MULTIPLE Gq-COUPLED RECEPTORS CONVERGE ON A COMMON PROTEIN SYNTHESIS-DEPENDENT LONG TERM DEPRESSION THAT IS AFFECTED IN FRAGILE X SYNDROME

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DEDICATION

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Activation of Gq-coupled group I metabotropic glutamate receptors (mGluR1, mGluR5) induces long-term synaptic depression (LTD) that requires rapid, dendritic protein synthesis. The significance of protein synthesis-dependent mGluR LTD to cognitive function is highlighted by the recent finding that mGluR-dependent LTD is enhanced and protein synthesis-*in*dependent in the mouse model of fragile X syndrome mental retardation (FXS, *Fmr1* KO mice). In fact, group I mGluR antagonism ameliorates some symptoms of FXS in model organisms. However, disagreement exists in the literature as to the specific roles of mGluR1 and mGluR5 in LTD.

Using pharmacological and genetic manipulations, I find that mGluR1 or mGluR5 activation is sufficient to induce LTD. In contrast, I see a selective role for persistent mGluR1 activity in expression of LTD induced with the group I mGluR agonist, DHPG. These data demonstrate a novel role for mGluR1 in induction and expression of LTD at hippocampal Schaffer collateral-CA1 synapses and confirm a role for mGluR5 in induction of LTD at this synapse.

LTD induced synaptically with paired-pulse low frequency stimulation (PP-LFS) is Gq- and protein synthesis-dependent and shares common signaling and expression

mechanisms with DHPG-induced LTD. However, I find that PP-LFS LTD persists in the presence of group 1 mGluR antagonists and in mGluR1 or mGluR5 knockout mice. These data led to the hypothesis that Gq-coupled receptors other than mGluRs are activated by PP-LFS to induce LTD, and LTD mediated by these receptors should share similar signaling and expression mechanisms with mGluR LTD.

A previous study shows that muscarinic acetylcholine receptors (mAChRs) activate protein synthesis in hippocampal CA1 dendrites. Data presented here demonstrate that PP-LFS activates both Gq-coupled mAChRs and mGluRs to induce LTD. Pharmacological activation of mAChRs induces LTD that requires rapid protein synthesis and activation of ERK and mTOR translational activation pathways. New proteins maintain mAChR-dependent LTD through a persistent decrease in surface AMPA receptors. In addition, mAChR LTD is enhanced and protein synthesis-*in*dependent in *Fmr1* knock-out mice. These data reveal that multiple Gq-coupled receptors converge on a common protein synthesis-dependent LTD mechanism that is aberrant in FXS. These findings suggest novel therapeutic strategies for FXS in the form of mAChR antagonists.

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LIST OF ABBREVIATIONS

4E-BP -- eIF4E binding protein

5'-TOP – 5' terminal oligopyrimidine tract

ACh -- acetylcholine

ACSF – artificial cerebrospinal fluid

AKT – protein kinase B

AMPAR -- α-**a**mino-3-hydroxy-5-**m**ethylisoxazole-4- **p**ropionic **a**cid sensitive glutamate receptor

AP5 – D(-)-2-amino-5-phosphonopentanoic acid

Arc – activity-regulated cytoskeletal protein

αCaMKII -- calcium/calmodulin-dependent protein kinase II

CCh – carbamoylcholine chloride

CHX -- cyclohexamide

CPE – cytoplasmic polyadenylation element

CPEB – cytoplasmic polyadenylation element binding protein

DAG -- diacylglycerol

DHPG – dihydroxyphenylglycine

D1/5R – dopamine receptor subtype 1/5

EF1α – elongation factor 1α

eIF4F – eukaryotic initiation factor 4F

eIF4E– eukaryotic initiation factor 4E

eIF4G– eukaryotic initiation factor 4G

eIF4A– eukaryotic initiation factor 4A

eCB – endocannabinoid

EF1α – elongation factor 1α

E-LTP/D – early phase long-term potentiation/depression

ERK – extracellular signal-related kinase

FMRP – Fragile X Mental Retardation Protein

FXS – Fragile X Syndrome mental retardation

GABA – gamma-aminobutyric acid

GluR1/2 – AMPAR subunit 1/2

GPCR – G-protein-coupled receptor

GR – glucocorticoid receptor

GRIP – glutamate receptor interacting protein

IA – inhibitory avoidance

IP3 – inositol triphosphate

KO – knock out

L-LTP/D – late phase long-term potentiation/depression

LTP – long term potentiation

LTD – long term depression

LY367385 – (S)-(+)-α-amino-4-carboxy-2-methlbenzeneacetic acid

LY341495 – (2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl)

propanoic acid

mAChR – muscarinic acetylcholine receptor

MAP1B – microtubule-associated protein 1B

mGluR – metabotropic glutamate receptor

MPEP – 2-methyl-6-(phenylethynyl)-pyridine

mPFC – medial pre-frontal cortex

mTOR – mammalian target of rapamycin

NMDAR – N-methyl-D-aspartate sensitive glutamate receptor

NAc – Nucleus Accumbens

PDE4B3 – type 4 phosphodiesterase B3

PDK – 3'-phosphoinositide-dependent kinase

PI3K – Phoshpinositide-3 kinase

PIP2 – phosphatidylinositol bisphosphate

PIP3 – phosphatidylinositol triphosphate

PKC -- diacylglycerol- regulated protein kinase C

PLC –phospholipase C

PP-LFS – paired pulse low frequency stimulation

PSD-95 – post-synaptic density protein of 95kDa size

S6K – ribosomal protein S6 kinase

STEP -- striatal-enriched protein tyrosine phosphatase

TPA – tissue plasminogen activator

UO126 – 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)-butadiene

UTR – untranslated region

VTA – ventral tegmental area

WT – wild type

CHAPTER ONE
Introduction

SYNAPTIC PLASTICITY: A CELLULAR MECHANISM FOR LEARNING AND MEMORY?

Understanding the cellular mechanisms resulting in adaptive cognitive processes such as learning, memory, and addiction is one of the fundamental questions of modern neuroscience. As early as 1949, Donald Hebb proposed that multiple iterations of coincident synaptic activity could result in long lasting changes in synaptic efficacy. It was almost 20 years later before the first experimental demonstrations that long-lasting changes in synaptic efficacy could occur as a result of patterned activity (reviewed in) (Kandel and Tauc, 1965; Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973; Miles et al., 2005). Though these first reports of synaptic plasticity described a long-term increase in synaptic strength, termed long-term potentiation (LTP), it was soon recognized that if LTP existed, there would likely be a mechanism for inducing long-lasting decreases in synaptic strength as well (long term depression, LTD). In 1977 Lynch et al were the first to report a persistent decrease in synaptic strength in response to synaptic stimulation (Lynch et al., 1977). After these initial discoveries, much effort was spent determining stimulation paradigms that could reliably induce synaptic plasticity as well as the receptors and signaling molecules involved in LTP and LTD.

The induction and expression mechanisms of LTP and LTD vary widely from one brain region to another. Reviewed in (Malenka and Bear, 2004; Massey and Bashir, 2007). N-methyl-D-aspartate receptor (NMDAR)-dependent LTP and LTD are the most

ubiquitous and well-studied forms of synaptic plasticity, but multiple neurotransmitter receptors, both ionotropic and metabotropic, induce LTP and LTD. Expression mechanisms also vary from alterations in pre-synaptic release efficacy to alterations in postsynaptic AMPA receptor number and/or channel properties. However, despite many correlative studies implicating LTP and LTD in memory, the "holy grail" of synaptic plasticity research, i.e. demonstrating that LTP and/or LTD truly are cellular mechanisms underlying learning and memory, remained elusive until very recently. In 2006, Whitlock et al. showed that a hippocampal-dependent learning task can induce LTP at hippocampal synapses, and Pastalkova et al. demonstrated that memories can be "erased" by a compound that reverses the maintenance of LTP suggesting that memory and LTP maintenance share a common mechanism (Pastalkova et al., 2006; Whitlock et al., 2006). Taken together, these are the first studies to provide strong evidence that LTP could be one of the molecular mechanisms underlying learning and/or memory.

Evidence supporting a role for LTD in learning and memory is still largely correlative at this point. Treatments that block or genetically inhibit NMDAR, metabotropic glutamate receptor (mGluR), muscarinic acetylcholine receptor (mAChR), and retinoid X receptor γ (RXRγ)-dependent LTD in various brain regions result in impairments in learning and memory (Aiba et al., 1994; Conquet et al., 1994; Ming-Yi Chiang et al., 1998; Koekkoek et al., 2003; Lee et al., 2003; Warburton et al., 2003; Welsh et al., 2005; Wietrzych et al., 2005; Power et al., 2006; Scheiderer et al., 2006; Volk et al., 2006; Herrera-Morales et al., 2007; Simonyi et al., 2007; Volk et al., 2007). However, in some cases it is not clear that these manipulations affect only LTD and not LTP. Though there are no studies definitively demonstrating LTD induction as a result

of a learning paradigm, LTD is induced in the nucleus accumbens (NAc) in response to chronic cocaine administration (specifically in animals displaying sensitization to cocaine), suggesting that LTD in the NAc may play a role in sensitization and/or addiction to cocaine (Thomas et al., 2001). Preliminary studies suggest that extinction of auditory fear conditioning induces mGluR-dependent LTD in the lateral amygdala (Kim et al., 2007). Also, in the perirhinal cortex, novelty recognition training results in fewer neurons being activated in response to familiar stimuli compared with novel stimuli, suggesting that an LTD-like mechanism may be involved in novelty recognition. Interestingly, this differential effect on neuronal activation is blocked by muscarinic acetylcholine receptor antagonists injected into the perirhinal cortex, and mAChR antagonists also block novelty recognition and mAChR-LTD in the perirhinal cortex, indicating a significant role for acetylcholine neurotransmission in learning and memory (Warburton et al., 2003).

SYNAPTIC PLASTICITY AND DENDRITIC PROTEIN SYNTHESIS

In the past few decades, dendritic protein synthesis has emerged as a key regulator of synaptic plasticity. New protein synthesis is also critical for the formation of long term memories, an idea put foreword almost 60 years ago that has since been supported by a large body of work (reviewed in) (Monné, 1948; Flexner et al., 1963; Sutton and Schuman, 2006). Alterations in protein synthesis-dependent synaptic plasticity correlate with deficits in cognitive function, supporting a critical role for

protein synthesis-dependent synaptic plasticity in normal cognitive function (Huber et al., 2002; Lee et al., 2005a).

Until recently, neuronal protein synthesis was thought to occur exclusively in the soma. However, the discovery of protein synthesis machinery, including polyribosomes, mRNA, and endoplasmic reticulum- and golgi-like structures, in dendrites prompted speculation that protein synthesis could occur locally in dendrites (reviewed in) (Job and Eberwine, 2001; Steward and Schuman, 2001; Sutton and Schuman, 2006). This local, dendritic protein synthesis could provide a mechanism that would allow neurons to make rapid and long-lasting changes at individual (or closely localized groups of) synapses in response to activity. In this view, dendritic protein synthesis would increase the temporal and spatial resolution of synaptic plasticity in an individual neuron, which would result in an increase in the information processing capacity of the neuron.

Several different neurotransmitter receptors stimulate the rapid *de novo* synthesis of proteins in dendrites. Activation of TrkB receptors (via brain derived neurotrophic factor, BDNF) or dopamine D1/5 receptors induces protein synthesis in dendrites as well as protein synthesis-dependent, transcription-independent LTP (Frey et al., 1991; Huang and Kandel, 1995; Kang and Schuman, 1996; Matthies et al., 1997; Aakalu et al., 2001). Stimulation of mGluRs or mAChRs also induces protein synthesis in dendrites, and activation of these receptors induces protein synthesis-dependent, transcription-independent LTD (Feig and Lipton, 1993; Weiler et al., 1996; Huber et al., 2000; Shin et al., 2004; Volk et al., 2007).

My thesis research focused on determining the signaling and expression mechanisms of protein synthesis-dependent mGluR and mAChR LTD.

Protein Synthesis-Dependent Plasticity: mGluRs

mGluRs: Overview

Metabotropic glutamate receptors are G protein-coupled glutamate receptors that are classified into three groups based on G protein coupling and sequence similarity (see Table 1), (reviewed in Conn and Pin, 1997). Group I mGluRs couple to the Gαq/11 class of heterotrimetic G proteins and are located primarily in the perisynaptic region of the postsynaptic density. Group II and Group III mGluRs couple to the Gαi/o class of heterotrimeric G proteins and are located primarily presynaptically. Group I mGluRs mediate persistent changes in neuronal and synaptic function and are important for many cognitive processes characterized by long lasting changes in neuronal function including learning and memory, drug addiction, and chronic pain (Chiamulera et al., 2001; Karim et al., 2001; Balschun and Wetzel, 2002; Huber et al., 2002; McGeehan and Olive, 2003; Szumlinski et al., 2003; Swanson et al., 2005; El-Kouhen et al., 2006; Lominac et al., 2006).

Classification	Group Members	Coupling	Localization
Group I	mGluR1 & 5	Gq	primarily postsynaptic (perisynaptic)
Group II	mGluR2 & 3	Gi/o	primarily presynaptic
GroupIII	mGluR4, 6, 7, & 8	Gi/o	primarily presynaptic

Table 1. mGluR Classification

In the hippocampus, activation of group I mGluRs has a number of effects on CA1 pyramidal neuron function, including increasing $[Ca^{2+}]_I$ (primarily due to release from intracellular stores), activation of a long lasting inward current, depolarization (due to a decrease in K+ leak conductance), suppression of I_{AHP} (Ca^{2+}-activated K^+ current), and potentiation of NMDAR currents. Interestingly, though mGluR1 and 5 are structurally similar Gq-coupled receptors activated by the same neurotransmitter with similar affinities for glutamate, there is evidence that these receptors can have distinct functions in the same neuron, and there is some debate as to the relative contributions of mGluR1 and mGluR5 to the above effects in CA1 pyramidal neurons (Mannaioni et al., 2001; Ireland and Abraham, 2002; Poisik et al., 2003; Rae and Irving, 2004).

In addition to direct effects on CA1 pyramidal neurons, activation of group I mGluRs on CA1 pyramidal neurons enhances release of endocannabinoids (eCBs) from the postsynaptic terminal which act on cannabinoid receptors (CB1) located on the presynaptic terminals of inhibitory interneurons (Edwards et al., 2006). Depending on the level of mGluR stimulation, this results in a transient or persistent decrease in GABA release from these interneurons

Consistent with a role in long-lasting modifications of neuronal function, mGluR activation induces LTD in multiple brain regions, facilitates NMDAR-dependent LTP at hippocampal SC-CA1 synapses, and induces a persistent prolongation of epileptiform bursts in the CA3 region of the hippocampus. Of particular importance is the fact that all of these processes rely on new protein synthesis (Cohen and Abraham, 1996; Merlin et al., 1998; Huber et al., 2000).

Group I mGluRs in Learning and Memory

Numerous studies implicate group I mGluR function in learning, memory, and behavior. Studies in the cerebellum demonstrate that mGluR1 activity is required for LTD at granule cell and climbing fiber synapses onto Purkinje cells and at the mossy fiber-deep cerebelar nucleus synapse, and implicate mGluR1-dependent LTD in motor learning (Ichise et al., 2000; Hirono et al., 2001; Zhang and Linden, 2006; reviewed in Ito, 2001). mGluR1 and mGluR5 are also implicated nociceptive plasticity in the spinal cord and the associated pain sensitivity (Karim et al., 2001; El-Kouhen et al., 2006). Group I mGluR activity is necessary for multiple hippocampal-dependent learning tasks: pharmacological blockade or genetic knockout of either mGluR1 or mGluR5 results in impairments in spatial learning (Conquet et al., 1994; Lu et al., 1997; Balschun and Wetzel, 2002; El-Kouhen et al., 2006) and pharmacological blockade of mGluR1 or mGluR5 specifically in the CA1 region of the hippocampus impairs inhibitory avoidance learning while blockade of mGluR1 impairs extinction learning of inhibitory avoidance memory (Simonyi et al., 2007). Surprisingly, mGluR5 has also been directly implicated in cocaine addiction; mGluR5 KO mice do not learn to self administer cocaine even though they show similar cocaine-induced increases of dopamine in the nucleus accumbens compared to WT controls (Chiamulera et al., 2001), and the mGluR5 antagonist MPEP selectively impairs the conditioned rewarding effects of cocaine but not other drugs of abuse (McGeehan and Olive, 2003). These studies highlight diverse roles of Group I mGluRs in regulating persistent changes in neuronal function.

mGluR LTD

Activation of the Gq-coupled group I mGluRs induces LTD at Schaffer collateral-CA1 (SC-CA1) synapses which requires rapid (within minutes) dendritic protein synthesis (Huber et al., 2000). MGluR LTD does not require NMDA receptor activation or transcription of new mRNA. Evidence supporting a role for dendritic protein synthesis in mGluR LTD include the demonstration that mGluR LTD is rapidly blocked by protein synthesis inhibitors, mGluR LTD is blocked by inclusion of a protein synthesis inhibitor the patch pipette, and most importantly, mGluR LTD is still elicited in dendrites that have been mechanically separated from their cell bodies, strongly suggesting that protein synthesis-dependent mGluR LTD can occur using proteins synthesized in dendrites. Further supporting the idea that mGluRs can induce protein synthesis in dendrites, mGluR activation induces protein synthesis in synaptoneurosomes (cellular fractions enriched for synapses), and local application of the group I mGluR agonist dihydroxyphenylgycine (DHPG) to distal hippocampal CA1 dendrites induces protein synthesis in the dendrites without a concomitant increase in protein levels in the soma (Weiler et al., 1996; Shin et al., 2004; Huang et al., 2005). Thus, mGluR LTD requires rapid translation of pre-existing mRNAs at or near the site of synaptic activation.

In addition to SC-CA1 synapses in the hippocampus, activation of group I mGluRs induces protein synthesis-dependent LTD in the cerebellum at mossy fiber-deep cerebellar nucleus synapses and granule cell-purkinje cell synapses, in the ventral

tegmental area, and in the dentate gyrus of freely moving rats (Karachot et al., 2001; Naie and Manahan-Vaughan, 2005; Zhang and Linden, 2006; Mameli et al., 2007).

mGluR LTD: Expression Mechanism

mGluR LTD can be elicited pharmacologically with the group I mGluR agonist DHPG (50-100μM, 5-20min.). Additionally, synaptic stimulation with paired pulse-low frequency stimulation (PP-LFS) induces LTD by co-activation of group I mGluRs and Gq-coupled mAChRs (see chapter 3). mGluR LTD in mature rodents is mediated by a persistent decrease in AMPAR surface expression. DHPG treatments that induce LTD also stimulate AMPA receptor endocytosis and a subsequent decrease in surface AMPA receptor expression. This decrease in AMPAR surface expression persists for at least one hour and requires new protein synthesis (Snyder et al., 2001; Xiao et al., 2001; Nosyreva and Huber, 2005). Inhibition of postsynaptic endocytosis blocks DHPG-induced LTD, further supporting a role for AMPAR endocytosis in mGluR LTD (Xiao et al., 2001). It should be noted however, that there is a developmental switch in the mechanism of mGluR LTD such that mGluR LTD at hippocampal Schaffer-collateral synapses in young rodents (younger than ~ 2-2.5 weeks) is mediated by a change in presynaptic function and is not protein synthesis-dependent (Bolshakov and Siegelbaum, 1994; Fitzjohn et al., 2001; Zakharenko et al., 2002; Feinmark et al., 2003; Rammes et al., 2003; Nosyreva and Huber, 2005)

mGluR Signaling and Regulation of Translation

Group I mGluRs are Gq-coupled glutamate receptors, and as such, activate the canonical Gq-dependent phospholipase C (PLC) signaling cascade. PLC hydrolyzes PIP2 to form IP3 and DAG, which in turn releases of Ca^{++} from internal stores and activates PKC respectively. However, mGluR LTD is unaffected by inhibition of PKC or chelation of intracellular Ca^{++}, demonstrating that this classical Gq signaling pathway is *not* necessary for mGluR-dependent LTD (Schnabel et al., 1999; Fitzjohn et al., 2001). mGluR stimulation in the hippocampus (and many other brain regions) activates the extracellular signal-regulated kinase 1/2 (ERK1/2) and phosphoinositide 3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) translational regulatory pathways, and both of these pathways are required for mGluR-dependent LTD (Gallagher et al., 2004; Hou and Klann, 2004). mGluR-dependent ERK activation is Gq-dependent, but like mGluR LTD, it is independent of the classical Gq-dependent PLC-PKC-Ca^{2+} signaling pathway, strengthening the argument that regulation of translation by mGluRs does not occur through this pathway (Peavy et al., 2001; Zhao et al., 2004; Wang et al., 2006). Studies in the hippocampus, striatum, and cultured astrocytes suggest group I mGluRs activate ERK via the Src family of tyrosine kinases (Peavy et al., 2001; Zhao et al., 2004; Mao et al., 2005b). In the striatum, mGluR-dependent ERK activation involves phosphorylation and inactivation of protein phosphatase 2A (PP2A) by a Src family tyrosine kinase (Mao et al., 2005b), and mGluR-dependent phosphorylation of α-CaMKII is also required for striatal ERK activation (Choe and Wang, 2001; Mao et al., 2005b). However, a role for Src in hippocampal mGluR LTD has not yet been established. The upstream signaling events in mGluR mediated PI3K-Akt-mTOR activation are less well defined, but

unpublished data from our lab indicate that mGluR interaction with the scaffolding protein Homer is necessary for mGluR-induced PI3K-Akt-mTOR signaling and LTD (Ronesi and Huber, 2007). Homer binds to mGluRs and to the protein PI 3-Kinase Enhancer (PIKE), which is a GTPase that binds PI 3-kinase and stimulates its lipid kinase activity (Ahn and Ye, 2005). Interestingly, mGuR-Homer interactions are also required for ERK activation in the striatum, but data from our lab demonstrate that mGluR-Homer interactions are not necessary for ERK activation in the hippocampus, suggesting that there is region specific regulation of these signaling pathways (Mao et al., 2005a; Ronesi and Huber, 2007).

ERK and mTOR signaling have been implicated in many forms of protein synthesis dependent plasticity. In addition to mGluR LTD, ERK and/or mTOR signaling are required for protein synthesis-dependent LTP induced by activation of β-adrenergic receptors, dopamine D1/5 receptors, and Trk B receptors, indicating that these signaling pathways may represent a conserved mechanism for regulating protein synthesis-dependent plasticity (Klann et al., 2004; Pfeiffer and Huber, 2006; Gelinas et al., 2007).

Regulation of translation can take place at either the initiation step or during peptide elongation. During initiation, most mRNAs bind to the ribosome via interaction of the 5'mRNA cap (5'm7GpppN, N = any nucleotide) with the cap-binding protein complex, eukaryotic initiation factor 4F (eIF4F). eIF4F is a heterotrimeric complex consisting of eIF4E, eIF4A and eIF4G. eIF4E binds to the 5' mRNA cap, eIF4A is a helicase that facilitates unwinding of RNA 2º structure, and eIF4G is a scaffolding proteins that binds both eIF4E and eIF4A, as well as other proteins that regulate translation initiation. mGluR LTD is blocked by inclusion of cap analogue, which blocks

cap-dependent translation, in the patch pipette, suggesting that cap-dependent translation is necessary for mGluR LTD (Huber et al., 2000). One way that cap-dependent translation is regulated is by binding of eIF4E-binding protein (4E-BP) to eIF4E. eIF4E that is bound to 4E-BP cannot associate with eIF4G. Phosphorylation of 4E-BP inhibits its interaction with eIF4E and allows translation to proceed. eIF4E is also directly phosphorylated. The physiological consequences of eIF4E phosphorylation are somewhat less clear, but in general eIF4E phosphorylation facilitates translation (Klann et al., 2004). Both mTOR and ERK1/2 phosphorylate 4E-BP (Lin et al., 1994; Beretta et al., 1996), and ERK phosphorylates Mnk1, which in turn phosphorylates eIF4E (Waskiewicz et al., 1997; Waskiewicz et al., 1999). See Figure 1.1.

