, 2001. 2(12): p. 889-98.
170. Carlisle, H.J. and M.B. Kennedy, *Spine architecture and synaptic plasticity.* Trends Neurosci, 2005. 28(4): p. 182-7.
171. Maletic-Savatic, M., R. Malinow, and K. Svoboda, *Rapid dendritic morphogenesis in CA1 hippocampal dendrites induced by synaptic activity.* Science, 1999. 283(5409): p. 1923-7.
172. Nagerl, U.V., et al., *Bidirectional activity-dependent morphological plasticity in hippocampal neurons.* Neuron, 2004. 44(5): p. 759-67.
173. Luscher, C., et al., *Synaptic plasticity and dynamic modulation of the postsynaptic membrane.* Nat Neurosci, 2000. 3(6): p. 545-50.
174. Symons, M. and N. Rusk, *Control of vesicular trafficking by Rho GTPases.* Curr Biol, 2003. 13(10): p. R409-18.
175. Allen, K.M., et al., *PAK3 mutation in nonsyndromic X-linked mental retardation.* Nat Genet, 1998. 20(1): p. 25-30.
176. Billuart, P., et al., *Oligophrenin-1 encodes a rhoGAP protein involved in X-linked mental retardation.* Nature, 1998. 392(6679): p. 923-6.
177. Kutsche, K., et al., *Mutations in ARHGEF6, encoding a guanine nucleotide exchange factor for Rho GTPases, in patients with X-linked mental retardation.* Nat Genet, 2000. 26(2): p. 247-50.
178. Frey, U. and R.G. Morris, *Synaptic tagging and long-term potentiation.* Nature, 1997. 385(6616): p. 533-6.
179. Banker, G. and K. Goslin, *Developments in neuronal cell culture.* Nature, 1988. 336(6195): p. 185-6.
180. Cho, K.O., C.A. Hunt, and M.B. Kennedy, *The rat brain postsynaptic density fraction contains a homolog of the Drosophila discs-large tumor suppressor protein.* Neuron, 1992. 9(5): p. 929-42.
181. Kaech, S., et al., *Isoform specificity in the relationship of actin to dendritic spines.* J Neurosci, 1997. 17(24): p. 9565-72.

182. Hufner, K., et al., *The verprolin-like central (vc) region of Wiskott-Aldrich syndrome protein induces Arp2/3 complex-dependent actin nucleation.* J Biol Chem, 2001. 276(38): p. 35761-7.
183. Rohatgi, R., et al., *The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly.* Cell, 1999. 97(2): p. 221-31.
184. Strasser, G.A., et al., *Arp2/3 is a negative regulator of growth cone translocation.* Neuron, 2004. 43(1): p. 81-94.
185. Watanabe, M., *Glial processes are glued to synapses via Ca(2+)-permeable glutamate receptors.* Trends Neurosci, 2002. 25(1): p. 5-6.
186. Frey, U., et al., *Anisomycin, an inhibitor of protein synthesis, blocks late phases of LTP phenomena in the hippocampal CA1 region in vitro.* Brain Res, 1988. 452(1-2): p. 57-65.
187. Matsuzaki, M., et al., *Structural basis of long-term potentiation in single dendritic spines.* Nature, 2004. 429(6993): p. 761-6.
188. Roelandse, M., et al., *Focal motility determines the geometry of dendritic spines.* Neuroscience, 2003. 121(1): p. 39-49.
189. Sajikumar, S. and J.U. Frey, *Anisomycin inhibits the late maintenance of long-term depression in rat hippocampal slices in vitro.* Neurosci Lett, 2003. 338(2): p. 147-50.
190. Smith, W.B., et al., *Dopaminergic stimulation of local protein synthesis enhances surface expression of GluR1 and synaptic transmission in hippocampal neurons.* Neuron, 2005. 45(5): p. 765-79.
191. Paulsen, O. and T.J. Sejnowski, *Natural patterns of activity and long-term synaptic plasticity.* Curr Opin Neurobiol, 2000. 10(2): p. 172-9.
192. Ashby, M.C., et al., *Removal of AMPA receptors (AMPARs) from synapses is preceded by transient endocytosis of extrasynaptic AMPARs.* J Neurosci, 2004. 24(22): p. 5172-6.
193. Nicoll, R.A., S. Tomita, and D.S. Bredt, *Auxiliary subunits assist AMPA-type glutamate receptors.* Science, 2006. 311(5765): p. 1253-6.
194. Pilpel, Y. and M. Segal, *Activation of PKC induces rapid morphological plasticity in dendrites of hippocampal neurons via Rac and Rho-dependent mechanisms.* Eur J Neurosci, 2004. 19(12): p. 3151-64.
195. Gu, Y., et al., *Impaired conditioned fear and enhanced long-term potentiation in Fmr2 knock-out mice.* J Neurosci, 2002. 22(7): p. 2753-63.