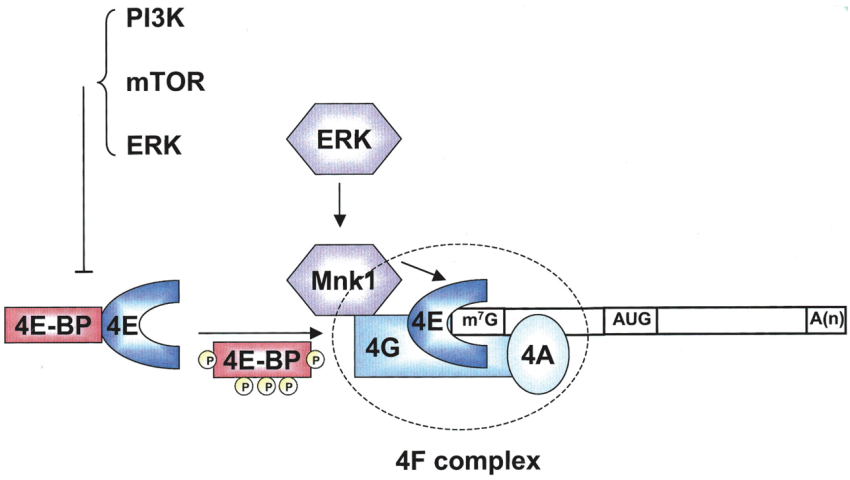


Figure 1.1, Regulation of cap-dependent translation initiation.
From (Klann et al., 2004)

In the hippocampus, mGluR activation results in mTOR-dependent phosphorylation of 4E-BP and ERK-dependent phosphorylation of Mnk1 and eIF4E, with a concomitant increase in eIF4F complex formation (Banko et al.,

2006). Consistent with role for 4E-BP regulation and cap-dependent translation in in mGluR LTD, mGluR LTD is enhanced in 4E-BP KO mice (Banko et al. 2005). In addition to regulating cap-dependent translation, ERK and mTOR regulate translation of "5'-TOP" mRNAs through activation of p70 S6 kinase (S6K) (Klann et al., 2004) 5'TOP mRNAs contain a 5'-terminal-oligopyrimidine tract and encode ribosomal proteins as well as other proteins involved in translation. Though the details of 5'TOP translation are not well understood, phosphorylation of p70 ribosomal S6 protin by S6K1 appears to be important. It is not known yet if 5'TOP mRNA translation plays a critical role in mGluR-dependent LTD, but mGluR stimulation does result in S6K1 activation (phosphorylation at the mTOR site), and synthesis of at least one 5'TOP mRNA, EF1α (Ronesi and Huber, 2007). Presumably, the synthesis of 5'TOP mRNAs would enhance translational capacity near activated synapses.

See Figure 1.2.

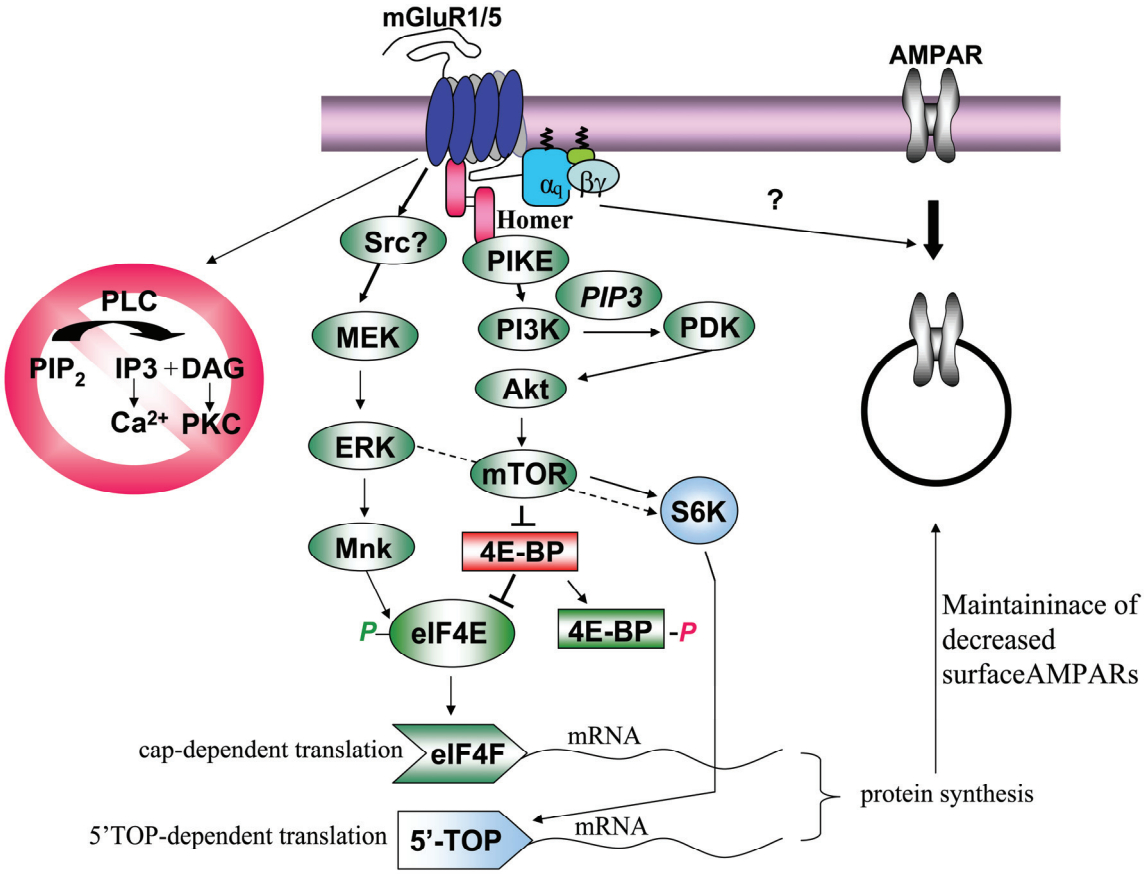


Figure 1.2, Signaling mechanisms in mGluR LTD

Translation initiation is also regulated by the RNA binding protein cytoplasmic polyadenylation element-binding protein, CPEB. CPEB binds to CPE sequences in the 3'-UTR of a subset of mRNAs. Under basal conditions CPEB binding inhibits translation via interaction with maskin, a specific 4E-BP that binds eIF4E and prevents formation of the initiation complex. Phosphorylation of CPEB leads to elongation of the poly(A)- tail which causes dissociation of maskin, recruitment of eIF4G and initiation complex formation (Klann et al., 2004; Pfeiffer and Huber, 2006; Wells, 2006). CPEB

phosphorylation and regulation of translation through polyadenylation has been implicated in some forms of protein synthesis-dependent LTP, but it has not been shown to be critical for mGluR LTD (Si et al., 2003; Alarcon et al., 2004). mGluR stimulation does induce polyadenylation and synthesis of the protein tissue plasminogen activator, tPA (Shin et al., 2004). tPA mRNA binds CPEB and is found in dendrites. Interestingly, the concentration of DHPG used to induce synthesis of tPA in this study is probably not sufficient to induce LTD, but is sufficient to induce mGluR and protein synthesis-dependent facilitation of NMDA receptor-dependent LTP (Cohen and Abraham, 1996; Huber et al., 2001). tPA is also necessary for protein synthesis-dependent L-LTP in the hippocampus (Huang et al., 1996; Pang et al., 2004). Taken together, these data suggest that mGluR-dependent regulation of translation through CPEB might contribute to protein synthesis-dependent LTP. Preliminary results from our lab indicate that mGluR LTD is moderately reduced in tPA knockout mice, but it is still unclear if translation regulation via CPEB and mRNA polyadenylation is involved in mGluR LTD.

In addition to inducing protein synthesis, mGluR activation induces internalization of AMPA receptors. While maintaining a persistent decrease in AMPAR surface expression requires new protein synthesis, the initial internalization step does not require new protein synthesis (Snyder et al., 2001; Nosyreva and Huber, 2005). Surprisingly, the signaling mechanisms responsible for mGluR-induced AMPAR internalization are not known. mGluRs still stimulate AMPAR internalization in *Fmr1* KO mice (see following section on fragile X mental retardation syndrome), but mGluR-dependent activation of the mTOR pathway is disrupted in these mice, so it is unlikely that mTOR signaling is required for mGluR-dependent AMPAR internalization (Ronesi

and Huber, 2007). ERK activation is intact in *Fmr1* KO mice, so it is possible that in addition to regulating translation, ERK functions in AMPAR endocytosis. Another candidate signaling mechanism is tyrosine phosphatase activity, which has been implicated in mGluR LTD and mGluR-induced AMPAR internalization, perhaps through dephosphorylation of the GluR2 subunit of AMPARs (Huang and Hsu, 2006; Moult et al., 2006).

How Does De Novo Dendritic Pprotein Synthesis Contribute to mGluR LTD?

Proteins synthesized in response to mGluR stimulation include the Fragile X Mental Retardation Protein (FMRP), microtubule-associated protein 1B (MAP1B), activity regulated cytoskeletal protein (Arc), Elongation Factor 1α (EF1α), α-calcium/calmodulin-dependent protein kinase II (αCaMKII), the AMPAR subunit GluR2, postsynaptic density protein of 95kDa (PSD-95), tissue plasminogen activator (tPA), and striatal-enriched protein tyrosine phosphatase (STEP) (Weiler et al., 1997; Todd et al., 2003; Shin et al., 2004; Huang et al., 2005; Hou et al., 2006; Mameli et al., 2007; Muddashetty et al., 2007; Waung et al., 2007). Very little data has been published describing a mechanism by which any of these proteins could function to maintain LTD. However, a number of recent studies presented at the 2007 Society for Neuroscience meeting provide some promising insight. Not surprisingly, it appears that the majority of proteins whose functions in mGluR LTD are being elucidated fall into two general categories: regulation of AMPA receptor trafficking/function (Arc, MAP1B, STEP, and GluR2) and regulation of translation (EF1α, αCaMKII (?), and FMRP).

Arc: Recent studies reported that Arc interacts with endocytosis machinery (endophilin and dynamin) and regulates AMPA receptor endocytosis (Chowdhury et al., 2006). Unpublished data from our lab demonstrate that Arc protein is rapidly synthesized in response to mGluR stimulation and that newly synthesized Arc is required for the persistent DHPG-induced increase in AMPA receptor endocytosis rate (Waung et al., 2007).

MAP1B: Davidkova and Carroll recently presented data suggesting that MAP1B protein is necessary for mGluR-induced AMPAR endocytosis. These authors implicate an mGluR-dependent increase in the interaction of MAP1B with glutamate receptor interacting protein 1(GRIP1) in this effect. GRIP is a scaffolding protein that binds to and regulates trafficking of AMPARs (Dong et al., 1997; Lu and Ziff, 2005).

STEP: Recently published work from Graham Collingridge's lab support a role for tyrosine phosphatases in mGluR LTD and mGluR-induced AMPAR internalization. This group reported that activation of mGluRs results in dephosphorylation of the GluR2 subunit of AMPARs and that pharmacological blockade of tyrosine phosphatase activity blocks mGluR LTD as well as mGluR-induced AMPA receptor internalization (Moult et al., 2006). However, whether mGluR-induced protein synthesis was involved in this regulation was not addressed. Data presented by Paul Lombroso's group demonstrate that the tyrosine phosphatase STEP is synthesized in hippocampal slices and synaptoneurosomes in response to mGluR stimulation (Kurup et al., 2007; Zhang et al., 2007). Inhibition of STEP activity increases tyrosine phosphorylation of GluR2 and blocks mGluR-induced AMPAR internalization and LTD. Interestingly, the phosphorylation state of GluR2 (on Ser880) is important in regulating interaction of

GluR2 with GRIP and another scaffolding protein, PICK. Phosphorylation of GluR2 on this residue prevents interaction with GRIP and results in GluR2 endocytosis (Dong et al., 1997; Seidenman et al., 2003; Lu and Ziff, 2005). It is tempting to speculate that tyrosine phosphorylation of GluR2 also regulates its interaction with scaffolding/endocytosis machinery to regulate AMPAR expression.

GluR2: In the ventral tegmental area (VTA) mGluR LTD requires rapid synthesis of GluR2. The mechanism of protein synthesis-dependent mGluR LTD in the VTA appears to differ from LTD in the hippocampus. As opposed to the reduction in surface AMPARs seen in hippocampal mGluR LTD, in the VTA mGluR LTD is expressed as a change in the subunit composition of AMPARs from GluR2-lacking (GluR1 homomers) to GluR2-containing receptors which have a lower single channel conductance (Mameli et al., 2007).

EF1α: EF1α is a component of translation machinery that promotes GTP-dependent binding of aminoacyl-tRNA to the ribosome during peptide elongation. As a component of cellular translation machinery, increases in EF1α protein levels might be expected to result in an increase translational capacity. Huang et al. found that dendritic EF1α synthesis did not result in an overall increase in dendritic translational capacity, but the possibility remains that EF1α synthesis may locally facilitate translation (Huang et al., 2005).

αCaMKII: Though αCaMKII certainly plays a critical role in mediating NMDAR -dependent (protein synthesis-independent) LTP through phosphorylation of AMPARs (Lee et al., 2000), the role of this kinase in protein synthesis-dependent plasticity is not clear. αCaMKII does phosphorylate CPEB1 in hippocampal neurons,

which would facilitate translation initiation (Wells, 2006). However, whether mGluR stimulation utilizes CPEB-dependent regulation of translation to maintain LTD is unclear.

FMRP: Fragile X Mental Retardation Protein derives its name from the fact that human fragile X syndrome mental retardation (FXS) results from a loss of function of FMRP (Pieretti et al., 1991; Verheij et al., 1993; O'Donnell and Warren, 2002). FMRP is an RNA-binding protein that regulates translation of its target mRNAs (Ashley et al., 1993; Khandjian et al., 1996; Tamanini et al., 1996; Brown et al., 1998; Sung et al., 2000; Brown et al., 2001; Schaeffer et al., 2001; Chen et al., 2003; Dolzhanskaya et al., 2003; Miyashiro et al., 2003; Sung et al., 2003). Specifically, several studies indicate that FMRP functions a translational repressor (Laggerbauer et al., 2001; Li et al., 2001). The Drosophila homologue of FMRP associates with RNA interference machinery, suggesting a mechanism by which FMRP may inhibit translation (Caudy et al., 2002). FMRP contains three RNA binding domains: two hnRNP-K homology domains (KH domains) and an arginine/glycine-rich RNA binding motif (RGG box)(Ashley et al., 1993; Gibson et al., 1993; Siomi et al., 1993). A point mutation in the KH2 domain of FMRP (Ile304Asn), discovered in a patient with severe FXS (De Boulle et al., 1993), prevents FMRP-dependent inhibition of translation, while deletion of either KH domain inhibits association of FMRP with polysomes, supporting a critical role for these domains in translation regulation(Mazroui et al., 2003).

MGluR stimulation results in rapid synthesis of FMRP (Weiler et al., 1997). In addition, mGluR LTD is enhanced in mice that lack FMRP (Fmr1 KO), resulting in the hypothesis that FMRP acts as a negative feedback regulator of mGluR-induced protein

synthesis and function (Huber et al., 2002). Recent data indicate that FMRP may play a more complex role in regulating translation at synapses. Phosphorylation of FMRP results in its selective association with "stalled", presumably untranslating, polyribosomes while dephosphorylated FMRP selectively associates with actively translating polyribosomes (Ceman et al., 2003). This raises the intriguing possibility that FMRP may dynamically regulate translation in response to neuronal activity. FMRP may also play a role in transport of mRNA to dendrites. A recent study reports that microtubule-associated FMRP is preferentially retained in translationally dormant, polyribosome-free messenger ribonucleoprotein (mRNP) complexes while soluble cytoplasmic FMRP is predominantly associated with elongating polyribosomes (Wang et al., 2007). Perhaps phosphorylated FMRP functions to transport mRNA into dendrites in a translationally silent state. When FMRP reaches an activated synapse, it could be dephosphorylated by an activity-dependent phosphatase, which would allow FMRP to recruit translation machinery to its associated mRNAs. This is of course speculation, and the specific details regarding how FMRP is regulated and how FMRP regulates translation at synapses remain to be elucidated. However, it is clear that FMRP plays a critical role in both synaptic and cognitive function.

Fragile X Mental Retardation Syndrome, mGluR LTD and the mGluR Theory of Fragile X Mental Retardation

Fragile X syndrome (FXS) is the most common inherited form of mental retardation, affecting ~1 in 1,450 males and 1 in 9,000 females, and is characterized by

moderate to severe mental retardation (IQ = 30-70), increased incidence of seizures, characteristics of autism, and other behavioral impairments. FXS is an X-linked recessive disorder resulting from loss of function of the fragile X mental retardation protein, FMRP. Most (>95%) cases of FXS result from expansion of a CGG trinucleotide repeat in the 5' untranslated region of *FMR1*, leading to methylation-induced transcriptional silencing of the gene and undetectable levels of FMRP, the gene product of *FMR1* (Kremer et al., 1991; Verkerk et al., 1991; Imbert et al., 1998). *Fmr1* KO mice were developed as a mouse model of FXS (Bakker, 1994). These mice show behavioral and physical phenotypes that are generally consistent with the human disease.

FXS, a disease of the synapse

Unlike many other forms of mental retardation, Fragile X patients exhibit no gross neuroanatomical deformities thought to give rise to cognitive deficits. Instead, structural and functional abnormalities in FXS occur at the level of the synapse. Cortical neurons of patients with Fragile X syndrome are characterized by a higher density of dendritic spines, an increased number of long, thin spines, and a reduction in mature, short and stubby spines (Rudelli et al., 1985; Hinton et al., 1991; Wisniewski et al., 1991; Irwin et al., 2001). Similar spine abnormalities exist in *Fmr1* KO mice (Comery et al., 1997; Nimchinsky et al., 2001). These long, thin spines resemble immature spines prevalent on developing neurons, suggesting that synapse number and/or function may be altered in FXS patients and *Fmr1* KO mice (Fiala et al., 1998). However, it was not until this year that FMRP was shown to play a direct role in regulating functional synapse number (Pfeiffer and Huber, 2007). Similar dendritic pathologies are associated with

other forms of mental retardation such as Down's or Rett syndrome suggesting that malfunctions of dendritic development and function may be a common mechanism which underlies mental retardation (Marin et al., 1997; Kaufmann and Moser, 2000).

Studies using the mouse model of FXS, *Fmr1* KO mice, reveal that FMRP is also critical for normal synaptic function. Long term potentiation is diminished in the cortex of *Fmr1*-KO mice, while mGluR-dependent LTD is enhanced in the hippocampus of these mice (Huber et al., 2002; Li et al., 2002).

Regulation of mGluR LTD by FMRP

Both DHPG and PP-LFS-induced LTD are enhanced in Fmr1 KO mice (Huber et al., 2002). In addition, both of these forms of LTD, which normally require rapid translation in WT mice, no longer require new protein synthesis in Fmr1 KO mice (Nosyreva and Huber, 2006). The precise mechanism by which loss of FMRP renders mGluR LTD protein synthesis-independent is not clear. mGluR-stimulated protein synthesis is absent in Fmr1 KO mice, and some reports suggest that basal levels of "LTD proteins" (proteins synthesized by mGluR stimulation in WT mice) are elevated in Fmr1 KO mice (Brown V, 2001; Todd et al., 2003; Hou et al., 2006; Muddashetty et al., 2007). Thus one possibility is that loss of FMRP leads to an unregulated increase in dendritic protein synthesis upon mGluR activation such that basal levels of LTD proteins are at a "ceiling" and further mGluR-dependent stimulation of protein synthesis is not possible. Alternatively, recent studies suggest that FMRP may act to either suppress or facilitate translation based on its phosphorylation state (Ceman et al., 2003). Thus it is possible that loss of FMRP results in a general increase in dendritic protein synthesis due to loss

of repression of unstimulated translation, and that FMRP activity is necessary for mGluR stimulation of protein synthesis.

mGluR LTD in mature Fmr1 KO mice is still mediated by a persistent decrease in surface AMPA receptor expression. However, like LTD in these mice, the mGluR-induced decrease in surface AMPAR expression does not require new protein synthesis (Nosyreva and Huber, 2006). This finding is particularly informative because it suggests that the abnormal mGluR-dependent plasticity observed in Fmr1 KO mice is not due to a delay in development. Recall that FXS patients have dendritic spine morphologies reminiscent of developmentally immature neurons and that mGuR LTD in young wild type animals (< 2.5 weeks) is also protein synthesis independent, raising the possibility that neuronal or synaptic development is delayed or incomplete in Fmr1 KO mice. However, mGluR LTD in young mice is mediated by a change in presynaptic function, not by decreased AMPAR surface expression (Nosyreva and Huber, 2006). These data are consistent with FMRP playing a critical role in regulating protein synthesis-dependent synaptic plasticity in mature rodents.

The mGluR theory of fragile X mental retardation

Mark Bear, Kim Huber and Stephen Warren put forth the idea that many of the psychiatric and neurological deficits seen in fragile X syndrome can be explained by overactive protein synthesis-dependent mGluR processes, which they termed the "mGluR theory of fragile X mental retardation" (Bear et al., 2004). In many respects this theory has proven to be extremely insightful. When this paper was published, it was known that hippocampal mGluR LTD was enhanced and no longer protein synthesis dependent in

Fmr1 KO mice. It was further known that FMRP was an RNA-binding protein that regulated translation. The mGluR theory posulated several predictions regarding unobserved FXS phenotypes which were based in part upon the following findings: weak activation of mGluRs facilitates NMDAR-dependent LTP, activation of mGluRs results in persistent prolongation of epileptiform bursts in the CA3 region of the hippocampus, and mGluRs induce LTD in the cerebellum (Cohen and Abraham, 1996; Merlin et al., 1998; Karachot et al., 2001; Lee et al., 2002). Most importantly, all of these long lasting, mGluR-dependent changes in neuronal function require new protein synthesis. A more recent study demonstrates that in the CA3 region of the hippocampus, blockade of inhibition results in robust, mGluR-dependent, prolonged epileptiform bursts in *Fmr1* KO mice but not in wild type mice (strong activation of group I mGluRs with DHPG does induce protein synthesis-dependent epileptiform bursts as described in the earlier study by Merlin et al.), confirming the prediction that protein synthesis-dependent mGluR processes are exaggerated in *Fmr1* KO mice (Chuang et al., 2005).

A direct result of this theory was the proposal that mGluR antagonists, specifically group I mGluR antagonists, might be an effective treatment for FXS. The mGluR5 antagonist MPEP has been tested in several FXS model systems including mice, *Drosophila,* and zebrafish. MGluR5 antagonism has proven effective in ameliorating a number of behavioral, physiological, and morphological abnormalities in these model systems, including the reduction of bicuculline-induced epileptiform bursts in hippocampal area CA3, reduction of audiogenic seizures, restoration of open field habituation in mice, restoration of courtship behavior and morphological abnormalities

in mushroom bodies in *Drosophila*, and rescue of axon branching deficits in zebrafish (Chuang et al., 2005; McBride et al., 2005; Yan et al., 2005; Tucker et al., 2006).

Protein Synthesis-Dependent Plasticity: mAchRs

mAChRs: Overview

mAChRs consist of the Gq-coupled M1, 3, and 5 receptors and the Gi/o-coupled M2 and M4 receptors. M1, M3, and M5 are predominantly postsynaptic receptors, while M2 and M4 are predominantly presynaptic (Porter et al., 2002). M2 is the primary Gi/o-coupled receptor in the hippocampus, while M1 is the primary Gq-coupled receptor in the hippocampus and cortex (Segal and Auerbach, 1997; Porter et al., 2002). In fact, Gq-specific GTP-γ-^{35}S binding, phosphoinositide hydrolysis, and ERK activation are all absent in the hippocampus of M1 mAChR knockout mice (Berkeley et al., 2001; Porter et al., 2002; Bymaster et al., 2003), but there is evidence that M3 receptors mediate some functions of ACh in the hippocampus (Ohno-Shosaku et al., 2003).

Activation of postsynaptic mAChRs depolarizes hippocampal CA1 pyramidal neurons while activation of presynaptic mAChRs decreases synaptic transmission (Cole and Nicoll, 1984; Nicoll et al., 1990; Fernandez de Sevilla and Buno, 2003). mAChR activation in CA1 pyramidal neurons blocks a K^+ leak current, the Ca^{2+}-activated K^+ current, I_{AHP}, and a noninactivating voltage-dependent K^+ current, I_M (Cole and Nicoll, 1984; Nicoll et al., 1990; Segal and Auerbach, 1997). M1 mAChR activation also potentiates NMDAR currents (Marino et al., 1998). In addition, like group I mGluR activation, activation of mAChRs facilitates eCB release and subsequent depression of

GABA release form inhibitory interneurons (Ohno-Shosaku et al., 2003; Edwards et al., 2006)

mAChR-LTD

In 1993 Feig and Lipton demonstrated, for the first time, that synaptic stimulation could induce synthesis of proteins in dendrites. They showed that coincident activation of mAChRs and NMDARs resulted in ^{3}H-Leu incorporation into the dendrites of hippocampal CA1 neurons (Feig and Lipton, 1993). It was later shown that pharmacological activation of mAChRs, specifically the Gq-coupled M1 receptor, induces LTD at the SC-CA1 synapses in the hippocampus (Kirkwood et al., 1999; Scheiderer et al., 2006), though the protein synthesis dependence of this LTD was not demonstrated until this year (Volk et al., 2007).

In addition to inducing LTD in the hippocampus, pharmacological or synaptic stimulation of mAChRs induces M1-dependent LTD in the visual cortex (Kirkwood et al., 1999; Origlia et al., 2006) and perirhinal cortex (Massey et al., 2001; Warburton et al., 2003), and synaptic stimulation (LFS) induces mAChR-dependent LTD at CA1-subicular synapses (Li et al., 2005).

Activation of the Gq-coupled M1 mAChRs via endogenous release of ACh facilitates (Shinoe et al., 2005) and lowers the threshold for (Ovsepian et al., 2004) hippocampal LTP. Shinoe et al also find that a very low concentration of the cholinergic agonist carbachol (CCH, 50nM), which has no effect on basal synaptic transmission, is sufficient to facilitate hippocampal SC-CA1 LTP. The protein synthesis-dependence of this priming effect was not investigated. Much stronger stimulation (50μM CCH) of

mAChRs is necessary to induce LTD (Kirkwood et al., 1999; Massey et al., 2001; Scheiderer et al., 2006). Of particular interest is the striking similarity between the level of M1 mAChR or group I mGluR stimulation and the effect on hippocampal plasticity. Weak activation of either group I mGluRs or M1 mAChRs facilitates or primes NMDAR-dependent LTP in the hippocampus, while strong activation of these Gq-coupled receptors directly induces LTD (Cohen and Abraham, 1996; Huber et al., 2001; Shinoe et al., 2005; Scheiderer et al., 2006; Volk et al., 2007). These similarities suggest that Gq-coupled mAChRs and mGluRs may utilize similar signaling mechanisms to modulate hippocampal plasticity.

mAChR Signaling

Until very recently, surprisingly little was known about the signaling mechanisms mediating mAChR-dependent LTD. Most studies find that mAChR activation induces LTD independently of NMDAR activation (Kirkwood et al., 1999; Massey et al., 2001; Li et al., 2005; Jo et al., 2006; McCoy and McMahon, 2007; Volk et al., 2007). However, mAChR activation has also been shown to facilitate or "gate" NMDAR-dependent LTD (Choi et al., 2005; Scheiderer et al., 2006). MAChR activation induces protein synthesis-dependent LTD in the perirhinal cortex, hippocampus and visual cortex, and in each of these studies, mAChR LTD was independent of NMDAR activation (Massey et al., 2001; McCoy and McMahon, 2007; Volk et al., 2007). In the hippocampus, protein synthesis-dependent mAChR LTD requires activation of the ERK and mTOR translation regulatory pathways. However, the upstream mechanism by which mAChRs induce ERK and mTOR activation in the hippocampus is not clear. In

nonneuronal cells mAChR-dependent ERK activation is PLC-dependent and requires increases in intracellular Ca^{2+} and Src family tyrosine kinase activation but not PKC activation, but whether this is the case in hippocampal neurons remains to be seen (Jiménez et al., 2002). In addition, mAChRs do not bind to Homer proteins, so it is unlikely that Homer-PIKE interactions are involved in mAChR-induced mTOR activation, suggesting that although Gq-coupled mGluRs and mAChRs induce similar signaling cascades to regulate translation (see Chapter Three), the upstream signaling events may be differentially regulated. On the other hand, modulation of NMDAR-dependent LTD by mAChRs (and other Gq-coupled receptors including group I mGluRs and α-adrenergic receptors) in the visual cortex does require activation of PLC and IP3 receptors (Choi et al., 2005). These data suggest that activation of PLC signaling pathways via mAChRs and other Gq-coupled can facilitate or "gate" NMDAR-dependent, protein synthesis-independent LTD, while activation of the ERK and mTOR translation regulatory pathways via mAChRs and mGluRs induces protein synthesis-dependent LTD. The physiological conditions under which these different signaling pathways are engaged and how they interact to regulate synaptic function and cognition are not yet clear.

Role of mAChRs in Learning and Memory

mAChR activity is clearly important for normal cognitive function. Pharmacological blockade of mAChRs in humans blocks encoding of new memories and interferes with working memory (Hasselmo, 2006). In rodents, local blockade of mAChRs in the hippocampus impairs spatial memory (Blokland et al., 1992). Infusion of

mAChR antagonists into the medial septum, which is the major cholinergic input into the hippocampus, decreases ACh release into the hippocampus and also impairs spatial learning and memory (Elvander et al., 2004a). Interestingly, increasing acetylcholine release into the hippocampus by infusion of a cholinergic agonist into the medial septum also impairs memory, suggesting that cholinergic activity must be tightly regulated to maintain normal cognitive function (Bunce et al., 2004a; Elvander et al., 2004a). Acetylcholine release into the medial prefrontal cortex (mPFC) and ventral hippocampus (VH) is transiently increased during inhibitory avoidance (IA) acquisition learning and recall, and ERK is activated in the mPFC and VH during IA acquisition (Giovannini et al., 2005). Blockade of mAChRs or ERK in the mPFC or VH blocks IA acquisition, suggesting that mAChR-induced ERK activation is important for some learning tasks.

The importance of cholinergic transmission and mAChR activity for cognitive function is highlighted by the selective loss of cholinergic neurons in the basal forebrain of Alzheimer's patients with a concomitant loss of cholinergic innervation to the cortex and hippocampus. This loss of cholinergic transmission is thought to be the major factor contributing to the cognitive impairments in Alzheimer's disease (AD) (Clader and Wang, 2005; Mikiciuk-Olasik et al., 2007). The role of hippocampal mAChRs in cognitive function is highlighted by the findings that hippocampal or medial septum lesions mimic the spatial memory deficits observed in AD patients (Kesner et al., 1989), and M1 mAChR agonists improve performance in cognitive tests in AD patients and improve cognition in animal models of AD, including rescuing deficits in spatial learning tasks (Clader and Wang, 2005; Caccamo et al., 2006).

Interestingly, maintenance of mAChR-dependent LTD is associated with "successful aging" in rats (Lee et al., 2005a). Successful aging refers to aged rats that maintain spatial learning and memory capabilities comparable with young animals. The researchers in this study found that they could divide aged rats in two categories based on those that performed well in a spatial memory task (aged unimpaired, AU) and those that showed impaired learning (aged impaired, AI). mAChR LTD was selectively decreased in AI animals compared to AU and young animals, whereas NMDAR-dependent LTD was not different between these groups.

Additional data supporting a role for mAChR LTD in learning and memory comes from work demonstrating that in a novelty recognition task, a subset of neurons in the perirhinal cortex are strongly activated when an animal is presented with a novel object, but when the animal is shown a familiar object the response is decreased (Warburton et al., 2003), consistent with an LTD-like mechanism. Novelty recognition and selective activation of neurons in response to novel stimiuli are blocked by infusions of a mAChR antagonist in the perirhinal cortex, and LTD in the perirhinal cortex is also dependent on mAChR activation.

Protein synthesis-Dependent Plasticity: NMDARs

Protein synthesis-dependent NMDAR LTD differs from protein synthesis-dependent mGluR and mAChR LTD in the time course of protein synthesis (Frey and Morris, 1997; Huber et al., 2000; Huber et al., 2001b). mGluR and mAChR LTD depend immediately on new protein synthesis, whereas NMDAR dependent LTP and LTD

exhibit two distinct phases: a protein synthesis-independent early phase and a protein synthesis-dependent late phase. Early LTP and LTD (E-LTP, E-LTD) are the most commonly studied forms of NMDAR-dependent plasticity. E-LTP and E-LTD are induced with a "weak" stimulus (see Table 1), typically last 1-3 hours, and are mediated by modification and trafficking of existing proteins, with a key step being selective phosphorylation and trafficking of AMPA receptors (Boehm et al., 206; Lee et al., 2000; Lee et al., 2003; Seidenman et al., 2003). In contrast, late LTP and LTD (L-LTD, L-LTP) are induced with "strong" stimulation, require new protein synthesis, and last 4+ hours. L-LTP and L-LTD stimuli given in the presence of protein synthesis inhibitors are converted to E-LTP and E-LTD respectively and decay to baseline by 1-3 hours (Sajikumar and Frey, 2004).

Type of Plasticity	**Duration**	**Requires Protein Synthesis?**	**Typical Stimulation Paradigm**
E-LTP	~1-3 hrs	No	1x 100 pulses at 100Hz
L-LTP	4+ hrs	Yes	3x 100 pulses at 100 Hz, 10min btw. bursts
E-LTD	~1-3 hrs	No	900 pulses at 1 Hz
L-LTD	4+ hrs	Yes	900 bursts at 1Hz, 1 burst = 3 pulses at 20Hz

Table 2. Stimulation Paradigms for Early and Late NMDAR LTP and LTD

While NMDA receptor antagonists block induction of late LTP and LTD, NMDAR activation alone is not sufficient to maintain late plasticity (Sajikumar and Frey, 2004). Maintenance of LTP and LTD requires activation of metabotropic receptors such as D1/5 dopamine receptors (Sajikumar and Frey, 2004). E-LTP can be converted into L-LTP by stimulation of cholinergic and noradrenergic inputs into the hippocampus, which activates mAChRs and β–adrenergic receptors respectively (Bergado et al., 2007). Taken

together, these data suggest that the protein synthesis component of late LTP and LTD is supplied by recruitment of GPCRs, and that NMDAR activation alone is insufficient to induce protein synthesis. However, though there is not a great deal of data demonstrating NMDAR-dependent dendritic protein synthesis, it should be noted that NMDAR activation does result in polyadenylation of α-CaMKII mRNA and dendritic translation of a GFP reporter fused to α-CaMKII 3' and 5' regulatory sequences, suggesting that NMDAR stimulation alone is capable of inducing synthesis of proteins in dendrites (Huang et al., 2002; Gong et al., 2006).

Are the new proteins required for L-LTP and L-LTD synthesized in dendrites? Protein synthesis-dependent L-LTP occurs in dendrites that are mechanically isolated from the cell body layer suggesting that protein synthesis is occurring in dendrites (Vickers et al., 2005). This is supported by multiple studies demonstrating that L-LTP and L-LTD do not require transcription for up to 8 hours (Otani et al., 1989; Manahan-Vaughan et al., 2000; Vickers et al., 2005; Sajikumar et al., 2007). However, there is some debate about the transcription dependence of L-LTP, as a number of studies do find that maintenance of L-LTP requires *de novo* mRNA synthesis (Nguyen et al., 1994; Frey et al., 1996; Alarcon et al., 2006). It may be that that L-LTP and L-LTD are maintained by local protein synthesis with no requirement for transcription for some time, but that transcription is eventually required. The amount of time that late phase plasticity persists without *de novo* mRNA synthesis likely depends on the nature of the stimulus as well as affective factors such as stress.

In 1997 Frey and Morris proposed a novel mechanism by which newly synthesized proteins facilitate synapse specific plasticity, which they term the "synaptic

tagging hypothesis" (Frey and Morris, 1997). In this model, "strong" stimulation induces protein synthesis that is spatially restricted by dendritic compartments (apical vs. basal in CA1 pyramidal neurons for example), but is *not* specific to the activated synapse. Instead, synapse specificity is generated by the setting of a "tag" via a protein synthesis independent process that occurs during both early and late plasticity induction paradigms. This model is based on data demonstrating that E-LTP can be converted into L-LTP if a strong, L-LTP-inducing stimulus is given in a separate pathway within ~1-2 hours of the weak E-LTP stimulus. Frey and Morris proposed that the tag set during weak stimulation "captures" proteins that are synthesized by the strong stimulus. This model predicts that the proteins synthesized by strong stimuli are not specific for the activated synapses and that it is the initial "tag" that confers synapse specificity. The discovery of "cross-tagging", which describes the observation that E-LTP can capture proteins synthesized by strong L-LTD stimuli, and visa versa, supports this idea (Sajikumar and Frey, 2004).

One interesting property of this bi-phasic NMDAR-dependent plasticity is that learning tasks or exploration of a novel environment convert E-LTP into L-LTP, suggesting that these experiences induce synthesis of proteins that can be "captured" and used by synapses that have experienced weak stimuli to induce a more persistent form of plasticity (Straube et al., 2003; Sajikumar et al., 2007). In addition, a behavioral correlate of synaptic tagging exists in which a transient inhibitory avoidance memory (IA, lasting < 60 min) is converted into a long lasting memory (24 hr duration) by exploration of a novel environment (Moncada and Viola, 2007). IA learning is a hippocampal dependent task (Isaacson and Wickelgren, 1962; Izquierdo et al., 1992; Lorenzini et al., 1996), and similar to biphasic NMDAR-dependent LTP at SC-CA1 synapses, conversion of transient

IA memory into long lasting IA memory relies on protein synthesis and dopamine signaling.

Multiple studies suggest that the phenomenon of synaptic tagging is spatially restricted such that tagged synapses can only capture proteins from stimuli in their own compartment (apical dendrites can't use proteins generated by a strong stimulus in basal dendrites and visa versa) (Alarcon et al., 2006; Sajikumar et al., 2007). However, very strong stimuli (e.g. 8x 100 pulses at 100Hz,(Alarcon et al., 2006)) or phosphodiesterase 4B3 (PDE4B3) inhibition, which results in activation of the cAMP/PKA pathway (Sajikumar et al., 2007), allows for cross-compartment protein capture. Interestingly, this breakdown of compartmentalization is accompanied by a gain of transcription dependence (Sajikumar et al., 2007). Furthermore, while both stressful (Morris water maze) and non-stressful (holeboarding) learning tasks convert E-LTP into L-LTP, only the stressful stimulus results in transcription-dependent L-LTP (Sajikumar et al., 2007). These data shed some light on the discrepancies seen in the transcriptional requirement of L-LTP and suggest that the strength of the stimulus as well as affective influences such as stress modulate the necessity for transcription. It may be that stress (or other stimuli with intense emotional content) immediately recruit nuclear transcription and induce a more widespread increase in protein synthesis, thus maintaining the neuron in a more responsive state.

Protein synthesis-dependent NMDAR, mGluR, and mAChR LTD are often studied in isolation, so the manner in which LTD induced by these different receptors interacts at the synapse is somewhat unclear. However, NMDAR and protein synthesis-dependent late phase LTD and LTP at SC-CA1 synapses require coactivation of D1/5

dopamine receptors (Sajikumar and Frey, 2004) or mGluR5 (Francesconi et al., 2004; Manahan-Vaughan, 1997) which both induce dendritic protein synthesis (Smith et al., 2005), and mAChR stimulation in the dentate gyrus is involved in converting E-LTP into L-LTP (Bergado et al., 2007), suggesting that activation of GPCRS coupled to various neurotransmitter systems can contribute to the dendritic protein synthesis needed for persistent forms of NMDAR-dependent plasticity. It is not clear if protein synthesis-dependent mGluR and mAChR LTD induced in the absence of NMDAR activity last longer than a few hours, or if coactivation of NMDARs with GPCRs coupled to dendritic protein synthesis is a requirement for persistent forms of synaptic plasticity.

MOTIVATION FOR STUDIES

Activity of group I mGluRs is critical for many processes relying on long lasting changes neuronal function. In particular, hippocampal protein synthesis-dependent mGluR LTD is aberrant in the mouse model for fragile X syndrome mental retardation, and mGluR5 antagonists are being tested as therapeutic treatment for FXS in model organisms. However, when I joined Dr. Huber's laboratory, it was still unclear if mGluR1 played a role in induction of LTD at hippocampal SC-CA1 synapses. In fact, there was some debate as to whether or not mGluR1 was even expressed in CA1 pyramidal neurons, though it has become clear in the past few years that mGuR1 does indeed play an important function at hippocampal SC-CA1 synapses. In addition, the relative roles for mGluR1 or mGluR5 activity in expression of LTD at this synapse were unknown. Considering both the general importance of understanding the mechanisms of

protein synthesis-dependent LTD and the specific interest in mGluR antagonists for use in treatment of human FXS, we sought to definitively determine the receptors necessary for mGluR LTD induction and expression in the rodent hippocampus (Chapter 2). Traditionally, mGluR LTD was induced pharmacologically with the group I mGluR agonist DHPG or synaptically with PP-LFS. Evidence that DHPG and PP-LFS activate the same receptors to induce LTD included the following: PP-LFS is blocked by a broad-range mGluR antagonist, saturation of PP-LFS, but not NMDAR-dependent LFS LTD, occludes DHPG LTD, and both DHPG and PP-LFS LTD are absent in Gq KO mice, are protein synthesis-dependent on the same time scale, require ERK activation, are developmentally regulated and are aberrant in the mouse model of fragile X syndrome mental retardation. Surprisingly, in investigating the relative contributions of mGluR1 and mGluR5 to mGluR LTD, I found that while both mGluR1 and 5 contribute to DHPG-induced LTD, PP-LFS LTD does not require activation of Group I mGluRs, or any mGluRs for that matter. Considering the many similarities in signaling and expression mechanisms for DHPG and PP-LFS LTD, and considering that both are absent in Gq KO mice, we hypothesized that PP-LFS activates Gq-coupled receptors other than, or in addition to, the known mGluRs, and that LTD induced by selective activation of these receptors should share similar signaling and expression mechanisms with mGluR (DHPG) LTD.

Over 10 years ago Feig and Lipton showed that coactivation of mAChRs and NMDARs induces protein synthesis in dendrites of hippocampal CA1 pyramidal cells (Feig and Lipton, 1993). In addition, pharmacological activation of Gq-coupled M1 mAChRs induces LTD in the hippocampus and induces protein synthesis-dependent LTD

in the perirhinal cortex (Massey et al., 2001; Scheiderer et al., 2006). Almost nothing was known about the signaling or expression mechanisms of mAChR LTD in the hippocampus prior to my studies, despite the clear role for mAChRs in learning and memory. I tested the hypothesis that PP-LFS activates M1 mAChRs to induce LTD and determined the signaling and expression mechanisms of hippocampal mAChR LTD. These studies led to the discovery of a conserved mechanism by which Gq-coupled receptors induce protein synthesis-dependent LTD, and to the finding that this mechanism is aberrant in the mouse model of fragile X syndrome mental retardation.

CHAPTER TWO
Results

DIFFERENTIAL ROLES FOR GROUP 1 MGLUR SUBTYPES IN INDUCTION AND EXPRESSION OF CHEMICALLY INDUCED HIPPOCAMPAL LONG-TERM DEPRESSION

Summary

Although group I metabotropic glutamate receptors (mGluR1 and mGluR5) are often found to have similar functions, there is considerable evidence that the two receptors also serve distinct functions in neurons. In hippocampal area CA1, mGluR5 has been most strongly implicated in long-term synaptic depression (LTD), whereas mGluR1 has been thought to have little or no role. Data presented in this chapter show that simultaneous pharmacological blockade of mGluR1 and mGluR5 is required to block induction of LTD by the group 1 mGluR agonist, (*RS*)-3,5-dihydroxyphenylglycine (DHPG). Blockade of mGluR1 or mGluR5 alone has no effect on LTD induction, suggesting that activation of either receptor can fully induce LTD. Consistent with this conclusion, mGluR1 and mGluR5 both contribute to activation of extracellular signal-regulated kinase (ERK), which was previously shown to be required for LTD induction. In contrast, selective blockade of mGluR1, but not mGluR5, reduces the expression of LTD and the associated decreases in AMPA surface expression. LTD is also reduced in mGluR1 knockout mice confirming the involvement of mGluR1. These data show a

novel role for mGluR1 in long-term synaptic plasticity in CA1 pyramidal neurons. In contrast to DHPG-induced LTD, LTD induced synaptically with paired-pulse low-frequency stimulation persists in the pharmacological blockade of group 1 mGluRs and in mGluR1 or mGluR5 knockout mice. This suggests different receptors and/or upstream mechanisms for chemically and synaptically induced LTD

Introduction

Group 1 metabotropic glutamate receptors (mGluRs) are composed of two subtypes, mGluR1 and mGluR5. Both receptors are coupled to the Gq subtype of heterotrimeric G proteins and activate phospholipase C. mGluR1 and mGluR5 both contribute to increased neuronal excitability, intracellular Ca^{2+} increases (Ireland and Abraham, 2002; Thuault et al., 2002; Rae and Irving, 2004), synaptic plasticity (Sung et al., 2001; Gubellini et al., 2003), and pain (Karim et al., 2001). However, although they activate similar signaling cascades, there is evidence that mGluR1 and mGluR5, expressed in the same neuron, can serve distinct functions (Mannaioni et al., 2001; Merlin, 2002; Thuault et al., 2002; Kettunen et al., 2003; for review see Valenti et al., 2002). Consistent with distinct functioning of these receptors, mGluR1 and mGluR5 function as dimers, but these receptors do not heterodimerize (Romano et al., 1996; Suzuki et al., 2004). In hippocampal CA1 pyramidal neurons, mGluR5 is the most highly expressed group I mGluR subtype (Romano et al., 1995; Lujan et al., 1996; Shigemoto et al., 1997). However, a role for mGluR1 in CA1 pyramidal neurons has recently been

elucidated using the selective mGluR1 antagonist LY367385 (Clark et al., 1997). mGluR1 contributes to many functions in CA1 pyramidal neurons including cell depolarization, intracellular Ca^{2+} increases, decreases in the afterhyperpolarization potential (AHP), short-term depression of excitatory postsynaptic currents (EPSCs), and extracellular signal-regulated kinase (ERK) activation (Mannaioni et al., 2001; Ireland and Abraham, 2002; Berkeley and Levey, 2003; Rae and Irving, 2004). Blockade of mGluR1 or mGluR5 in the CA1 region of the hippocampus inhibits consolidation of inhibitory avoidance learning, while mGluR1, but not mGluR5, is required for extinction of inhibitory avoidance memory, supporting a role for mGluR1 and mGluR5 localized to the CA1 region of the hippocampus in learning and memory (Simonyi et al., 2007).