196. Gu, Y. and D.L. Nelson, *FMR2 function: insight from a mouse knockout model.* Cytogenet Genome Res, 2003. 100(1-4): p. 129-39.
197. Balschun, D., et al., *Deletion of the ryanodine receptor type 3 (RyR3) impairs forms of synaptic plasticity and spatial learning.* Embo J, 1999. 18(19): p. 5264-73.
198. Futatsugi, A., et al., *Facilitation of NMDAR-independent LTP and spatial learning in mutant mice lacking ryanodine receptor type 3.* Neuron, 1999. 24(3): p. 701-13.
199. Tang, Y.P., et al., *Genetic enhancement of learning and memory in mice.* Nature, 1999. 401(6748): p. 63-9.
200. Tang, Y.P., et al., *Differential effects of enrichment on learning and memory function in NR2B transgenic mice.* Neuropharmacology, 2001. 41(6): p. 779-90.

201. Malleret, G., et al., *Inducible and reversible enhancement of learning, memory, and long-term potentiation by genetic inhibition of calcineurin.* Cell, 2001. 104(5): p. 675-86.
202. Pavlov, I., et al., *Role of heparin-binding growth-associated molecule (HB-GAM) in hippocampal LTP and spatial learning revealed by studies on overexpressing and knockout mice.* Mol Cell Neurosci, 2002. 20(2): p. 330-42.
203. Silva, A.J., et al., *Impaired spatial learning in alpha-calcium-calmodulin kinase II mutant mice*, in *Science*. 1992. p. 206-11.
204. Aiba, A., et al., *Reduced hippocampal long-term potentiation and context-specific deficit in associative learning in mGluR1 mutant mice.* Cell, 1994. 79(2): p. 365-75.
205. Brambilla, R., et al., *A role for the Ras signalling pathway in synaptic transmission and long-term memory.* Nature, 1997. 390(6657): p. 281-6.
206. Bourtchuladze, R., et al., *Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein.* Cell, 1994. 79(1): p. 59-68.
207. Giese, K.P., et al., *Hippocampus-dependent learning and memory is impaired in mice lacking the Ras-guanine-nucleotide releasing factor 1 (Ras-GRF1).* Neuropharmacology, 2001. 41(6): p. 791-800.
208. Davare, M.A., et al., *Inhibition of calcium/calmodulin-dependent protein kinase kinase by protein 14-3-3.* J Biol Chem, 2004. 279(50): p. 52191-9.
209. Maletic-Savatic, M., T. Koothan, and R. Malinow, *Calcium-evoked dendritic exocytosis in cultured hippocampal neurons. Part II: mediation by calcium/calmodulin-dependent protein kinase II.* J Neurosci, 1998. 18(17): p. 6814-21.
210. Shirke, A.M. and R. Malinow, *Mechanisms of potentiation by calcium-calmodulin kinase II of postsynaptic sensitivity in rat hippocampal CA1 neurons.* J Neurophysiol, 1997. 78(5): p. 2682-92.
211. Andrasfalvy, B.K. and J.C. Magee, *Distance-dependent increase in AMPA receptor number in the dendrites of adult hippocampal CA1 pyramidal neurons.* J Neurosci, 2001. 21(23): p. 9151-9.
212. Song, I., et al., *Interaction of the N-ethylmaleimide-sensitive factor with AMPA receptors.* Neuron, 1998. 21(2): p. 393-400.
213. Carroll, R.C., et al., *Dynamin-dependent endocytosis of ionotropic glutamate receptors.* Proc Natl Acad Sci U S A, 1999. 96(24): p. 14112-7.
214. Luscher, C., et al., *Role of AMPA receptor cycling in synaptic transmission and plasticity.* Neuron, 1999. 24(3): p. 649-58.
215. Noel, J., et al., *Surface expression of AMPA receptors in hippocampal neurons is regulated by an NSF-dependent mechanism.* Neuron, 1999. 23(2): p. 365-76.
216. Turrigiano, G.G., *AMPA receptors unbound: membrane cycling and synaptic plasticity.* Neuron, 2000. 26(1): p. 5-8.
217. Andrasfalvy, B.K. and J.C. Magee, *Changes in AMPA receptor currents following LTP induction on rat CA1 pyramidal neurones.* J Physiol, 2004. 559(Pt 2): p. 543-54.

218. Otani, S. and W.C. Abraham, *Inhibition of protein synthesis in the dentate gyrus, but not the entorhinal cortex, blocks maintenance of long-term potentiation in rats.* Neurosci Lett, 1989. 106(1-2): p. 175-80.
219. Ju, W., et al., *Activity-dependent regulation of dendritic synthesis and trafficking of AMPA receptors.* Nat Neurosci, 2004. 7(3): p. 244-53.