Activation of group 1 mGluRs with the selective agonist, (*RS*)-3,5-dihydroxyphenylglycine (DHPG), or with low-frequency (1–5 Hz) synaptic stimulation causes long-term depression (LTD) of excitatory synaptic transmission (mGluR-LTD) (Bolshakov and Siegelbaum, 1994; Oliet et al., 1997; Palmer et al., 1997; Fitzjohn et al., 1999; Huber et al., 2000; Huber et al., 2001). mGluR-LTD in mature rodents is mediated by a persistent reduction in the number of postsynaptic amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPARs) (Snyder et al., 2001; Xiao et al., 2001; Nosyreva and Huber, 2005). However, mGluR LTD undergoes a developmental switch such that mGluR LTD in young rodents (< 2.5 weeks) is mediated by a presynaptic mechanism and does not require new protein synthesis (Bolshakov and Siegelbaum, 1994; Fitzjohn et al., 2001; Zakharenko et al., 2002; Feinmark et al., 2003; Rammes et al., 2003; Nosyreva and Huber, 2005) In addition, there is evidence for presynaptic contributions to mGluR-LTD (Oliet et al., 1997; Watabe et al., 2002) but see

(Zhang et al., 2006). One of the most interesting properties of mGluR-LTD is that it relies on rapid (within minutes) dendritic protein synthesis (Huber et al., 2000). Likewise, the long-term decrease in AMPAR surface expression requires protein synthesis (Snyder et al., 2001; Nosyreva and Huber, 2005). Recent work has revealed the signaling pathways that activate translation in response to mGluRs. The ERK and PI3K/ mTOR pathways that regulate translation initiation in many cell types, including neurons, are activated by DHPG and required for mGluR-LTD (Gallagher et al., 2004; Hou and Klann, 2004; for review see Kelleher et al., 2004; Klann and Dever, 2004). LTD induced with DHPG or synaptic stimulation [paired-pulse low-frequency stimulation (PP-LFS)] are both absent in Gq knockout mice, are both blocked by the broad range mGluR antagonist, LY341495, occlude each other, and both rely on protein synthesis and ERK activation, suggesting that these two methods of LTD induction represent the same or a similar LTD mechanism (Huber et al., 2000; Huber et al., 2001; Kleppisch et al., 2001; Gallagher et al., 2004).

mGluR-LTD is absent in mGluR5 knockout (KO) mice, suggesting a requirement for this subtype in induction or expression of LTD (Huber et al., 2001). Consistent with this work, the mGluR5 antagonist MPEP reduces or abolishes mGluR-LTD in rats and mice (Gasparini et al., 1999; Faas et al., 2002; Hou and Klann, 2004; Huang et al., 2004; Huang and Hsu, 2006). In contrast, selective mGluR1 blockade has been reported to have either a partial or no effect on DHPG-induced LTD in area CA1 (Fitzjohn et al., 1999; Faas et al., 2002; Hou and Klann, 2004). Recent work demonstrates that mGluR1 activity is required for the acute, short-term depression of excitatory synaptic transmission induced with DHPG (Mannaioni et al., 2001; Faas et al., 2002), suggesting that both mGluR1 and mGluR5 regulate excitatory synaptic transmission onto CA1 neurons.

Remarkably, the expression of DHPG-induced LTD can be reversed by broad-range mGluR antagonists, even when applied hours after the induction stimulus (Palmer et al., 1997; Fitzjohn et al., 1999; Watabe et al., 2002), indicating that sustained mGluR activation is required for the expression of mGluR-LTD. How is sustained receptor activity maintained in the absence of agonist? Many GPCRs are able to exist in a conformation that displays agonist independent, or constitutive, activity. Both mGluR1 and mGluR5 exhibit constitutive activity (Pagano et al., 2000; Pula et al., 2004), and correlative evidence suggests that constitutive activity of mGluRs can be regulated by activity (Ango et al., 2001; Bottai et al., 2002). Constitutive activity of mGluRs is regulated by binding to the scaffolding protein Homer. Homer proteins consist of constitutively expressed long forms ($Homer_L$), which couple mGluRs to intracellular signaling molecules, and an activity-inducible short form of Homer (Homer1a), which acts in a dominant negative manner to disrupt mGluR-$Homer_L$ interactions (Duncan et al., 2005). Disruption of mGluR-$Homer_L$ interactions promotes constitutive activity of mGluR1 and mGluR5 (Ango et al., 2001). It is important to note that the mGluR1-specific antagonist LY367385 and the mGluR5-specific antagonist MPEP used in this study are actually classified as inverse agonists, meaning that in addition to blocking agonist-induced receptor activity, they also block constitutive receptor activity. In contrast, the mGluR1 antagonist CPCCOEt is not an inverse agonist (Pagano et al., 2000; Pula et al., 2004). At the time of this study, the mechanism underlying reversal of LTD and the specific receptor(s) responsible for this effect were unknown.

mGluR-LTD is enhanced in the mouse model of fragile X syndrome, and it has been suggested that group 1 mGluR antagonists may serve as potential therapies for

Fragile X syndrome patients (Huber et al., 2002; Bear et al., 2004b). In FXS model organisms, promising results have been obtained using the mGluR5 antagonist MPEP, suggesting that this is a valid avenue of investigation. Determination of the specific mGluR subtypes required for LTD induction and expression may facilitate development of more effective pharmaceutical treatments for Fragile X syndrome mental retardation.

In light of accumulating data for mGluR1 function in CA1 pyramidal neurons and synaptic function, I evaluated the role of mGluR1 in LTD at Schaffer collateral-CA1 synapses. Unexpectedly, I find a role for mGluR1 in the induction and expression of DHPG-induced LTD. Activation of mGluR1 or mGluR5 alone can induce the full complement of LTD. Consequently, simultaneous blockade of mGluR1 and mGluR5 is required to abolish DHPG-induced LTD and the associated ERK activation. However, mGluR1 is selectively required for the expression of DHPG-induced LTD. In contrast, synaptically induced LTD (with PP-LFS), is unaffected by blockade or genetic knockout of group 1 mGluRs. These data demonstrate a clear role for mGluR1 in plasticity at hippocampal SC-CA1 synapses and suggests that different neurotransmitter receptors induce chemically and synaptically induced LTD.

Materials and Methods

Drugs

D,L-AP5 (Tocris, Ellisville, MO) was prepared fresh in artificial cerebrospinal fluid (ACSF). *R,S*-DHPG or *S*-DHPG, LY367385, LY341495, MPEP, (Tocris), tetraethylammonium chloride, and TTX (Sigma) were prepared as stocks in water or

equimolar NaOH (LY367385 and LY341495), aliquoted, and frozen for no more that 10 days. We observed no differences in the effectiveness of fresh or frozen MPEP or LY367385. CPCCOEt (Tocris) was prepared fresh in 0.1% DMSO each day.

Electrophysiology

Hippocampal slices were prepared from Long Evans hooded or Sprague-Dawley rats or mGluR1 or mGluR5 knockout (KO) or wildtype (WT) mice. Rats were obtained from Charles River Laboratories (Boston, MA). mGluR1 KO mice were originally from Francois Conquet (Conquet et al., 1994). mGluR5 KO mice were from John Roder (Lu et al., 1997) and were mated with WT C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) to obtain heterozygotes. Experiments in KO mice were compared with those in WT littermates. Hippocampal slices (400 μm) were prepared from 9-15-day old ("immature") or 21-55-day-old animals ("mature"). Rats used in AHP experiments were 19-24 days old. Rats or mice were anesthetized with pentobarbital sodium (50 mg/kg) and decapitated soon after the disappearance of corneal reflexes. The brain was removed, dissected, and sliced in ice-cold dissection buffer containing (in mM) 2.6 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 0.5 $CaCl_2$, 5 $MgCl_2$, 212 sucrose, and 10 dextrose, using a vibratome (Leica VT 1000S). The slices were transferred into a reservoir chamber filled with ACSF containing (in mM) 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$, and 10 dextrose. Slices were allowed to recover for 2–5 h at 30°C. ACSF and dissection buffer were equilibrated with 95% O_2-5% CO_2.

For recording, slices were transferred to a submerged recording chamber, maintained at 30°C, and perfused continuously with ASCF at a rate of 2–3 ml/min. Field

potentials (FPs) were recorded with extracellular recording electrodes (1 MΩ) filled with ACSF and placed in stratum radiatum of area CA1. FPs were evoked by monophasic stimulation (200-μs duration) of Schaffer collateral/commissural afferents with a concentric bipolar tungsten stimulating electrode (FHC, Bowdoinham, ME). Stable baseline responses were collected every 30 s using a stimulation intensity (10–30 μA) yielding 50–60% of the maximal response. The initial slope of the FPs was used to measure stability of synaptic responses and quantify the magnitude of LTD. Chemically induced mGluR-LTD was elicited by application of 100 μM DHPG for 5 or 20 min as indicated. Synaptically induced LTD was induced using PP-LFS (50-ms interstimulus interval) pulses at 1 Hz for 15 (for rats) or 20 min (for mice). The group data were analyzed as follows: *1*) the initial slope of the FPs were expressed as percentages of the pre-DHPG baseline average, *2*) the time scale in each experiment was converted to time from the onset of DHPG, and *3*) the time-matched, normalized data were averaged across experiments and expressed in the text and figures as means ± SE. The effects of all pharmacological treatments on LTD were evaluated by comparing interleaved control and treated slices. Significant differences were determined by a Student's independent *t*-test. Paired *t*-tests were used to determine significance of reversal effects on LTD expression (Fig. 2). Probability values of $P < 0.05$ were considered to represent significant differences.

For I_{AHP} measurements, whole cell voltage-clamp recordings were performed from visualized CA1 pyramidal neurons under IR-DIC optics. Whole cell pipettes (3–7 MΩ) were fabricated from thick wall (1.5 mm OD, 0.86 mm ID, Sutter Instruments) borosilicate glass and filled with (in mM) 135 K-methanesulfonate, 8 KCl, 4 NaCl, 10

HEPES, 4 MgATP, and 0.4 TrisGTP, pH 7.25, 300 mOsm. To elicit and measure the I_{AHP}, cells were voltage clamped at –50 mV, and depolarizing steps (+60 mV; 200 mS) were applied every 30 s to elicit an unclamped Ca^{2+} action current. The resulting outward tail current (10–15 ms after the offset of the depolarizing step) was measured as the I_{AHP}. This would be considered the medium AHP as previously described (Pedarzani and Storm, 1993; Mannaioni et al., 2001). Series and input resistance were monitored throughout the experiment. Only cells that maintained a stable series resistance (<15% change) were included in the analysis. FP and I_{AHP} records were filtered at 2 kHz, acquired, and digitized at 10 kHz on a PC using custom software (Labview, National Instruments, Austin, TX). Paired *t*-tests were used to make within-cell comparisons of the effects of MPEP on DHPG-induced suppression of I_{AHP}.

Biochemical measurements of surface expressed AMPA receptors and ERK phosphorylation

Hippocampal slices were prepared as for electrophysiology experiments. After a 2- to 3-h recovery period in ACSF, slices (containing area CA1 and dentate gyrus, CA3 was cut-off) were maintained in a static incubation chamber in ACSF (containing 100 μM D,L-AP5) at 30°C and aerated with 95% O_2-5% CO_2. Slices were preincubated in antagonist and treated with DHPG (5 min) or ACSF (control). For phosphospecific (P)-ERK measurements, slices were frozen immediately after DHPG treatment and stored at –80°C as previously described (Gallagher et al. 2004). Slices were homogenized in lysis buffer containing 50 mM HEPES, pH 7.3, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% glycerol, 0.2 mM NaVO4, 100 mM NaF, 50 mM β-glycerophosphate, 1 mM

dithiothreitol, 1 mM benzamindine, 0.01 mg/ml leupeptin, 0.1 mg/ml aprotonin, 0.5 µg/ml pepstatin A, and 1% Triton. Protein concentrations were measured with a BCA Protein Assay (Pierce). Samples containing 20–35 µg of protein were resolved on 10% SDS-PAGE in duplicate and transferred to nitrocellulose. Membranes were blocked and incubated with P-ERK (Thr202/Tyr204, Promega; 1:5,000 dilution) or total ERK (1:1,000; Cell Signaling Technologies) according to manufacturer's protocol.

Biotinylation experiments were performed as previously described (Chung et al., 2000; Heynen et al., 2003; Nosyreva and Huber, 2005). From each rat, two to three slices were pooled together for one condition. Fifteen minutes after DHPG treatment, slices were placed on ice to stop endocytosis, washed with ice-cold ACSF, and incubated in ACSF containing 1 mg/ml Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) for 10 min on ice. To quench the biotin reaction, slices were washed three times with Tris-buffered saline (TBS) and homogenized in a modified radioimmunoprecipitation (RIPA) buffer containing: 50 mM Tris-HCl, pH 7.4, 1% Triton X100, 0.1% SDS, 0.5% Na-deoxycholate, 150 mM NaCl, 2 mM EDTA, 50 mM NaH_2PO_4, 50 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, and protease inhibitor cocktail III (Calbiochem, La Jolla, CA). The homogenates were centrifuged at 14,000*g* for 10 min at 4°C. Fifteen micrograms of protein was removed for total (T) protein measurements; 150 µg of protein was mixed with 150 µl of UltraLink immobilized NeutrAvidin beads (Pierce) by rotating for 2 h at 4°C. The beads were washed with 10 volumes of RIPA buffer, and proteins were eluted with SDS-PAGE sample buffer supplemented with 50 mM dithiothreitol for 20 min at 90°C. Both total and biotinylated proteins were resolved by SDS-PAGE transferred to nitrocellulose membranes and probed with anti-GluR1 C-terminal antibody (1:5,000;

Upstate Biotechnology). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) captured on autoradiography film (Kodak). Digital images were produced by densitometric scans of autoradiographs on a ScanJet 4300C (Hewlett Packard) and quantified using Scion Image software. Multiple film exposures were performed and quantified to insure that the values were in the linear range of the ECL reaction. A subset of GluR1 biotinylation experiments were quantified both using chemiluminense (ECL) and chemifluorescence using ECL Plus (Amersham) and quantified using a Storm 860 scanner (Molecular Dynamics) that yielded similar results.

The P-ERK/total ERK or surface/total GluR1 ratio was calculated for each condition. For the GluR1 ratios, duplicate conditions within one animal were averaged to obtain an animal average for that condition. Therefore the *n* values for the biotinylation experiments (Fig. 4) represent the number of rats as opposed to slices. Significant differences between surface/total ratios of treated slices and within-animal control slices were determined using a Wilcoxon signed-rank test. Although the raw ratio values were used for statistical comparisons, the group data are presented in Fig. 4 as a percent of condition control to compare across different treatment conditions.

All experiments were conducted according to a protocol approved by the Institutional Animal Use and Care Committee at UT Southwestern Medical Center.

Results

Combined blockade of mGluR1 and mGluR5 is necessary to block DHPG-induced LTD

mGluR-LTD can be induced chemically with the specific group 1 mGluR agonist, DHPG (Ito et al., 1992). LTD induced with 5 min of DHPG is absent in mGluR5 KO mice (Huber et al., 2001). I first attempted to confirm that pharmacological blockade of mGluR5 inhibits DHPG-induced LTD (Faas et al., 2002; Hou and Klann, 2004; Huang et al., 2004) in hippocampal slices prepared from Long Evans rats. Extracellular field potential recordings elicited by Schaffer collateral stimulation were obtained in area CA1. These and all subsequent LTD experiments were performed in the *N*-methyl-D-aspartate (NMDA) receptor antagonist D,L-AP5 (100 μM) to prevent induction of NMDA receptor-dependent LTD (Dudek and Bear, 1992). AP5 had no effect on DHPG-induced LTD (100 μM; 20 min; data not shown) (Huber et al., 2001). MPEP is the most potent and selective commercially available mGluR5 antagonist, with an IC_{50} = 32 nM for mGluR5 and >100 μM for mGluR1 (Gasparini et al., 1999). Surprisingly, in contrast to previous reports, MPEP (10 μM), applied during the baseline and DHPG application, had no effect on the acute or long-lasting depression induced with DHPG (**Fig. 2.1A,** *100 μM; 5 min; MPEP = 77 ± 2% of pre-DHPG baseline, n = 6, measured 60–65 min after DHPG application; interleaved control slices = 77 ± 3%, n = 6, P = 0.99).* To test the role of mGluR1 in the induction of LTD, the mGluR1 antagonist LY367385 (100 μM) was applied before and during DHPG (100 μM; 5 min). As previously described, LY367385 reduced the acute depression observed during DHPG application but did not affect LTD (**Fig. 2.1B,** *acute depression; LY367385 = 64 ± 4%, n*

= 5; interleaved controls = 37% ± 5%, n = 7, P < 0.01; LTD; LY367385 = 82 ± 4%, n = 5; interleaved control; 82 ± 4%, n = 7, P = 0.92) (Fitzjohn et al., 1999; Mannaioni et al., 2001; Hou and Klann, 2004). However, preincubation in both MPEP (10 μM) and LY367385 (100 μM) completely blocked mGluR-LTD induced with DHPG (**Fig. 2.1C**, *LY367385 + MPEP = 97 ± 2%, n = 7; interleaved controls = 83 ± 4%, n = 6, P < 0.0*). These data suggest that activation of mGluR1 or mGluR5 is sufficient to induce LTD and consequently inhibition of both receptors is required to block LTD induction. Interestingly, LY367385 and MPEP did not completely block the acute depression observed during DHPG application (**Fig. 2.1C**, *60 ± 6% of pre-DHPG baseline, n = 7*). It is unlikely that the residual acute depression was caused by subsaturating concentrations of LY367385 or MPEP, because increasing LY367385 to 150 μM did not further inhibit the acute depression during DHPG compared with 100 μM LY367385 (62 ± 6%, $n = 6$; $P = 0.8$), and MPEP, at ~100x its IC50, had no affect on the acute depression when applied alone (**Fig. 2.1A**) or in the presence of LY367385 (**Fig. 2.1B, and C**)

mGluR1 and mGluR5 contribute to DHPG-induced phosphorylation of ERK

DHPG induces phosphorylation of the mitogen-activated kinase, ERK, in hippocampal area CA1 (Roberson et al., 1999; Berkeley and Levey, 2003; Gallagher et al., 2004), and ERK activation is required for mGluR-LTD (Gallagher et al., 2004). Therefore, ERK activation may be a biochemical measure of the LTD induction signaling cascade. Because activation of mGluR1 or mGluR5 in isolation induces LTD, activation of mGluR1 or mGluR5 alone should induce phosphorylation of ERK. Previously our lab reported that combined application of LY367385 and MPEP blocks DHPG-induced (100

μM; 5 min) phosphorylation of ERK (Gallagher et al., 2004). In contrast to their effects on DHPG-induced LTD, MPEP or LY367385 alone inhibited DHPG-induced ERK phosphorylation (**Fig. 2.1D**, *ACSF + DHPG; 470 ± 64% of basal levels, n = 6; MPEP + DHPG = 195 ± 24%, n = 5; LY367385 + DHPG = 214 ± 45%, n = 7*). A one-way ANOVA and subsequent multiple comparison test (Fisher's PLSD) indicated that the ACSF (DHPG only) group was different from either MPEP- or LY367385-treated groups [$F(2,15) = 11.01$, $P = 0.002$; MPEP; $P = 0.005$; LY367385; $P = 0.005$]. Although LY367385 and MPEP reduce the levels of phosphorylated ERK, DHPG still increased ERK phosphorylation over basal levels in the presence of each drug (MPEP, $P = 0.01$; LY367385, $P = 0.04$; *t*-test). Taken together, the data in Fig. 1 suggest that this moderate level of ERK activation is sufficient to induce the full level of LTD.

mGluR1 activity is required for LTD expression

Previous data show inconsistency in the role of mGluR1 in DHPG-LTD: pharmacological blockade of mGluR1 either reduces or has no effect on DHPG-LTD (Fitzjohn et al., 1999; Faas et al., 2002; Hou and Klann, 2004). I noticed that a key difference in these studies seemed to be the duration of LY367385 application. Studies that observed no effect of mGluR1 antagonism on DHPG LTD washed the antagonist out immediately after DHPG application, whereas mGluR1 antagonist was persistently in the bath in studies that observed a reduction in LTD. Consistent with this observation, I found that the presence of LY367385 throughout the experiment reduced DHPG-LTD by ~50% using either brief (5 min) or prolonged (20 min) DHPG applications (**Fig. 2.5A,** *5 min; LY367385; 91 ± 1%; n = 6; control 79 ± 2%; n = 7; P = 0.002*; **Fig. 2.2A**, *20 min;*

LY367385 = 88 ± 4% measured at 75–80 min, n = 6; controls = 66 ± 2%, n = 6, P < 0.01). Because DHPG-LTD is unaffected when LY367385 is washed out of the slice immediately after DHPG (**Fig. 2.1B**), these data suggest a role for mGluR1 in LTD expression. Previous reports have shown that DHPG-induced LTD can be transiently "reversed" by applying broad range mGluR antagonists after LTD has been established (Palmer et al., 1997; Fitzjohn et al., 1999; Watabe et al., 2002). To further examine the role of mGluR1 in expression of LTD, I tested the ability of LY367385 to reverse LTD. LY367385 was applied for 20 min beginning 60 min after DHPG application. LY367385 reversed ~50% of LTD expression (**Fig. 2.2B$_1$**, *LTD, 55–60 min post-DHPG = 69 ± 2%; LY367385 reversal, 75–80 min post-DHPG = 86 ± 4%, n = 12; P < 0.01*). After LY367385 washout, LTD was re-established and not different from LTD before LY367385 (*100–105 min post-DHPG = 73 ± 4%, n = 12, P = 0.1*). LY367385 did not facilitate baseline synaptic transmission before DHPG application (**Fig. 2.2B$_2$**, *95 ± 2%; n = 8; P = 0.07,* ○), suggesting that LY367385 is specifically reversing an LTD process. In addition, application of LY367385 vehicle (NaOH) 60 min after DHPG had no effect on LTD, confirming that reversal is mediated by the actions of LY367385 (*LTD, 55–60 min post-DHPG = 51 ± 5%; NaOH reversal, 75–80 min post-DHPG = 50 ± 6%, n = 5; P = 0.4*). To determine if reversal of LTD is specific to postsynaptically mediated mGluR LTD, I repeated the reversal experiment in young rats (P9-P15). Recall that mGluR LTD is protein synthesis-independent and mediated by a presynaptic mechanism in immature rodents. LTD was induced in the presence of anisomycin (20μM) in young rats, but LY367385 failed to reverse LTD (**Fig. 2.2C,** *LTD, 55–60 min post-DHPG = 79 ± 6%; LY367385 reversal, 75–80 min post-DHPG = 75 ± 7%, n = 5; P=0.03*). In fact,

LY367385 slightly but significantly enhanced LTD in this experiment. LY367385 also failed to reverse LTD in immature rodents in the absence of anisomycin (*LTD, 55–60 min post-DHPG = 78 ± 4%; LY367385 reversal, 75–80 min post-DHPG = 82 ± 6%, n = 16; P=0.09*). The lack of LTD reversal in young animals suggests that reversal is mediated by a postsynaptic mechanism, perhaps modulation of AMPAR trafficking. In addition, these data argue against LTD reversal being a pharmacology artifact resulting from DHPG failing to wash out of the slice.

To confirm the role for mGluR1 in expression of DHPG-induced LTD, I evaluated LTD in mGluR1 knockout mice. In agreement with the pharmacological data, the DHPG-induced acute depression and LTD were reduced by ≈50% in mGluR1 KO mice (**Fig. 2.2D**, *acute depression measured 15–20 min: mGluR1 KO = 59 ± 2%; mGluR1 WT littermates = 34 ± 4%, P < 0.01; LTD: mGluR1 KO = 81 ± 3%; mGluR1 WT littermates = 64 ± 4%, P = 0.01 measured 75–80 min after DHPG application*). I also examined the ability of the mGluR1 antagonist LY367385 to reverse LTD in WT and mGluR1 KO mice. Consistent with the data in rats, LY367385 transiently reversed LTD in WT mice when applied after LTD had been established (**Fig. 2.2D,** *80–100 min after DHPG application; mGluR1 WT, reversal; 79 ± 5% measured at 95–100 min after DHPG application; P < 0.05 compared with LTD at 75–80 min*). However, LY367385 had no effect in mGluR1 KO mice, confirming that LY367385 is selective for mGluR1 (**Fig. 2.2D**, *mGluR1 KO reversal = 83 ± 3% measured at 95–100 min after DHPG application; P = 0.44 compared with LTD at 75–80 min*). Interestingly, LY367385 reversed LTD in the mGluR1 WT up to the level of LTD in the mGluR1 KO such that that there is no difference in the magnitude of LTD between the mGluR1 KO and WT

during LY367385 application (**Fig. 2.2D,** *P = 0.61*). Overall, these results implicate sustained mGluR1 activity in the expression of LTD. To test the hypothesis that the requirement for sustained mGluR1 activity in LTD expression represents induction of constitutively active mGluR1 by DHPG, I attempted to reverse LTD with the mGluR antagonist CPCCOEt, which does not display inverse agonist activity (Pula et al., 2004). Application of CPCCOEt (50-100μM) after induction of LTD resulted in small but significant reversal of LTD (**Fig. 2.2E,** *LTD, 56–60 min post-DHPG = 57 ± 3%; CPCCOEt reversal, 78–82 min post-DHPG = 67 ± 2%, n = 13; P < 0.01*). However, DMSO also produced a small but significant reversal of LTD (**Fig. 2.2E,** *LTD, 56–60 min post-DHPG = 57 ± 2%; CPCCOEt reversal, 78–82 min post-DHPG = 61 ± 3%, n = 5; P = 0.04*) and the magnitude of LTD was not different between CPCCOEt and DMSO treated slices either before or immediately after reversal (**Fig.2.2E**, *LTD at 56-60min, CPCCOEt vs. DMSO, P = 0.97; LTD at 78-82min., CPCCOEt vs. DMSO, P = 0.17*). While these data are not conclusive, CPCCOEt does have a much smaller effect than LY367385 when applied after induction of LTD, consistent with the idea that reversal of LTD by LY367385 acts through inhibition of constitutively active mGluR1. However, more experiments are required to determine if a DHPG- induced increase in constitutive mGluR1 activity is necessary for expression of LTD.

In contrast to mGluR1, blockade of mGluR5 with 10 μM MPEP throughout the experiment had no effect on LTD (**Fig. 2.3A,** *MPEP = 64 ± 3%, n = 7; interleaved controls = 62 ± 4%, n = 4; P = 0.7*). In addition, blockade of mGluR5 60–80 min after DHPG failed to reverse LTD expression (*LTD, 55–60 min post-DHPG application = 53 ± 4%; MPEP reversal, 75–80 min post-DHPG = 55 ± 4%, n = 10; P = 0.5*). Prolonging

the MPEP application from 20 to 60 min also did not reverse LTD expression (**Fig. 2.3B,** *LTD, 55–60 min post-DHPG = 57 ± 4%; MPEP reversal, 115–120 min post-DHPG = 64 ± 8%, n = 5; P = 0.29*). Perfusion of MPEP and LY367385 after 60min. after LTD induction only partially reversed LTD (**Fig2.3C,** *LTD, 55–60 min post-DHPG = 67 ± 3%; MPEP + LY367385 reversal, 75–80 min post-DHPG = 89 ± 3%, n = 8; P < 0.01*) and was not different from reversal with LY367385 alone ($P = 0.78$), suggesting that persistent mGluR5 activity is not necessary for expression of LTD, or MPEP is ineffective in blocking persistent mGluR5 activity.

MPEP inhibits the DHPG-induced suppression of IAHP in hippocampal CA1 neurons but not DHPG-induced LTD

MPEP inhibits ERK phosphorylation (**Fig. 2.1D**) and blocks LTD induction when combined with mGluR1 blockade (**Fig. 2.1C**). However, in light of our negative results with MPEP alone on LTD, I wanted to confirm an effect of MPEP on an electrophysiological measure in CA1 pyramidal neurons in our slice preparation. I tested the ability of MPEP to block DHPG-induced suppression of the AHP. The AHP can be divided into three components, a fast, medium, and slow AHP, which are mediated by activation of different potassium conductances (Storm, 1990). DHPG suppresses both the medium and slow AHP, which are either partially or completely blocked by MPEP (Mannaioni et al., 2001; Ireland and Abraham, 2002). Whole cell patch-clamp recordings were obtained from visualized CA1 neurons from rat hippocampal slices. Cells were voltage clamped at –50 mV, and the Ca^{2+} activated potassium current that mediates the AHP (I_{AHP}) was elicited by applying a depolarizing step to +60 mV. An outward tail

current was measured as the I_{AHP} as described (Pedarzani and Storm, 1993; Mannaioni et al., 2001) (**Fig. 2.4A**). DHPG application (100 μM) reduced I_{AHP} (*baseline = 382 ± 32 pA; DHPG = 221 ± 21 pA, n = 10; P < 0.01*). MPEP (10 μM) reversed the effects of DHPG on the I_{AHP} (*DHPG + MPEP = 266 ± 19 pA, n = 10; P < 0.01*). Similar results were observed with 30 μM DHPG (*baseline = 366 ± 32 pA; DHPG = 161 ± 27 pA, P < 0.01 compared with baseline; DHPG+MPEP = 229 ± 29 pA, n = 9, P < 0. 01 compared with DHPG alone*). Although MPEP did not completely reverse the effects of DHPG in every cell tested, on average, it reduced DHPG induced suppression of the I_{AHP}. This result is consistent with that of Ireland and Abraham (2002), who found that both mGluR1 and mGluR5 mediate IAHP suppression in CA1 neurons and suggests that our inability to block LTD is not caused by inactive MPEP.

Strain variations in LTD and mGluR expression have been noted in mice and rats (Manahan-Vaughan, 2000b, a; Chen et al., 2005). To determine if our inability to block LTD with MPEP was caused by the particular rat strain used in our experiments, I tested the ability of MPEP to block LTD in a different strain of rats and in mice. Previous work has shown that MPEP blocks LTD in hippocampal slices prepared from Sprague-Dawley (SD) rats induced with the mixed isomer *R,S*-DHPG (100 μM; 20 min) or the active form *S*-DHPG (50 μM; 5 min) (Faas et al., 2002; Huang et al., 2004). Consistent with my data in Long Evans rats, I found that in slices prepared from Sprague-Dawley rats, MPEP (10 μM) had no effect on LTD induced with *R,S*-DHPG (**Fig. 2.4B,** *100 μM; 20 min; MPEP = 60 ± 4%, n = 8; interleaved controls = 62 ± 3%, n = 8, P = 0.78*) or *S*-DHPG (50 μM; 5 min; (*MPEP = 85 ± 9%, n = 2; control = 88 ± 2%, n = 2, P = 0.73*). I also saw no effect of MPEP on DHPG-induced LTD (100 μM; 20 min) in slices prepared from

C57BL6 mice (**Fig. 2.4C,** *MPEP = 74 ± 5%, n = 8; control = 69 ± 5%, n = 4, P = 0.55*). Finally, slices (from Long Evans rats) that were perfused with 25 μM MPEP throughout the experiment had normal DHPG-induced LTD (*DHPG, 100 μM; 5 min; MPEP = 87 ± 3%, n = 6; control = 84 ± 2%, n = 8, P = 0.44*). Therefore, I found that under several experimental conditions in both mice and rats, MPEP was ineffective in blocking mGluR-LTD.

mGluR1 is required for DHPG-induced decreases in AMPA receptor surface expression

DHPG treatment of hippocampal neurons results in a rapid endocytosis and persistent decrease in the surface expression of postsynaptic AMPA receptors that are thought to mediate LTD (Snyder et al., 2001; Xiao et al., 2001; Nosyreva and Huber, 2005). The mGluR subtype that mediates DHPG-induced decreases in AMPAR surface expression is unknown. Receptor biotinylation was used to study the role of mGluR1 and mGluR5 in DHPG-induced decreases in GluR1 surface expression. Slices were preincubated in antagonists (15–30 min) before DHPG or ACSF application and throughout the experiment. Slices were treated with DHPG (100 μM; 5 min), and surface proteins were biotinylated 15 min after DHPG application. **Figure 2.5A** shows the time-course for receptor biotinylation overlaid onto the electrophysiological equivalent of **Fig 2.5D**. Like LTD, the combined application of LY367385 and MPEP blocked DHPG-induced decreases in GluR1 surface expression (**Fig. 2.5C,** *LY367385 + MPEP + DHPG = 109 ± 12% of control slices, n = 7, P = 0.7*). Because LTD is reduced by the continued presence of LY367385, but not MPEP, I predicted that decreases in GluR1 surface expression should also rely on mGluR1. Incubation in LY367385, but not MPEP, blocked

DHPG induced decreases in GluR1 surface expression (**Fig. 2.5C**, *MPEP + DHPG = 84 ± 4% of condition control, n = 9, P = 0.02; ACSF + DHPG = 77 ± 4%, n = 8, P = 0.02*; **Fig. 2.5D**, LY367385 + DHPG = 106 ± 12% of condition control, $n = 12$, $P = 0.53$; ACSF = 80 ± 5%, $n = 10$, $P < 0.01$). These results are consistent with our findings that mGluR1 antagonists alone reduce expression of DHPG-LTD (**Fig. 2.5A**), whereas blockade of mGluR5 alone has no effect (**Fig. 2.5D**).

Synaptically induced LTD does not require group I mGluR activation

Previously it was shown that mGluR-dependent LTD can be elicited by synaptic stimulation using PP-LFS (50-ms interstimulus interval; 1 Hz) (Kemp and Bashir, 1999; Huber et al., 2000). This conclusion was based on the finding that the broad range mGluR antagonist LY341495 (100 μM; **Fig. 2.6E**) blocks PP-LFS-induced LTD (Huber et al., 2000). However, there is very little data addressing the specific mGluR(s) required for PP-LFS–induced LTD (but see Faas et al. 2002). I next examined the role of group 1 mGluRs in synaptically induced LTD using PP-LFS. All experiments were performed in 100 μM D,L-AP5. PP-LFS-induced LTD is normal in mGluR1 KO mice (**Fig. 2.6A,** *mGluR1 KO = 85 ± 3%, n = 15; mGluR1 WT littermates = 84 ± 3%, n = 11, P = 0.78*) and in mGluR5 KO mice (**Fig.2.6B$_1$**, *mGluR5 KO = 73 ± 6%, n = 5; mGluR5 WT littermates = 75 ± 6%, n = 6, P = 0.77*). This result is in stark contrast to the effect of mGluR1 KO (**Fig. 2C**) and mGluR5 KO (**Fig. 2.6B$_2$**, *mGluR5 KO = 96 ± 1%, n = 5; mGluR5 WT littermates = 61 ± 4%, P < 0.01*) (Huber et al. 2001) on DHPG-induced LTD. I next sought to determine if inhibition of both mGluR1 and mGluR5 was necessary to block PP-LFS-induced LTD. Blockade of mGluR1 with 100 μM LY367385

in mGluR5 KO mice had no effect on PP-LFS-induced LTD (**Fig. 2.6C,** *mGluR5 KO + LY367385 = 81 ± 5%, n = 10, P = 0.2 compared with mGluR5 KO without antagonists*). In addition, pharmacological blockade of group I mGluRs in Long Evans rats with 10 μM MPEP and 100 μM LY367385 had no effect on PP-LFS-induced LTD (**Fig.2.6D,** *LY367385 + MPEP = 76 ± 4%, n = 11; interleaved controls = 79 ± 3%, n = 11, P = 0.47*). In light of the surprising finding that group I mGluR activation is not required for induction of PP-LFS LTD, we confirmed that LY341495 (100 μM) inhibits PP-LFS-induced LTD (**Fig. 2.6E,** *100 μM LY341495 = 93 ± 4%, n = 5; interleaved controls = 61 ± 4%, n = 5, P < 0.01*). Taken together, this data suggests that specific blockade of group II and III mGluRs or combined blockade of all three mGluR groups is required to block PP-LFS–induced LTD. While LY341495 inhibits group I, II, and III mGluRs at 100 μM, it is effective against primarily group II and III mGluRs at 20 μM (Kingston et al., 1998; Capogna, 2004). Selective blockade of group II and III mGluRs with 20 μM LY341495 had no effect on PP-LFS LTD in Long Evans rats (**Fig. 2.6F,** *20 μM LY341495 = 76 ± 3%, n = 6*). A cocktail of 10 μM MPEP, 100 μM LY367385, and 20 μM LY341495 was used to block all three mGluR groups, and this also was ineffective against PP-LFS-induced LTD (**Fig. 2.6F,** *20 μM LY341495 + LY367385 + MPEP = 71 ± 5%, n = 6, P = 0.45 compared with 20 μM LY341495 alone*). These data suggest that blocking all mGluRs may not be sufficient to block PP-LFS LTD and that 100 μM LY341495 may have effects at other as yet unidentified mGluRs or other neurotransmitter receptors. Future experiments are required to determine the identity of these receptors.

Discussion

Data presented in this chapter indicate a novel and unexpected role for mGluR1 in DHPG-induced synaptic plasticity at CA1 excitatory synapses. Activation of either mGluR1 or mGluR5 can induce the full complement of LTD. Consistent with these data, both mGluR1 and mGluR5 induced activation of ERK, which is required for mGluR-LTD (Gallagher et al. 2004). Furthermore, mGluR1 is required for the expression of DHPG-induced LTD and the associated decrease in AMPAR surface expression.

Previous work in the hippocampus and other brain regions show that mGluR1 and mGluR5 can mediate similar functions in neurons (Gubellini et al. 2003; Ireland and Abraham 2002; Karim et al. 2001; Lee et al. 2002; Merlin 2002; Rae and Irving 2004). However, in these studies, mGluR1 or mGluR5 blockade alone had a partial or complete effect on the physiological or behavioral measure. I found that blockade of either mGluR1 or mGluR5 had no effect on LTD induction, whereas the combined blockade completely prevented LTD (Fig. 2.1). This result suggests that mGluR1 and mGluR5 can fully substitute for each other and that both receptors can activate the signaling cascades required for LTD induction. The fact that both mGluR1 and mGluR5 induced ERK activation supports this conclusion and suggests that this level of activation is sufficient for maximal LTD.

In my attempts to block DHPG-induced LTD with MPEP, I tested mice and two different rat strains, used 5 or 20 min of DHPG application, used either *R,S*-DHPG or *S*-DHPG, and used different durations of MPEP application and two different concentrations (10 and 25 μM). All of these conditions yielded no effect of MPEP on

LTD. Like previous studies, I used 10 μM MPEP for most of these experiments, which is almost 300 times the IC_{50} value for MPEP against mGluR5. Furthermore, MPEP is a noncompetitive antagonist for mGluR5, and at 10 μM, its effects on agonist-stimulated phosphoinositide turnover are saturating. MPEP (10 μM) inhibits >95% of agonist-stimulated phosphoinositide turnover at cloned mGluR5 and >75% in rat hippocampus (Gasparini et al. 1999). It is unlikely that differences between this and other published work are caused by developmental changes in the mGluR subtype required for LTD, because the age range of rats and mice used in this study is similar to that used in previous studies (3–6 wk) (Faas et al. 2002; Hou and Klann 2004; Huang and Hsu 2006; Huang et al. 2004). My inability to block DHPG-induced LTD with MPEP cannot be explained by inactive MPEP in my slice preparation because I observed effects of MPEP on ERK activation (Fig. 2.1D), suppression of the I_{AHP} (Fig. 2.4), and LTD induction when combined with LY367385 (Fig. 1C). There are other examples or reports that differ in their findings of the contribution of mGluR1 and mGluR5 to other mGluR-dependent functions in CA1 and striatal neurons, suggesting that group I mGluR expression or function may be particularly sensitive to regulation by a number of physiological or pathophysiological conditions (Gubellini et al. 200; Ireland and Abraham 2002; Mannaioni et al. 2001; Rae and Irving 2004; Sung et al. 2001). Recent findings demonstrate that elevated glutamate levels associated with excitotoxicity result in NMDAR-dependent cleavage of mGluR1 but not mGluR5 (Xu et al., 2007). Cleavage of mGluR1 results in a redistribution of mGluR1 from dendrites to axons and prevents activation of PI3K/Akt/mTOR signaling, which is required for group I mGluR-mediated LTD. Brain slices are vulnerable to excitotoxic damage during the slicing procedure,

suggesting that if care is not taken to minimize excitotoxic damage, cleavage of mGluR1 may occur resulting in slices that have less functional mGluR1 and therefore rely on mGluR5 for effects mediated by group I mGluRs. In contrast to results with MPEP, LTD induced with a either a brief (50 μM; 5 min) (Huber et al. 2001) or prolonged (100 μM; 20 min) DHPG application is completely absent in mGluR5 KO mice, (Fig. 2.6B_2). These results suggest that mGluR1 can compensate for mGluR5 when it is blocked pharmacologically, but not in the absence of mGluR5 protein. My findings also suggest that there may be alterations in mGluR1 expression, localization, or function in CA1 neurons of mGluR5 KO mice.

Although my data show that mGluR1 and mGluR5 can both induce LTD, I only see a role for mGluR1 in expression of LTD and decreases in surface GluR1 (Figs. 2.2 and 2.5). This finding is consistent with studies that have found that mGluR1 and mGluR5 mediate distinct functions in neurons (for review, see Valenti et al. 2002). Behavioral data also support distinct roles for mGluR1 and mGluR5 in the same brain region. Infusion of mGluR1 or mGluR5 antagonists into the CA1 region of the hippocampus impairs inhibitory avoidance memory, whereas mGluR1 but not mGluR5 in is required for extinction of inhibitory avoidance learning (Simonyi et al., 2007). The reversal of DHPG-induced LTD with nonselective mGluR antagonists has been previously shown (Fitzjohn et al. 1999; Palmer et al. 1997; Watabe et al. 2002). Data with pharmacological blockade or genetic knockout of mGluR1 indicate that activation of mGluR1 is important for the expression of LTD (Fig. 2.2), suggesting that persistent mGluR1 activity contributes to LTD expression. Other studies have discovered that mGluR1 activity is required for the expression of long-term potentiation in medial

vestibular neurons and epileptiform bursts in CA3, suggesting that mGluR1 may be a common mechanism to sustain mGluR-dependent plasticity in the brain (Grassi et al., 2002; Merlin, 2002). In support of this, my data demonstrating that the inverse agonist LY367385 is much more effective in reversing LTD expression than the antagonist CPCCOEt suggest that induction of LTD with DHPG induces constitutive mGluR1 activity (Fig.2.2B, E). These findings support studies that have shown a role for mGluR1 in hippocampal-dependent learning and suggest that LTD in CA1 may contribute to these behaviors (Aiba et al., 1994; Petersen et al., 2002; Maciejak et al., 2003; Simonyi et al., 2007).

What is the physiological mechanism underlying reversal of LTD? LY367385 fails to reverse LTD in immature rats, suggesting that constitutive mGluR1 activity regulates a postsynaptic LTD mechanism, most likely AMPAR endocytosis. However, I did not test the relative contributions of mGluR1 and mGluR5 to LTD in young animals, so additional studies are needed to confirm that mGluR1 plays a role in LTD in immature animals. A recent study by Huang et al. finds that DHPG LTD is reversed by the mGluR5 antagonist MPEP, but not the mGluR1 antagonist LY367385 (Huang and Hsu, 2006). Interestingly, they find that tyrosine phosphatase inhibitors also reverse LTD and that reversal by the non-selective mGluR antagonist MCPG is associated with a transient increase in GluR2 phosphorylation. GluR2 dephosphorylation is associated with DHPG-induced AMPAR endocytosis (Moult et al., 2006). Taken together, these data support a role for constitutive mGluR activity in maintaining LTD through persistent regulation of AMPAR endocytosis. It is unclear why different groups see varying contributions of mGluR1 and mGluR5 to hippocampal LTD, but regardless of the receptor required, my

data and other recently published studies shed light on a mechanism by which group I mGluRs maintain synaptic plasticity.

Previous work using single cell recordings of CA1 pyramidal neurons established that mGluR1 has many functions in these neurons, including acute depression of EPSCs, cell depolarization (or inward current), increases in intracellular [Ca^{2+}], and suppression of I_{AHP} (Ireland and Abraham 2002; Mannaioni et al. 2001; Rae and Irving 2004). Furthermore, an immunohistochemical study reported that DHPG induced phosphorylation of ERK in CA1 pyramidal neurons was inhibited by LY367385 or MPEP, consistent with the western blotting results in Fig2.1D (Berkeley and Levey 2003). These studies and my present work have relied on the specificity of LY367385 to make conclusions regarding mGluR1 function (Clark et al. 1997; Valenti et al. 2002). The fact that LTD reversal is not observed with LY367385 in the mGluR1 KO mouse strongly supports that its effects are specific for mGluR1 (Fig. 2.2D). Many studies have shown a functional role of mGluR1 in CA1 pyramidal neurons, but showing the presence of mGluR1 protein has been more elusive (Ferraguti et al. 2004; Lujan et al. 1996; Martin et al. 1992). The many functions attributed to mGluR1 in CA1 neurons may be mediated by a low diffuse expression of mGluR1. Alternatively, the currently available antibodies may not detect the relevant mGluR1 isoforms.

Studies from our lab and others have demonstrated that DHPG results in an endocytosis and persistent decrease in the surface expression of AMPARs that is thought to mediate LTD in mature neurons (Nosyreva and Huber 2005; Snyder et al. 2001; Xiao et al. 2001). In the present study, mGluR1 blockade completely blocked DHPG-induced decreases in GluR1 surface expression (Fig. 2.5). Based on my LTD, I expected that

LY367385 would only partially block the decrease in GluR1 surface expression. It is likely that partial effects of LY367385 on GluR1 surface expression were undetectable because of large variability in the receptor biotinylation assay compared with LTD measurements. Alternatively, the mGluR1-dependent GluR1 endocytosis may only mediate part of the LTD. Based on the effects of mGluR1 blockade on LTD (Fig. 2.5A), I interpret this result as an effect of LY367385 on the expression (as opposed to induction) of GluR1 surface decreases.

At the time of this study DHPG- and PP-LFS-induced LTD (in AP5) were believed to represent the same LTD mechanism. Both are blocked by LY341495, absent in the Gαq KO mouse, rely on protein synthesis and ERK activation, are enhanced in the fragile X syndrome mouse model, and are similarly developmentally regulated (Huber et al. 2000, 2001, 2002; Nosyreva and Huber 2005; Zho et al. 2002). While I find that group I mGluR activity is not required for PP-LFS LTD, these data, and the fact that PP-LFS-induced LTD occludes DHPG-induced LTD indicate that the two forms of plasticity converge on a common protein synthesis-dependent mechanism (Huber et al. 2001). Because PP-LFS-induced LTD is absent in Gαq knockout mice, it is possible that other Gq-coupled neurotransmitter receptors are sufficient to induce LTD when group1 mGluRs are blocked (Kirkwood et al. 1999; Kleppisch et al. 2001; Scheiderer et al. 2004), and this is addressed in Chapter Three. It is unclear why 100 μM LY341495 blocked PP-LFS-induced LTD. At high concentrations, LY341495 may have nonspecific effects at these other receptor types, or there may be other mGluR subtypes yet to be identified (Fitzjohn et al. 1998).