220. Kacharmina, J.E., et al., *Stimulation of glutamate receptor protein synthesis and membrane insertion within isolated neuronal dendrites.* Proc Natl Acad Sci U S A, 2000. 97(21): p. 11545-50.
221. Karachot, L., et al., *Induction of long-term depression in cerebellar Purkinje cells requires a rapidly turned over protein.* J Neurophysiol, 2001. 86(1): p. 280-9.
222. Broutman, G. and M. Baudry, *Involvement of the secretory pathway for AMPA receptors in NMDA-induced potentiation in hippocampus.* J Neurosci, 2001. 21(1): p. 27-34.
223. Misumi, Y., et al., *Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes.* J Biol Chem, 1986. 261(24): p. 11398-403.
224. Klausner, R.D., J.G. Donaldson, and J. Lippincott-Schwartz, *Brefeldin A: insights into the control of membrane traffic and organelle structure.* J Cell Biol, 1992. 116(5): p. 1071-80.
225. Oda, K., et al., *Brefeldin A arrests the intracellular transport of a precursor of complement C3 before its conversion site in rat hepatocytes.* FEBS Lett, 1987. 214(1): p. 135-8.
226. Wu, G.Y., K. Deisseroth, and R.W. Tsien, *Spaced stimuli stabilize MAPK pathway activation and its effects on dendritic morphology.* Nat Neurosci, 2001. 4(2): p. 151-8.
227. Job, C. and J. Eberwine, *Identification of sites for exponential translation in living dendrites.* Proc Natl Acad Sci U S A, 2001. 98(23): p. 13037-42.
228. Wilshaw, D., Dayan, P., Neural Comput., 1990. 2: p. 85-93.
229. Dudek, S.M. and M.F. Bear, *Bidirectional long-term modification of synaptic effectiveness in the adult and immature hippocampus.* J Neurosci, 1993. 13(7): p. 2910-8.
230. Kirov, S.A., et al., *Dendritic spines disappear with chilling but proliferate excessively upon rewarming of mature hippocampus.* Neuroscience, 2004. 127(1): p. 69-80.
231. Kirov, S.A., K.E. Sorra, and K.M. Harris, *Slices have more synapses than perfusion-fixed hippocampus from both young and mature rats.* J Neurosci, 1999. 19(8): p. 2876-86.
232. Izaki, Y. and J. Arita, *Long-term potentiation in the rat hippocampal CA1 region is inhibited selectively at the acquisition stage of discriminatory avoidance learning.* Brain Res, 1996. 723(1-2): p. 162-8.
233. Schroeder, B.W. and P. Shinnick-Gallagher, *Fear learning induces persistent facilitation of amygdala synaptic transmission.* Eur J Neurosci, 2005. 22(7): p. 1775-83.
234. Lebel, D., Y. Grossman, and E. Barkai, *Olfactory learning modifies predisposition for long-term potentiation and long-term depression induction in the rat piriform (olfactory) cortex.* Cereb Cortex, 2001. 11(6): p. 485-9.

235. Rioult-Pedotti, M.S., D. Friedman, and J.P. Donoghue, *Learning-induced LTP in neocortex.* Science, 2000. 290(5491): p. 533-6.
236. Terashima, A., et al., *Regulation of synaptic strength and AMPA receptor subunit composition by PICK1.* J Neurosci, 2004. 24(23): p. 5381-90.
237. Lu, W. and E.B. Ziff, *PICK1 interacts with ABP/GRIP to regulate AMPA receptor trafficking.* Neuron, 2005. 47(3): p. 407-21.
238. Bortolotto, Z.A. and G.L. Collingridge, *A role for protein kinase C in a form of metaplasticity that regulates the induction of long-term potentiation at CA1 synapses of the adult rat hippocampus.* Eur J Neurosci, 2000. 12(11): p. 4055-62.
239. Gisabella, B., M.J. Rowan, and R. Anwyl, *Mechanisms underlying the inhibition of long-term potentiation by preconditioning stimulation in the hippocampus in vitro.* Neuroscience, 2003. 121(2): p. 297-305.
240. Wu, J., M.J. Rowan, and R. Anwyl, *Synaptically stimulated induction of group I metabotropic glutamate receptor-dependent long-term depression and depotentiation is inhibited by prior activation of metabotropic glutamate receptors and protein kinase C.* Neuroscience, 2004. 123(2): p. 507-14.
241. Weiler, I.J., et al., *Fragile X mental retardation protein is necessary for neurotransmitter-activated protein translation at synapses.* Proc Natl Acad Sci U S A, 2004. 101(50): p. 17504-9.
242. Schenck, A., et al., *CYFIP/Sra-1 controls neuronal connectivity in Drosophila and links the Rac1 GTPase pathway to the fragile X protein.* Neuron, 2003. 38(6): p. 887-98.