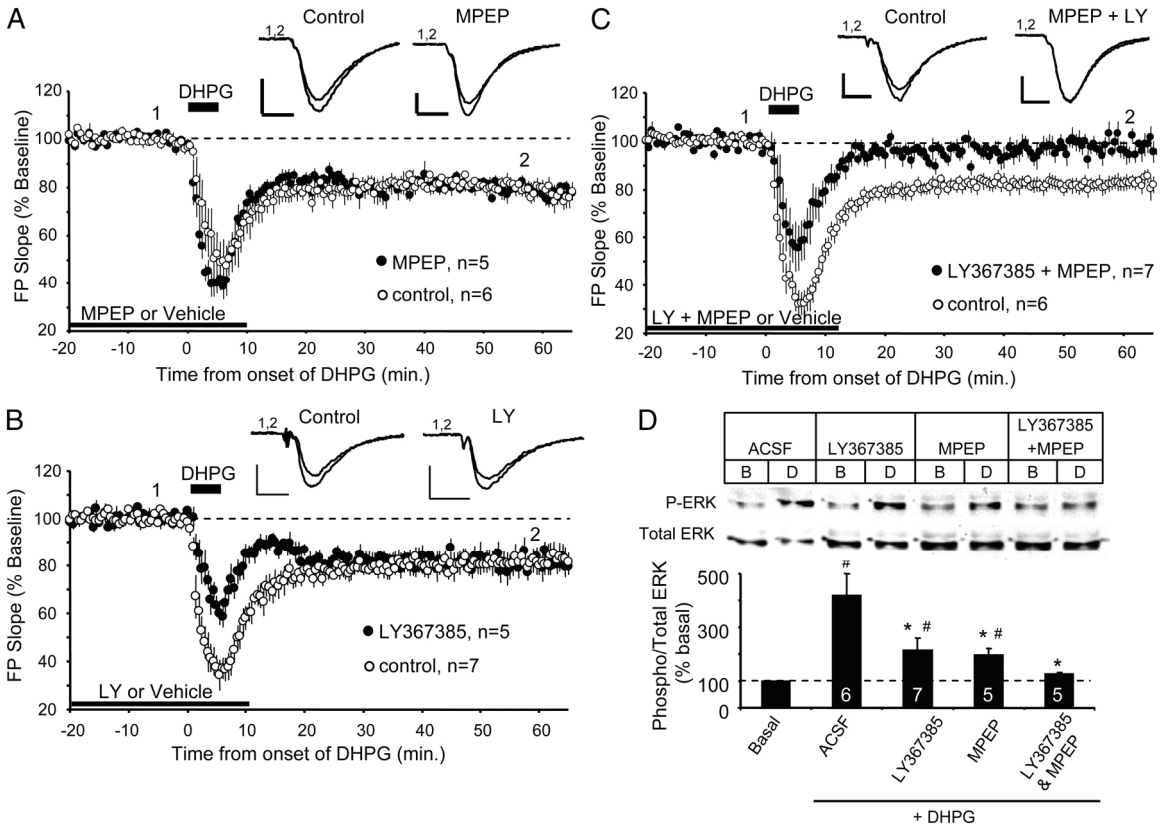


Figure 2.1, Activation of mGluR1 or mGluR5 is sufficient to induce LTD and activate ERK.

In all experiments, *N*-methyl-D-aspartate (NMDA) receptors were blocked with 100 µM D,L-AP5. ***A–C***: plotted are the average (±SE) initial slope values of field potentials (FPs) normalized to the pre-(*RS*)-3,5-dihydroxyphenylglycine (DHPG) baseline. LTD was induced with a 5-min application of 100 µM DHPG.

A: acute application of the mGluR5-specific antagonist MPEP (10 µM; solid line) during the baseline and DHPG application had no effect on LTD induction.

B: likewise, acute application of the mGluR1-specific antagonist LY367385 (100 µM; solid line) during the baseline and DHPG application had no effect on LTD induction.

C: combined, acute blockade of mGluR1 and mGluR5 (10 µM MPEP + 100 µM LY367385) abolished LTD. *Inset*: FPs (averages of 4–10 waveforms) from a representative experiment were taken at the times indicated by the numbers on the graph. For all panels, scale bars = 0.5 mV/5 ms.

D: representative Western blot of phosphorylated (P)-ERK and Total ERK under basal (*B*; untreated) or DHPG (*D*; 100 µM; 5 min) treated conditions in the absence of

antagonist [artificial cerebrospinal fluid (ACSF)] or in the presence of mGluR1 (LY367385) or mGluR5 (MPEP) antagonists. Group data show that the DHPG-induced increase in the ratio of P-ERK/total ERK is reduced by LY367385 or MPEP alone and the combined application of LY367385 and MPEP (*$P < 0.01$: LY367385, MPEP, or LY367385 + MPEP compared with ACSF). However, in the presence of LY367385 or MPEP, DHPG induces a significant increase in P-ERK (#$P < 0.05$). Data showing that LY367385 + MPEP block the DHPG-induced increase in P-ERK is replotted from (Gallagher et al., 2004a) for comparison to each antagonist alone.
**Note that experiments in panel D of this figure were performed by Christine Daly.

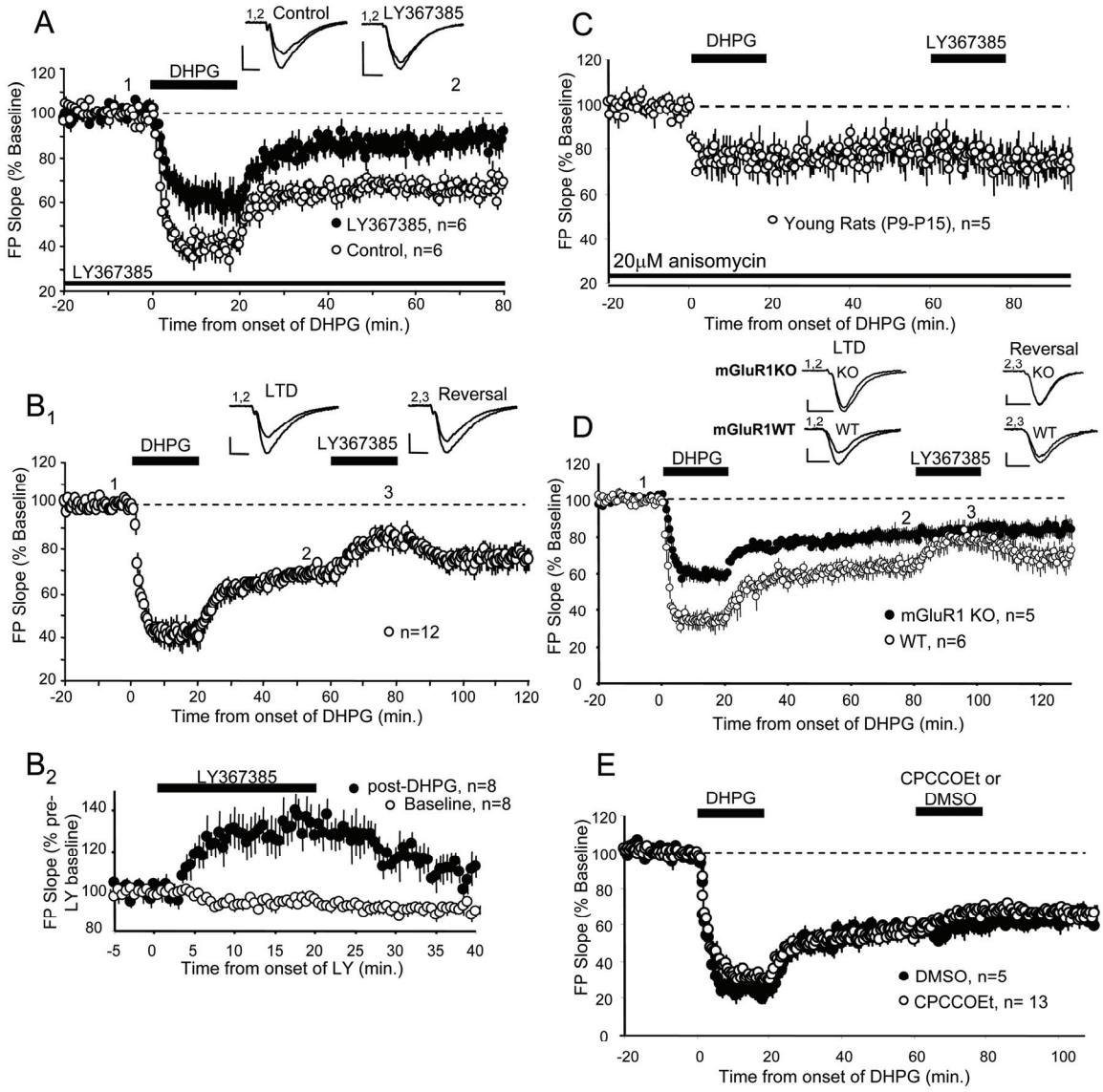
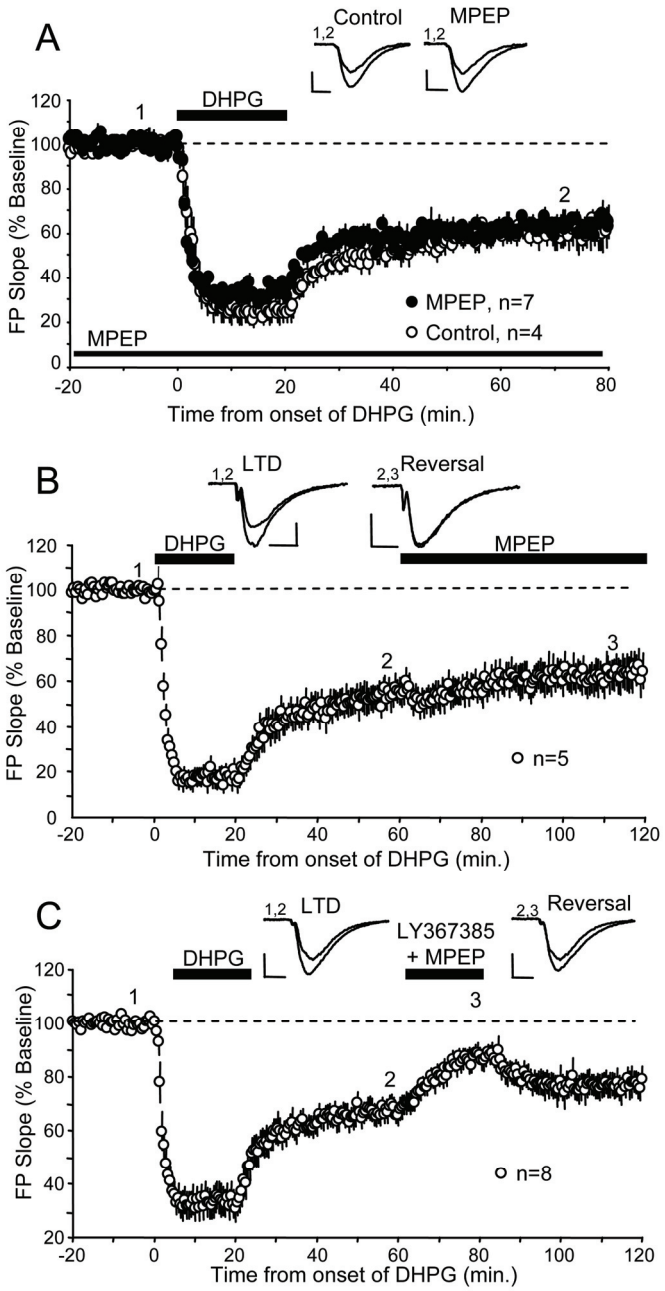


Figure 2.2, mGluR1 activity is required for LTD expression.

In all experiments, NMDA receptors were blocked with 100 μM D,L-AP5. *A, B_1*, and *C–F*: average (±SE) initial slope values of FPs normalized to the pre-DHPG baseline. LTD was induced with a 20-min application of 100 μM DHPG.
A: presence of the mGluR1-specific antagonist LY367385 (100 μM) for the duration of the experiment reduces DHPG-induced LTD ($P < 0.01$, LY vs. control at 70–75 min).
B_1: LY367385 (100 μM) application 60–80 min after DHPG transiently reverses LTD.
***B_2*:** LY367385 (20 min, 100 μM) was applied during the baseline (◌) and 60–80 min after DHPG (●) in the same slice. Plotted are the average (±SE) initial slope values of

FPs normalized to the pre-LY baseline. LY367385 (100 μM) has no significant effect on basal synaptic transmission (○). In contrast, LY367385 significantly increases synaptic transmission after LTD has been induced with DHPG (●).
C*:* LY367385 (100 μM) application 60–80 min after DHPG has no effect on LTD in immature rats.
D: LTD is reduced in mGluR1 knockout (KO) mice. LY367385 significantly reverses LTD in wildtype (WT) mice but not in mGluR1 KO mice. During LY367385 application, LTD is not different in mGluR1 KO vs. WT mice.
E: Application of the mGluR1 antagonist CPCCOEt, which does not display inverse agonist activity, 60-80min after DHPG has a minimal effect on LTD expression.



A: presence of the mGluR5-specific antagonist MPEP (10 μM) for the duration of the experiment has no effect on DHPG-induced LTD.
B: application of MPEP (60 min) does not reverse LTD expression.
C: reversal of LTD by co-application of LY367385 and MPEP is not different from LY367385 alone (as in Fig2.2B). *Inset*: FPs (averages of 4–10 waveforms) from a representative experiment are taken at the times indicated by the numbers on the graph. For all panels, scale bars = 0.5 mV/5 ms.

Figure 2.3, DHPG-LTD is unaffected by the mGluR5 antagonist MPEP.

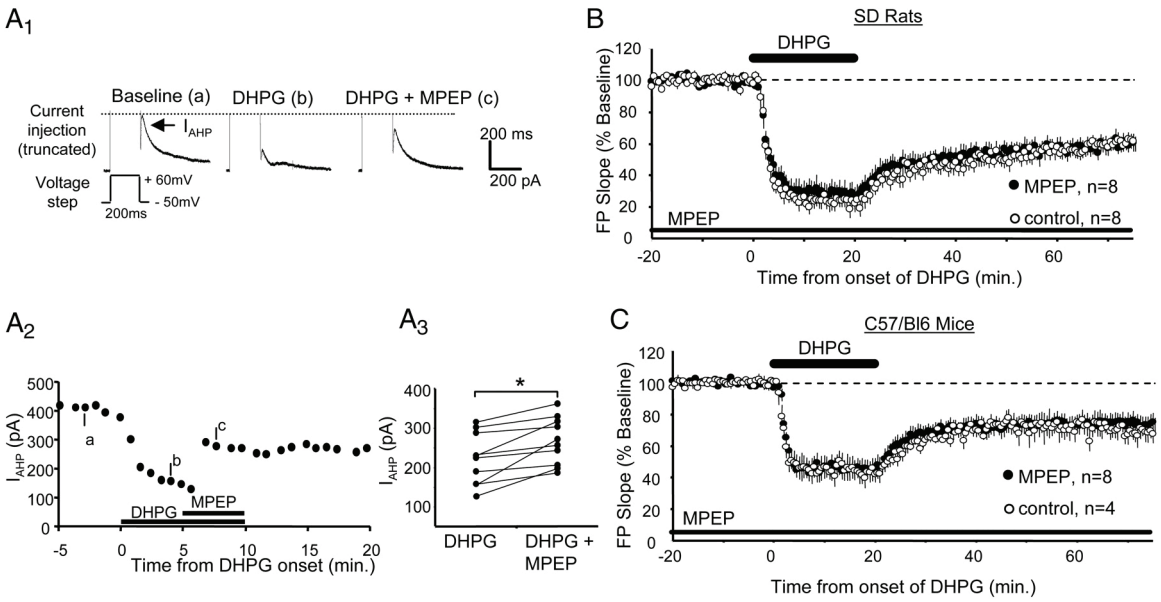
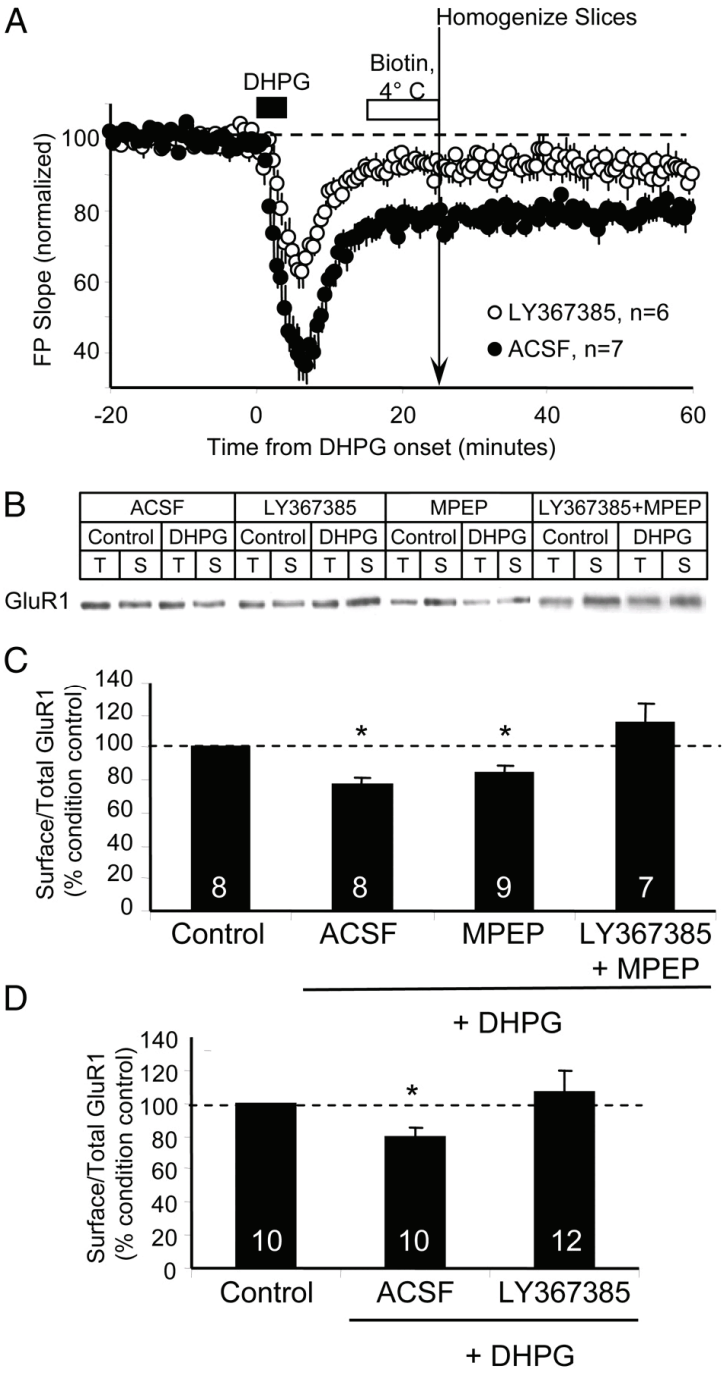


Figure 2.4, MPEP is effective at blocking the DHPG-induced suppression of I_{AHP} but not DHPG-induced LTD.

A1 and ***A2***: time-course of I_{AHP} in a representative experiment. I_{AHP} was elicited by applying a 110-mV (–50- to +60 mV) voltage step in the presence of 0.5 μM TTX and 1 mM TEA. Example waveforms (*A1*) indicate that I_{AHP} was measured as the outward current peak immediately (10–15 ms) after the voltage step; 100 μM DHPG significantly reduced the I_{AHP}, and subsequent wash-on of MPEP reversed this effect in the continued presence of DHPG.
A3: average I_{AHP} values from each cell in 100 μM DHPG and on reversal in MPEP (*$P <$ 0.01: DHPG vs. DHPG + MPEP).
B* and *C: LTD induced with 20 min DHPG is not affected by MPEP in multiple rat strains (*B*, Sprague-Dawley rats, and Long Evans hooded rats as in Figs. 2.1A, 2.3A), or in mice (*C*, C57BL/6 mice).



A: presence of LY367385 (100 μM) before, during, and after DHPG (100 μM; 5 min) reduces LTD. Time-course of biotin application (open bar) and slice homogenization (↓) is indicated.
B: sample Western blots of total (T) and surface (S) GluR1 subunits of the AMPA receptor for each condition.
C and *D*: average ratios of surface to total GluR1 normalized to condition control slices taken 15 min after DHPG application (100 μM; 5 min). Control slices, like DHPG-treated slices, were preincubated in ACSF or antagonists. Number of experiments per group is denoted on each bar.
*Significantly different from within animal controls ($P < 0.05$). DHPG treatment of hippocampal slices results in a decrease of GluR1 surface expression using receptor biotinylation. Preincubation in LY367385 (100 μM), but not MPEP (10 μM), blocks DHPG-induced decreases in GluR1 surface expression.
** Note that experiments in panel A were performed by Sean Gallagher and panels B-E were performed by Christine Daly

Figure 2.5, mGluR1 is required for DHPG-induced decreases in AMPA receptor surface expression.

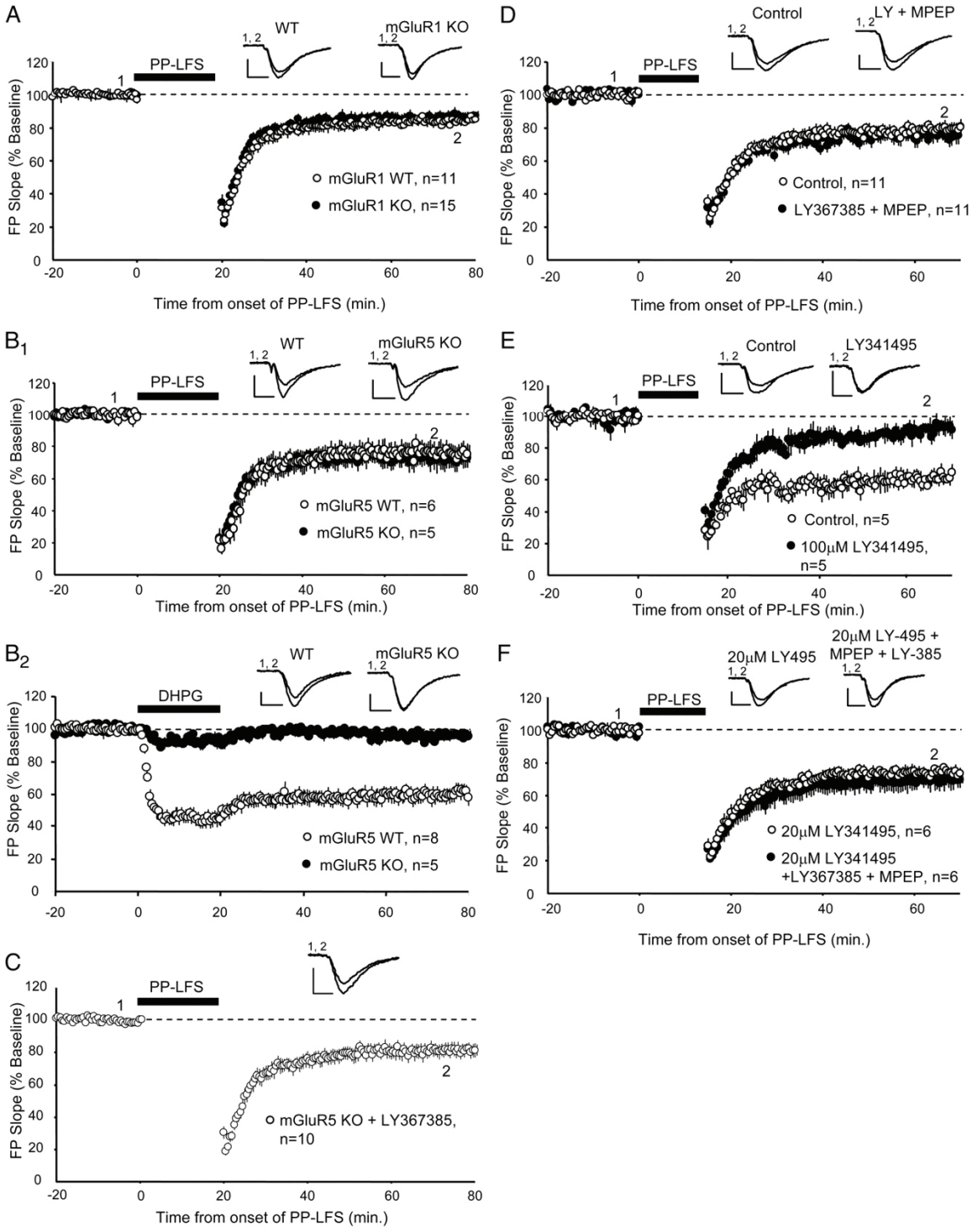


Figure 2.6, Group I mGluR activation is not required for synaptically induced LTD.

In all experiments, NMDA receptors were blocked with 100 μM D,L-AP5. ***A–F***: average (±SE) initial slope values of FPs normalized to the pre-DHPG or pre-paird-pulse low-frequency stimulation (PP-LFS) baseline. LTD was induced with PP-LFS (2 pulses with 50-ms interstimulus interval at 1 Hz) for 15 min. *D–F*: or 20 min. ***A***, ***B*** $_1$, and ***C***: or with 20 min of 100 μM DHPG (***B*** $_2$).
A: synaptically induced LTD is normal in mgluR1 KO mice.
B: synaptically induced LTD is normal in mgluR5 KO mice (B_1). In contrast, LTD induced with 20 min of DHPG is drastically reduced in the mGluR5 KO mouse (B_2).
C: synaptically induced LTD in mGluR5 KO mice is not affected by the addition of the mGluR1 antagonist LY367385 (100 μM).
D: pharmacological blockade of group I mGluRs with LY367385 (100 μM) and MPEP (10 μM) has no effect on LTD in Long Evans rats.
E: pharmacological inhibition of all mGluRs with the broad range mGluR antagonist LY341495 (100 μM) blocks synaptically induced LTD in Long Evans rats.
F: inhibition of groups II and III mGluRs with 20 μM LY341495 or inhibition of groups I, II, and III mGluRs with 20 μM LY341495 + 100 μM LY367385 + 10 μM MPEP has no effect on synaptically induced LTD.

CHAPTER THREE
Results

MULTIPLE GQ-COUPLED RECEPTORS CONVERGE ON A COMMON PROTEIN SYNTHESIS-DEPENENT LONG-TERM DEPRESSION THAT IS AFFECTED IN FRAGILE X SYNDROME MENTAL RETARDATION

Summary

Gq-coupled, M_1 muscarinic acetylcholine receptors (mAChRs) facilitate hippocampal learning, memory, and synaptic plasticity. M_1 mAChRs induce long-term synaptic depression (LTD), but little is known about the underlying mechanisms of mAChR-dependent LTD and its link to cognitive function. Data in this chapter demonstrate that chemical activation of M_1 mAChRs induces LTD in hippocampal area CA1, which relies on rapid protein synthesis, as well as the extracellular signal-regulated kinase and mammalian target of rapamycin translational activation pathways. Synaptic stimulation of M_1 mAChRs, alone, or together with the Gq-coupled glutamate receptors (mGluRs), also results in protein synthesis-dependent LTD. New proteins maintain mAChR-dependent LTD through a persistent decrease in surface AMPA receptors. mAChRs stimulate translation of the RNA-binding protein, Fragile X mental retardation protein (FMRP) and FMRP target mRNAs. In mice without FMRP (*Fmr1* knock-out), a model for human Fragile X syndrome mental retardation (FXS), both mGluR- and mAChR-dependent protein synthesis and LTD are affected. These results reveal that multiple Gq-coupled receptors converge on a common protein synthesis-dependent LTD

mechanism, which is aberrant in FXS. These findings suggest novel therapeutic strategies for FXS in the form of mAChR antagonists.

Introduction

Activity of Gq-coupled, muscarinic acetylcholine receptors regulates hippocampal-dependent learning and memory consolidation (for review, see Hasselmo, 2006). Consequently, loss of cholinergic innervation to the hippocampus during Alzheimer's disease (AD) is thought to contribute to the cognitive deficits observed in AD (Kesner et al., 1989; Caccamo et al., 2006). M_1 muscarinic acetylcholine receptors (mAChRs), the primary Gq-coupled mAChRs in hippocampus, contribute to hippocampal-dependent memory, and M_1 agonists reverse the cognitive deficits in AD mouse models (Anagnostaras et al., 2003; Caccamo et al., 2006). M_1 mAChRs may facilitate memory and cognition in part through modulation or induction of plasticity at excitatory synapses (Anagnostaras et al., 2003; Shinoe et al., 2005). Understanding the mechanisms by which mAChRs mediate hippocampal-dependent synaptic plasticity will provide insight into cholinergic-dependent cognitive enhancement.

Activation of M_1 mAChRs induces long-term depression (LTD) of excitatory synaptic transmission (mAChR-LTD) in CA1 and neocortex (Kirkwood et al., 1999; Massey et al., 2001; Choi et al., 2005; Jo et al., 2006; Scheiderer et al., 2006), but little is known of the cellular mechanisms underlying mAChR-dependent LTD or how it is related to LTD induced by other Gq-coupled receptors. The cholinergic agonist carbachol (CCh) stimulates new protein synthesis in CA1 dendrites, suggesting a mechanism by

which mAChRs establish long-lasting changes at synapses (Feig and Lipton, 1993). A form of LTD induced by pharmacological activation of Gq-coupled group 1 metabotropic glutamate receptors (mGluRs) has been characterized previously and relies on rapid, dendritic protein synthesis (Palmer et al., 1997; Fitzjohn et al., 1999; Huber et al., 2000, Fitzjohn et al., 2001). Paired pulses of low-frequency synaptic stimulation (1 Hz; PP-LFS) delivered to Schaffer collateral axons also induces protein synthesis-dependent LTD (Huber et al., 2000). Although initial studies observed that PP-LFS and other synaptic stimulation paradigms induce an mGluR-dependent LTD in CA1 (Oliet et al., 1997; Kemp and Bashir, 1999; Bolshakov and Siegelbaum, 1994), more recent work has failed to confirm a role for group 1 mGluRs in LTD induced specifically with PP-LFS (Volk et al., 2006). The importance of protein synthesis-dependent LTD to human cognition is highlighted by the finding that it is selectively enhanced in the mouse model of human Fragile X syndrome mental retardation, *Fmr1* knock-out (KO) mice (Huber et al, 2002; Koekkoek et al., 2005; Hou et al., 2006). Fragile X mental retardation protein (FMRP), the product of *Fmr1*, is an RNA-binding protein that functions as a regulator of translation (for review, see (Garber et al., 2006)). It is unknown whether FMRP specifically regulates plasticity through group 1 mGluRs or generally affects protein synthesis-dependent plasticity in response to other neurotransmitters.

Here, I find that synaptic or pharmacological activation of M_1 mAChRs induces LTD in hippocampal area CA1, which requires rapid protein synthesis, and we identify a role for these newly synthesized proteins in AMPA receptor (AMPAR) trafficking. To examine whether FMRP generally regulates Gq- and protein synthesis-dependent plasticity, I investigated mAChR-dependent LTD in *Fmr1* KO mice. Our results indicate

that the Gq-coupled acetylcholine and glutamate receptors converge on a common protein synthesis-dependent LTD mechanism, which is abnormal in Fragile X syndrome (FXS). These results provide a link between altered cholinergic-dependent synaptic plasticity and cognitive dysfunction, as well as suggest novel therapeutic strategies for FXS, mental retardation, and related autistic disorders (Bear et al., 2004).

Materials and Methods

Drugs.

Carbamoylcholine chloride (CCh), TTX, pirenzepine, anisomycin, atropine, picrotoxin (Sigma, St. Louis, MO), U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-amino-phenylthio]butadiene), 2-methyl-6-(phenylethynyl)-pyridine (MPEP), (*S*)-(+)- α -amino-4-carboxy-2-methylbenzeneacetic acid (LY367385) (Tocris Bioscience, Ellisville, MO), and rapamycin (Calbiochem, San Diego, CA) were prepared as stocks in water, DMSO (U0126, rapamycin, actinomycin D, picrotoxin), or equimolar NaOH (LY367385), aliquoted and frozen for no more than 10 d. D,L-AP-5 (Tocris Bioscience) was prepared fresh in artificial CSF (ACSF). *N*-ethyl bromide quaternary salt (QX-314) Cl- (Alomone Labs, Jerusalem, Israel) was dissolved directly into the internal solution.

Electrophysiology.

Hippocampal slices were prepared from postnatal day 25 (P25) to P45 Long Evans hooded rats (Charles River, Wilmington, MA) or the C57BL/6 congenic strain of *Fmr1* KO and wild-type mice (obtained from Dr. Steve Warren, Emory University,

Atlanta, GA). Rats or mice were anesthetized with the barbiturate pentobarbital (50 mg/kg) and decapitated soon after the disappearance of corneal reflexes. For whole-cell recordings, rats were transcardially perfused with ice-cold dissection buffer for 1 min before decapitation. The brain was removed, and the hippocampus was dissected and sliced at 400 μM (300 μM for intracellular recordings) in slushed (partially frozen) dissection buffer containing the following (in mM): 2.6 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 0.5 $CaCl_2$, 5 $MgCl_2$, 212 sucrose, and 10 dextrose, using a vibratome (VT 1000S; Leica, Nussloch, Germany). CA3 was cut off slices immediately after slicing to prevent any CCh-induced oscillations of CA3 neurons from contaminating evoked synaptic responses in CA1 (Scheiderer et al., 2006). The slices were transferred into a reservoir chamber filled with ACSF containing the following (in mM): 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$, and 10 dextrose for field recordings or 119 NaCl, 2.5 KCl, 1 NaH_2PO_4, 26 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$, and 11 dextrose (290–300 mOsm) for intracellular recordings. Slices were allowed to recover for 2–5 h at 30°C. ACSF and dissection buffer were continuously aerated with 95% O_2/5% CO_2.

For intracellular recording, slices were transferred to a submerged recording chamber (Warner Instruments, Hamden, CT), maintained at 30°C and perfused continuously with ASCF at a rate of 2.5–3 ml/min. Picrotoxin (20 μM) was added to the ACSF immediately before transferring slices to the recording chamber. Whole-cell voltage-clamp recordings were obtained from CA1 pyramidal neurons in slices visualized using IR-DIC optics(Gibson et al., 2006). Neurons were clamped at –60 mV through whole-cell recording pipettes (~3–7 MΩ) filled with an intracellular solution containing the following (in mM): 130 K-gluconate, 6 KCl, 3 NaCl, 10 HEPES, 10 sucrose, 2 QX-

314, 0.2 EGTA, 4 ATP-Mg, 0.3 GTP-Na, 14 phosphocreatine-Tris, pH 7.2, 290–310 mOsm. EPSCs were evoked every 10 s using a single bipolar electrode placed in stratum radiatum of area CA1 (along the Schaffer collaterals) 50–200 μm from the recorded neurons with monophasic current pulses (5–25 μA, 200 μs). Series resistance (SR) and input resistance were measured in voltage clamp with a 400 ms, –10 mV step from a –60 mV holding potential (filtered at 30 kHz, sampled at 50 kHz). Series resistance did not change significantly over the course of the experiment for the cells used (paired Student's *t* test, $p = 0.2$; baseline SR vs SR at 60 min). Average SR values for the cells used ranged from 16 to 34 MΩ. Initial input resistance values ranged from 126 to 282 MΩ. Data were not corrected for junction potential.

For extracellular recordings, slices were placed in a submersion recording chamber (BSC-ZT; Harvard Apparatus, Holliston, MA), perfused at 3 ml/min, and field EPSPs (fEPSPs) were recorded using electrodes (1–2 MΩ) filled with ACSF and placed in stratum radiatum of area CA1. Field EPSPs were evoked by monophasic stimulation (200 μs duration) of Schaffer collateral/commissural afferents with a concentric bipolar tungsten stimulating electrode (FHC, Bowdoinham, ME). Baseline presynaptic stimulation was delivered once every 30 s in rat (see Figs. 1–4) and once every 5 s in mice (see Fig. 6) (Scheiderer et al., 2006) using a stimulation intensity (10–30 μA) yielding 50–60% of the maximal response. The initial slope of the FP was used to measure stability of synaptic responses and quantify the magnitude of LTD. LTD was elicited by application of 50 μM CCh for 10 min as indicated. Synaptically induced LTD was induced using paired pulses (50 ms interstimulus interval) of low-frequency (1 Hz) stimulation (PP-LFS) for 15 or 20 min as indicated (1800 or 2400 pulses) (Huber et al.,

2000). Note that synaptic responses are not collected during PP-LFS, so no data points are plotted during this time. Theta-burst stimulation (θ) consisted of 10 bursts at 5 Hz, each burst containing four pulses at 100 Hz, given four times at 10 s. Group data were analyzed as follows: (1) the initial slope (fEPSPs) or amplitude (EPSCs; 1 min moving average) was expressed as a percentage of the baseline average, (2) the time scale in each experiment was converted to time from the onset of CCh or PP-LFS, and (3) the time-matched, normalized data were averaged across experiments and expressed in the figures as the means (±SEM). LTD was calculated as a 5 min average of the normalized fEPSP slope values 1 h after CCh or PP-LFS. The effects of all pharmacological treatments on LTD were evaluated by comparing interleaved control and treated slices. For LTD experiments, significant differences were determined by a Student's independent *t* test versus control measured at 1 h after LTD induction. Probability values of $p < 0.05$ were considered to represent significant differences.

Dissociated culture and immunocytochemistry.

Dissociated CA3–CA1 hippocampal cultures were prepared from P0–P2 rats using modified, previously published protocols (Brewer et al., 1993). Neurons were plated at a density of 250 neurons/mm^2 on poly-D-lysine/laminin or matrigel-coated coverslips. Experiments were performed at 18–22 d *in vitro*. All AMPA receptor internalization and surface expression results were confirmed with the experimenter blind to the treatment condition. For AMPA receptor internalization assays: live cultures were pretreated with 1 μM TTX for 15 min, labeled with anti-GluR$_1$ N-terminal antibody (1:10; Calbiochem, La Jolla, CA) for 15 min, followed by treatment with 50 μM CCh or

water (±75 nM pirenzepine) for 10 min. Internalization was allowed to proceed for 15 min after washout of CCh. Cells were then fixed in 4% PFA/4% sucrose for 15 min at 37°C. Surface $GluR_1$ was blocked with unlabeled 2° donkey anti-rabbit antibody [1 h, room temperature (RT)], followed by permeabilization with 0.2% Triton-X (10 min, RT). Cells were blocked with TBS/10% goat serum (1 h, RT) and then treated with Alexa Fluor 2° antibody (1:300, 1 h, RT; Invitrogen, Eugene, OR) to visualize internalized $GluR_1$. For surface $GluR_1$ experiments, cells were pretreated with 1 μM TTX (± anisomycin where indicated) for 20 min followed by treatment with 50 μM CCh or water for 10 min. Cells were then fixed (as described above) but not permeabilized 10 or 60 min after washout of CCh. Cells were blocked in TBS/10% goat serum (1 h, RT), treated with anti-$GluR_1$ N-terminal antibody (1:50; 1 h, RT) washed, and then treated with Alexa Fluor 2° antibody. For M_1 mAChR staining, cultures were fixed as described above and treated with anti-M_1 mAChR antibody (1:100; Millipore, Billerica, MA).

Fluorescence was detected using a Nikon (Tokyo, Japan) TE2000 inverted microscope equipped with a cooled CCD camera (Roper CoolSnapHQ). Images were analyzed and quantitated using MetaMorph software (Universal Imaging, West Chester, PA). For synaptic staining, immunoreactive puncta are defined as discrete points along a dendrite (within 50 μm from the soma) with fluorescence intensity at least twice the background staining of a region adjacent to the dendrite. For all group data, the average number of puncta was determined for all control cells within each culture (1–2 coverslips, 5–20 cells per condition per culture). The number of puncta in each control and treated cell was then normalized to this value. Averages ± SEM are plotted, and *n* (number of cells) is on each bar (*$p < 0.05$; **$p < 0.01$).

Western blotting.

Hippocampal slices were prepared as for electrophysiology experiments. After a 2–3 h recovery period in ACSF, slices were maintained in a static incubation chamber in ACSF at 30°C and aerated with 95% O_2/5% CO_2. Slices were treated with CCh (10 min) or ACSF (control). In experiments where anisomycin was used, slices were preincubated with anisomycin (30–60 min) before treatment. Slices were frozen on dry ice immediately after treatment and stored at –80°C until processing (3 d or less). In a subset of experiments (see Figs. 1F, 6F) CA1 was microdissected on wet ice in slushed ACSF before freezing. Quantitative Western blots were performed on slice homogenates using antibodies against FMRP (1:2500; Millipore IC3), EF1αα (1:10,000; Upstate Biotechnology, Lake Placid, NY), αCa^{2+}/calmodulin-dependent protein kinase II (α-CaMKII) (1:200,000; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-p70 S6 kinase (Thr389; 1:1000; Cell Signaling Technology, Beverly, MA), or phospho-ERK (Thr202/Tyr204; 1:1000; Cell Signaling Technology) as described previously (Gallagher et al., 2004; Volk et al., 2006). Homogenates were also probed for total ERK (1:2000; Cell Signaling Technology) or vasoline-containing peptide (VCP; 1:10,000; gift from Dr. Thomas Südhof, University of Texas Southwestern Medical Center, Dallas, TX) as loading controls. Data are presented as percentage control (all samples have within animal controls). Significant differences were determined by a Student's independent *t* test versus control (see Fig.1*F*) or by a one-sample *t* test as percentage control (see Fig. 6*E,F*).

Results

The cholinergic agonist carbachol induces protein synthesis-dependent LTD in hippocampal area CA1

Previous work has demonstrated that the general cholinergic agonist CCh (50 μM; 10 min) induces long-term depression of extracellularly recorded population EPSPs or fEPSPs (Kirkwood et al., 1999; Massey et al., 2001; Lee et al., 2005; Scheiderer et al., 2006). To determine whether this protocol induces LTD of EPSCs and whether this is associated with changes in passive membrane properties, whole-cell voltage clamp (at −60 mV) recordings of CA1 pyramidal neurons in rat acute hippocampal slices were performed. After a stable baseline of EPSC amplitudes was established, CCh application (50 μM; 10 min) induced a robust, acute depression followed by a long-lasting depression of EPSCs (Fig. 3.1*A*). CCh also induced a trend toward a long-term increase in input resistance (baseline R_m, 190 ± 28MΩ, 60 min after CCh; R_m, 244 ± 39MΩ, $p = 0.09$) consistent with the findings that M_1 mAChRs reduce a number of membrane currents (Dutar and Nicoll, 1988).

Pharmacological activation of mAChRs induces protein synthesis in CA1 dendrites (Feig and Lipton, 1993). To determine whether newly synthesized proteins are required for mAChR-dependent LTD in CA1, acute rat hippocampal slices were preincubated (20–40 min) in the protein synthesis inhibitor anisomycin (25 μM). Consistent with previous studies, LTD was induced in interleaved control slices with the general cholinergic agonist CCh (50 μM; 10 min) (Kirkwood et al., 1999; Massey et al., 2001; Lee et al., 2005; Scheiderer et al., 2006). We refer to this as CCh-LTD. Anisomycin did not affect the acute synaptic depression observed with CCh but dramatically reduced

the magnitude of LTD (Fig. 3.1*B*). Similarly, preincubation of slices with a mechanistically different protein synthesis inhibitor, cycloheximide (60 μM), blocked CCh-LTD (Fig.3.1*C*). These results reveal that newly synthesized proteins are required for mAChRs to establish LTD. To determine whether CCh-LTD requires transcription of new mRNAs, I pretreated slices in the transcription inhibitor actinomycin D (25 μM). Interleaved control slices were treated with vehicle (0.1% DMSO). CCh-LTD is unaffected by actinomycin D (Fig. 3.1*D*), suggesting that protein synthesis from pre-existing mRNAs is sufficient to support CCh-LTD for at least 1 h.

Translation in neurons is regulated through two major signaling pathways, the mammalian target of rapamycin (mTOR) and extracellular signal-regulated kinase (ERK) pathways, via phosphorylation of factors known to stimulate translation initiation such as eukaryotic initiation factor 4E (eIF4E), 4E binding protein (4EBP), and ribosomal S6 protein (for review, see Kelleher et al., 2004; Klann and Dever, 2004). To determine whether the mTOR or ERK translational regulatory pathways were required for CCh-LTD, slices were pretreated with either an inhibitor of mTOR (20 nM rapamycin) or the upstream activating kinase of ERK, mitogen-activated protein/ERK kinase (MEK; 20 μM U0126). Interleaved control slices were incubated in vehicle (0.1% DMSO). Rapamycin reduced the magnitude of CCh-LTD, similar to anisomycin (Fig. 3.1*F*), and U0126 completely blocked CCh-LTD (Fig. 3.1*E*). Consistent with a role for ERK and mTOR in CCh-LTD, treatment of hippocampal slices with CCh (50 μM; 10 min) induced activation of ERK and mTOR as measured by phosphorylation of ERK (Thr202/Tyr204) and p70 S6 kinase, at the mTOR site (Thr389) (Fig. 3.1*G*) (Roberson et al., 1999; Rosenblum et al., 2000; Berkeley et al., 2001). These results strengthen the data that protein synthesis

is required for mAChR-dependent LTD and suggest that mAChRs stimulate translation initiation through the ERK and mTOR pathways.

LTD induced by pharmacological stimulation of mAChRs with carbachol is independent of presynaptic activity, NMDA receptors, or group 1 mGluRs

The initial study demonstrating that mAChRs stimulate dendritic protein synthesis observed that synaptic coactivation of NMDA receptors (NMDARs) were also necessary (Feig and Lipton, 1993). More recent studies found that NMDARs are required for mAChR-dependent LTD in some brain regions but not others (Kirkwood et al., 1999; Massey et al., 2001; Jo et al., 2006; Scheiderer et al., 2006). To determine whether NMDARs are required for protein synthesis-dependent CCh-LTD in CA1, slices were preincubated in the NMDAR antagonist D,L-AP-5 (100 μM). Normal levels of CCh-LTD were observed in AP-5 compared with interleaved control slices (Fig. 3.2*A*). AP-5 was active in the slice preparation, because long-term potentiation (LTP) was blocked in all slices subsequent to mAChR-LTD (Fig. 3.2*A*, θ-burst). Because CCh -LTD does not require NMDARs, it may also occur independently of synaptic stimulation. To test this, presynaptic stimulation was ceased immediately before CCh application and was not resumed until 30 min after CCh washout (Fig. 3.2*B*, CCh no stim). Control slices were placed in the same recording chamber simultaneously but received low-frequency baseline stimulation throughout the experiment (CCh + stim). Normal levels of CCh-LTD were observed in slices that did not receive presynaptic stimulation (Fig. 3.2*B*). Together, these results indicate that CCh-LTD is independent of synaptic NMDAR activation and are consistent with previous work indicating that rapid protein synthesis is required for

Gq-dependent LTD but not NMDAR-dependent LTD (Huber et al., 2000; Massey et al., 2001).

The Gq-coupled group 1 mGluRs induce LTD in CA1, which relies on rapid protein synthesis (Huber et al., 2000). Therefore, mAChRs may induce protein synthesis-dependent LTD indirectly by enhancing neuronal excitability, glutamate release, and activation of group 1 mGluRs (Benardo and Prince, 1982). To test whether group 1 mGluR activity was required for CCh-LTD, slices were preincubated in $mGluR_1$ and mGluR5 antagonists, LY367385 (100 μM) and MPEP (10 μM), which block LTD induced by the group 1 mGluR agonist dihydroxyphenylglycine (DHPG) (Volk et al., 2006). Group 1 mGluR blockade had no effect on CCh-LTD (Fig. 3.2*C*). Together, these data suggest that mAChRs induce protein synthesis-dependent LTD directly through signaling of mAChRs and not through activation of NMDARs or group 1 mGluRs.

Synaptic activation of M1 mAChRs induces protein synthesis-dependent LTD

I next determined whether synaptic activation of M_1 mAChRs induced protein synthesis-dependent LTD. There are few cholinergic neurons intrinsic to the hippocampus, but extracellular stimulation in CA1 regions of hippocampal slices elicits acetylcholine release from fibers originating from the septal nucleus (Cole and Nicoll, 1983; Frotscher et al., 1986; Schafer et al., 1998; Shinoe et al., 2005). Protein synthesis-dependent LTD can be elicited by electrical stimulation of Schaffer collateral axons using paired pulses of low-frequency stimulation (50 ms interstimulus interval, 1800–2400 pulses at 1 Hz; PP-LFS) (Huber et al., 2000; Nosyreva and Huber, 2005, 2006). PP-LFS experiments were performed in D,L-AP-5 (100 μM) to prevent induction of NMDA

receptor-dependent LTD and isolate Gαq-dependent LTD. Both the broad range mAChR antagonist atropine (5 μM) (Fig. 3.3*A*) and the M_1-selective antagonist pirenzepine (Dorje et al., 1991; Scheiderer et al., 2006) (75 nM) (Fig. 3.3*B*) significantly reduced LTD induced with PP-LFS. To implicate endogenous release of acetylcholine in LTD induction, I applied the acetylcholine esterase inhibitor eserine (2 μM) during PP-LFS to enhance acetylcholine concentrations in the slice (Fig. 3.3*C*). Eserine significantly facilitated the LTD, suggesting that either PP-LFS activates cholinergic terminals or accumulation of basal acetylcholine in the slice is sufficient to enhance LTD. Eserine application alone caused a transient depression but had no long-lasting effects on synaptic transmission (80–85 min average; 98 ± 2%; $p = 0.54$ compared with baseline). Antagonism of M_1 mAChRs reduces PP-LFS induced LTD but does not completely block it, suggesting involvement of another Gq-coupled receptor, because PP-LFS LTD is completely abolished in Gαq knock-out mice (Kleppisch et al., 2001). Because PP-LFS induced LTD shares a common expression mechanism with LTD induced by pharmacological activation of group I mGluRs, it is likely that group I mGluRs play a role in PP-LFS induced LTD (Huber et al., 2001). Here, I observe that combined application of an M_1 mAChR selective antagonist (75 nM pirenzepine; "P") and group I mGluR antagonists ($mGluR_5$-specific antagonist MPEP; "M"; 10 μM and $mGluR_1$-specific antagonist LY367385; "L", 100 μM) blocks PP-LFS induced LTD (P,M,L) (Fig. 3.3*D*). Because PP-LFS induced LTD is unaffected by group 1 mGluR antagonists alone (Volk et al., 2006), but completely blocked by the addition of pirenzepine (Fig. 3.3*D*), this indicates that synaptic activation of M_1 mAChRs is sufficient to induce the full level of

protein synthesis-dependent LTD. It is only when M_1 mAChRs are blocked that group 1 mGluRs are required for LTD induction.

To determine whether pharmacological activation of mAChRs and synaptic stimulation of mGluRs and mAChRs (using PP-LFS) use a common expression mechanism, I performed an occlusion experiment (Fig. 3.4*A*). Synaptically induced LTD was first saturated by delivering three episodes of PP-LFS followed by CCh application to the slice (AP-5 was present throughout the experiment). Saturation of PP-LFS-induced LTD greatly reduced subsequent CCh-LTD compared with CCh-LTD in naive interleaved control slices (Fig. 3.4*A*). In contrast, saturation of NMDA receptor-dependent LTD (induced with 15 min of 1 Hz single pulse stimulation or "LFS" in the absence of AP-5 (Dudek and Bear, 1992) did not occlude induction of LTD with CCh (Fig. 3.4*B*) demonstrating that LTD induced by activation of mAChRs does not share a common expression mechanism with NMDA receptor-dependent LTD. The fact that saturation of PP-LFS LTD also occludes LTD induced by chemical activation of group I mGluRs (Huber et al., 2001) supports the assertion that M_1 mAChRs and group 1 mGluRs converge on a common, protein synthesis-dependent LTD expression mechanism, which is distinct from LTD induced by activation of NMDARs.

M1 mAChRs induce AMPAR endocytosis and protein synthesis-dependent decreases in surface AMPARs

How is mAChR-dependent LTD expressed and what is the role of newly synthesized proteins in this expression mechanism? I investigated this question by measuring the effects of mAChR activation on trafficking of postsynaptic AMPARs using

immunocytochemistry in dissociated CA3 and CA1 hippocampal neuron cultures (Fig. 3.5*A–D*). Because our cultures do not contain the medial septum, the major cholinergic projection to the hippocampus, I first confirmed that the cultured neurons expressed M_1 muscarinic receptors (Fig. 3.5*E*). Cultures were treated with CCh (50 μM; 10 min) in the presence of TTX (1 μM) to prevent action potential-induced glutamate release. CCh application to hippocampal neurons in culture caused an increase in endocytosis of the AMPAR subunit $GluR_1$, which was blocked by the M_1 mAChR selective antagonist, pirenzepine (75 nM) (Fig. 3.5*A*,*B*). The endocytosis of $GluR_1$ in response to CCh resulted in decreases in $GluR_1$ surface expression at 10 and 60 min after CCh application (Fig. 3.5*C*,*D*). Like CCh-LTD, the persistent decrease in $GluR_1$ surface expression required protein synthesis, because it was blocked by anisomycin (25 μM) (Fig. 3.5*C*,*D*). These results suggest that the newly synthesized proteins maintain LTD through a persistent decrease in AMPAR surface expression and most likely function to regulate trafficking of endocytosed AMPARs.

mAChR-dependent LTD is abnormal in the mouse model of Fragile X syndrome

FMRP is an RNA-binding protein that regulates dendritic protein synthesis as well as plasticity through the Gq-coupled group 1 mGluRs (Huber et al., 2002; Weiler et al., 2004; Chuang et al., 2005; Koekkoek et al., 2005; Hou et al., 2006). Loss of function mutations in FMRP in humans leads to a form of mental retardation termed Fragile X syndrome (Bagni and Greenough, 2005). To determine whether FMRP generally regulates Gq- and protein synthesis-dependent synaptic plasticity, I evaluated CCh-LTD in the mouse model of FXS, *Fmr1* KO mice. I observed that CCh-LTD is enhanced in

Fmr1 KO mice compared with wild-type (WT) mice (Fig. 3.6*A*). Although the magnitude of CCh-induced LTD is smaller in WT mice compared with rats (Fig. 3.1), it is still blocked by anisomycin (Fig. 3.6*B*). In contrast, CCh-LTD no longer requires protein synthesis in *Fmr1* KO mice (Fig. 3.6*C*,*D*). Gq-dependent LTD induced by chemical activation of group 1 mGluRs or PP-LFS is similarly affected in *Fmr1* KO mice (Fig. 3.6*D*) (Huber et al., 2002; Nosyreva and Huber, 2006) suggesting that FMRP generally acts to regulate Gq- and protein synthesis-dependent plasticity.

Although cholinergic stimulation is known to activate dendritic protein synthesis, the specific mRNAs that are translated are unknown (Feig and Lipton, 1993). The mRNA for FMRP is present in dendrites, where FMRP is translated in response to group 1 mGluR stimulation (Weiler et al., 1997; Antar et al., 2004). It has been hypothesized that rapid synthesis of FMRP then regulates translation of its target mRNAs such as those required for LTD (Huber et al., 2002; Bear et al., 2004; Weiler et al., 2004; Hou et al., 2006). Here, I investigated whether mAChRs stimulate the rapid synthesis of FMRP as well as FMRP target mRNA, elongation factor 1α (EF1α) (Sung et al., 2003). Acute hippocampal slices from WT mice were treated with CCh (50 μM; 10 min) and quantitative Western blotting was performed on homogenates of CA1 for FMRP and EF1α. CCh stimulates a rapid (10 min) increase in FMRP and EF1α levels, which was blocked by anisomycin (Fig. 3.6*E*). To determine whether FMRP is required for mAChR-dependent protein synthesis, we measured CCh-induced synthesis of EF1α and that of another FMRP target and dendritic mRNA, CaMKII in CA1 of *Fmr1* KO mice (Burgin et al., 1990; Zalfa et al., 2003). CCh induced rapid increases in EF1α and CaMKII protein in WT mice, which was not observed in *Fmr1* KO littermates (Hou et al., 2006) (Fig.

3.6*F*). In support of previous studies, I observed a trend toward an increase in EF1α and αCaMKII levels in isolated CA1 slices of *Fmr1* KO mice (EF1α, 124 ± 20% of WT; p = 0.27; αCaMKII, 136 ± 23% of WT; p = 0.14) (Sung et al., 2003; Zalfa et al., 2003; Hou et al., 2006). These data demonstrate that mAChRs regulate translation of FMRP as well as FMRP target mRNAs. In turn, FMRP regulates mAChR induced protein synthesis and LTD. These results suggest that Gq-coupled and protein synthesis-dependent plasticity is generally elevated and misregulated in FXS and may contribute to the cognitive deficits associated with the disease.

Discussion

Here, I demonstrate that the Gq-coupled muscarinic acetylcholine receptors activate rapid protein synthesis and induce protein synthesis-dependent LTD. Newly synthesized proteins contribute to mAChR-dependent LTD by maintaining a persistent decrease in AMPAR surface expression. Synaptic or pharmacological activation of group 1 mGluRs or M_1 mAChRs elicits protein synthesis-dependent LTD, and these forms of LTD occlude each other (Figs. 3.1, 3.4) (Huber et al., 2000; Huber et al., 2001). These results indicate that different Gq-coupled receptors converge onto a common protein synthesis-dependent LTD mechanism. Data also indicate that the RNA binding protein FMRP is synthesized in response to different Gq-coupled receptors and, in turn, FMRP regulates Gq-dependent translation of FMRP target mRNAs and LTD (Fig. 3.6) (Weiler et al., 1997, 2004; Huber et al., 2002; Koekkoek et al., 2005; Hou et al., 2006). I

demonstrate that LTD induced through at least two different Gq-coupled receptors is abnormal in the mouse model of FXS, which suggests that strategies targeting these Gq-coupled receptors may be efficacious in treatment of FXS.

Feig and Lipton (1993) demonstrated that activation of mAChRs with CCh induced ^{3}H leucine incorporation in CA1 dendrites. Since then, little progress has been made in understanding the role of local protein synthesis in mAChR-dependent synaptic plasticity. In neocortex, application of CCh or exogenous acetylcholine induces LTD, which is reduced by the protein synthesis inhibitor anisomycin (Massey et al., 2001; McCoy and McMahon, 2007). Importantly, in CA1, I demonstrate that pharmacological or synaptic activation of M_1 mAChRs (Fig. 3.1*B*) induces LTD, which relies on rapid (~20 min) protein synthesis from pre-existing mRNA (Figs. 3.1, 3.3) (Huber et al., 2000; Volk et al., 2006). These findings, combined with the fact that mAChRs induce protein synthesis in CA1 dendrites, suggest that mAChR-dependent LTD utilizes locally synthesized proteins from dendritic mRNA (Feig and Lipton, 1993). I also found that the major signaling pathways known to regulate translation in neurons, ERK and mTOR, are activated by mAChRs and are required for mAChR-dependent LTD (Fig. 3.1*D–F*) (Dufner and Thomas, 1999; Kelleher et al., 2004b; Klann and Dever, 2004). Current evidence points to a role for ERK and mTOR in stimulation of translation initiation during induction of mGluR-LTD and NMDAR-dependent LTP through phosphorylation of eIF4E, 4E binding protein and ribosomal S6 protein (Hou and Klann, 2004; Kelleher et al., 2004b; Banko et al., 2005, 2006; Tsokas et al., 2007). These data imply that similar translation initiation factors are regulated by mAChRs, which lead to LTD (Fig. 3.7). Very recent work finds a role for ERK, but not mTOR, in CCh-induced LTD in the visual

cortex suggestive of differential translational regulation by mAChRs across brain regions (McCoy and McMahon, 2007). The fact that a residual LTD persists in both rapamycin and protein synthesis inhibitors, but not in U0126, suggests an additional role for ERK. For example, ERK has recently been implicated in the capture of newly synthesized proteins required for LTD maintenance (Sajikumar et al., 2007). Findings presented in this chapter are consistent with previous work in neocortex and subiculum demonstrating that mAChR-dependent LTD can occur independently of NMDARs (Kirkwood et al., 1999; Massey et al., 2001; Jo et al., 2006; McCoy and McMahon, 2007) and support the hypothesis that NMDA and Gq-coupled receptors activate distinct forms of LTD, the latter of which is regulated by protein synthesis and FMRP (Oliet et al., 1997; Huber et al., 2000, 2002). Elegant work has demonstrated that M_1 mAChRs also facilitate induction of NMDAR-dependent LTD by activation of phospholipase C and release of Ca^{2+} from intracellular stores (Choi et al., 2005; Scheiderer et al., 2006), but it is unclear whether the LTD is protein synthesis dependent under these conditions. mAChRs may facilitate the induction of both an NMDAR-dependent, protein synthesis-independent LTD and the strictly Gq and protein synthesis-dependent LTD described here (Marino et al., 1998; Choi et al., 2005). The extent to which either occurs may depend on experimental conditions (see Materials and Methods).

Previous work implicated mGluRs in LTD induced synaptically with PP-LFS using the broad range mGluR antagonist LY341495 (100 μM) (Kemp and Bashir, 1999; Huber et al., 2000; Volk et al., 2006). However, PP-LFS induced LTD is unaffected with selective pharmacological blockade or genetic deletion of group 1 mGluRs (Volk et al., 2006). Here, I find that coapplication of M_1 mAChR and group I mGluR antagonists

completely blocks PP-LFS-induced LTD, indicating that both receptor types contribute to LTD, and M_1 mAChRs fully induce LTD in group 1 mGluR blockade (Fig. 3.3). This suggests that high concentrations of LY341495 may antagonize synaptic activation of mAChRs or interfere with Gq signaling. PP-LFS-induced LTD also occludes subsequent LTD induced by pharmacological activation of mAChRs or group 1 mGluRs, suggesting that these three induction methods converge on a common pathway to induce and/or express LTD (Figs. 3.4, 3.7*A*) (Huber et al., 2001).

Data presented in this chapter reveal that the newly synthesized proteins required for CCh-induced LTD function to maintain decreases in surface AMPAR expression (Fig. 3.4). These results also imply that CCh-induced LTD is expressed postsynaptically, consistent with the fact that CCh-LTD does not alter paired-pulse facilitation (Scheiderer et al., 2006). Similar changes in AMPAR trafficking occur with group 1 mGluR activation, further supporting a common expression mechanism for LTD induced by multiple Gq-coupled receptors (Snyder et al., 2001; Xiao et al., 2001; Moult et al., 2006).

The importance of the Gq- and protein synthesis-dependent LTD mechanism to cognition and cognitive disorders is underscored by our findings that both mAChR and mGluR-LTD are enhanced in the mouse model of human Fragile X syndrome mental retardation, *Fmr1* KO mice (Fig. 3.6) (Huber et al., 2002; Koekkoek et al., 2005; Hou et al., 2006; Nosyreva and Huber, 2006). These results indicate that the defect in *Fmr1* KO mice may lie downstream of mGluRs, within the Gq or translational regulatory pathways. Related to this idea, there is a report of abnormal mAChR regulation of GABA release in *Fmr1* KO mice, suggesting that other aspects of mAChR function are altered (D'Antuono et al., 2003). I also present evidence that mAChRs induce rapid translation of FMRP

target mRNAs, which are expressed dendritically and are known to be synthesized in response to mGluRs, such as FMRP, EF1α, and CaMKII (Burgin et al., 1990; Weiler et al., 1997; Sung et al., 2003; Zalfa et al., 2003; Antar et al., 2004; Huang et al., 2005; Hou et al., 2006). FMRP is an RNA-binding protein and regulates translation of its mRNA targets, including itself and EF1α (Garber et al., 2006). In support of this idea, I find that mAChR induced synthesis of EF1α and CaMKII are absent in *Fmr1* KO mice (Fig. 3.6*E*), similar to mGluR-induced synthesis, suggesting that FMRP is a common regulator of Gq-dependent protein synthesis and/or Gq signaling to translation machinery is generally affected in *Fmr1* KO mice (Weiler et al., 2004; Hou et al., 2006). Multiple studies suggest that the observation of enhanced Gq-dependent LTD in *Fmr1* KO mice despite the absence of mGluR and mAChR-dependent protein synthesis stems from the loss of FMRP-mediated translational suppression, which leads to a steady state elevation of "LTD proteins" and protein synthesis independent LTD in the *Fmr1* KO mice (Figs. 3.6, 3.7*B*) (Zalfa et al., 2003; Hou et al., 2006).

Recent evidence indicates that mGluR-dependent plasticity may be generally enhanced in multiple brain regions of *Fmr1* KO mice (Huber et al., 2002; Chuang et al., 2005; Koekkoek et al., 2005; Nosyreva and Huber, 2006). Consequently, mGluR antagonists have been suggested to reverse some phenotypes of FXS (Bear et al., 2004; Bear, 2005) for which there is experimental support in animal models (McBride et al., 2005; Yan et al., 2005; Tucker et al., 2006). Both a deficit and elevation of hippocampal cholinergic activity is associated with learning and memory deficits in rodents, suggesting that a critical range of cholinergic activity is required for optimal hippocampal function(Bunce et al., 2004b, a; Elvander et al., 2004b) (for review, see Hasselmo, 2006).

Therefore, antagonism of M_1 mAChRs may actually facilitate cognitive function in FXS, alone or in combination with group 1 mGluR antagonists.

Here, I demonstrated that cholinergic and glutamatergic Gq-coupled receptors activate a common, protein synthesis-dependent LTD mechanism. An interesting possibility is that other Gq-coupled receptors known to induce LTD in CA1, such as α1 adrenergic and 5-HT_2 receptors, do so through the same mechanism (Scheiderer et al., 2004; Choi et al., 2005). *In vivo*, Gq-dependent LTD may be induced by moderate, coincident, activation of multiple Gq-coupled receptors (Fig. 3.3*A–D*). Septohippocampal inputs may facilitate memory during arousal or emotional states through modulation of Gq-dependent LTD (McGaugh, 2004). In support of this idea, the magnitude of hippocampal Gq-dependent LTD is positively correlated with spatial memory performance in aged rats (Lee et al., 2005) and loss of hippocampal cholinergic input, such that occurs in Alzheimer's disease, leads to deficits in hippocampal-dependent memory and mAChR dependent LTD (Kesner et al., 1989; Scheiderer et al., 2006). Elucidation of Gq- and protein synthesis-dependent LTD mechanisms will contribute to our understanding of how extrahippocampal neuromodulatory systems contribute to memory and cognitive disorders.

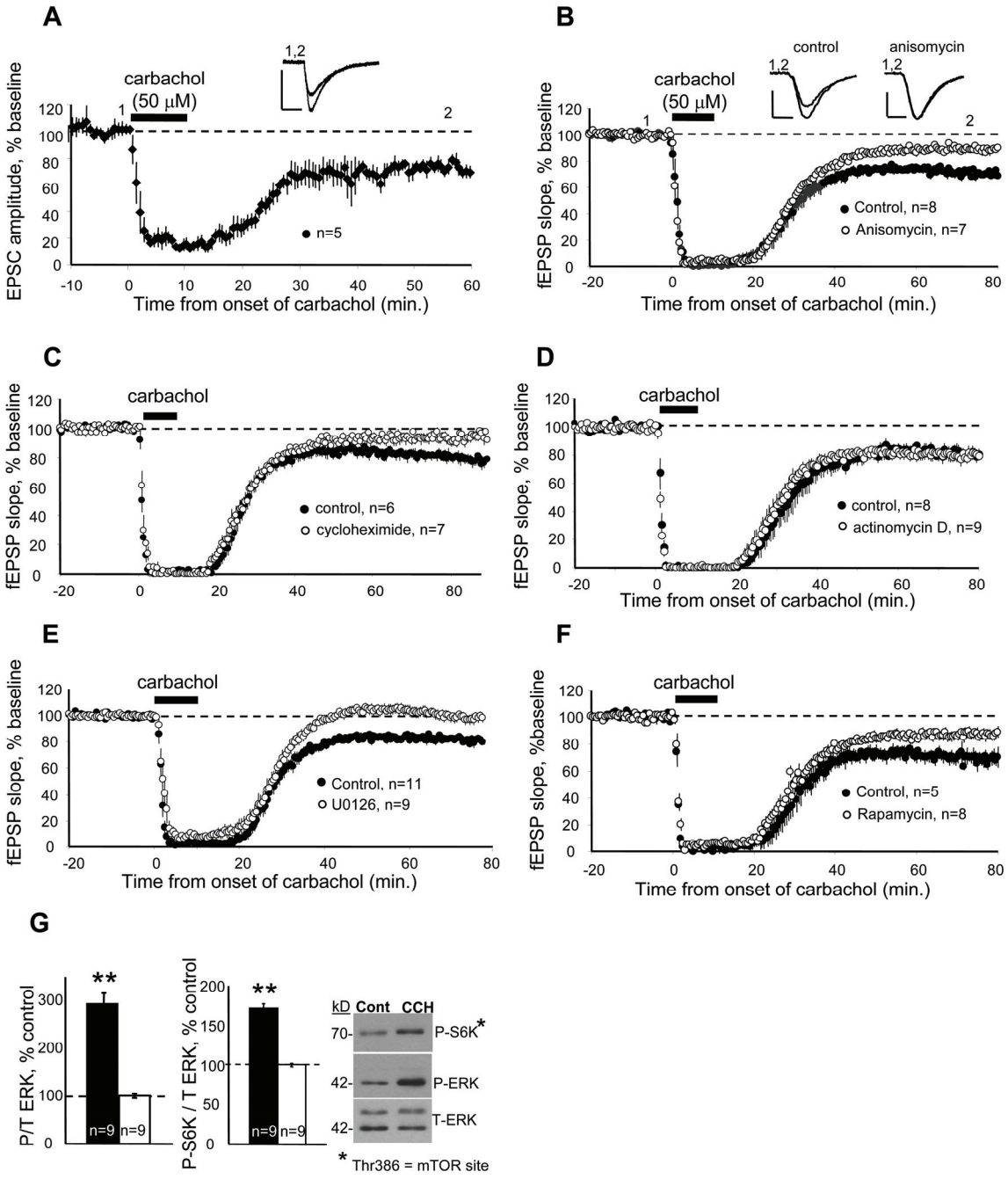


Figure 3.1, Muscarinic acetylcholine receptor-induced long-term synaptic depression requires protein synthesis, ERK, and mTOR activity.

A–E, Application of the muscarinic acetylcholine receptor agonist CCh (50 μM, 10 min) to acute hippocampal slices from mature rats (P25–P41) induces a long-term depression of EPSCs (***A***) and fEPSPs (***B–F***). Plotted are average (±SEM) amplitude values of EPSCs or average initial slope values of fEPSPs normalized to the pre-CCh baseline. All drugs except CCh were present in the bath throughout the experiment. All experiments are compared with interleaved controls.
A, Stimulation of mAChRs induces LTD (EPSC amplitude at 55–60 = 71 ± 4% of baseline; $p = 0.002$), which does not require cell depolarization (cells were clamped at –60 mV for the duration of the experiment). Calibration: 100 pA, 10 ms.
B, The protein synthesis inhibitor anisomycin (25 μM) significantly reduces CCh-induced LTD (control, 70 ± 2%; anisomycin, 87 ± 3%; $p < 0.001$). LTD is quantified as a 5 min average taken 1 h after CCh washout, and all statistical comparisons are made at this time point (see Materials and Methods). Inset, fEPSP waveforms (average of 4–6 traces) from a representative experiment are taken at the time points indicated on the graph (1, 2). Calibration: 0.5 mV, 5 ms.
C, The protein synthesis inhibitor cycloheximide (60 μM) blocks CCh-LTD [LTD measured 75 min after CCh washout; control, 78 ± 3%, $n = 6$; cycloheximide, 94 ± 4%; $p = 0.001$, control vs cycloheximide; $p = 0.1$, cycloheximide baseline vs 75 min after CCh washout].
D, The transcription inhibitor actinomycin D (25 μM) has no effect on CCh-LTD [control (0.1% DMSO), 81 ± 4%; actinomycin D, 80 ± 3%; $p = 0.8$].
E The MEK inhibitor U0126 (20 μM) blocks CCh-induced LTD [control (0.1% DMSO), 82 ± 2%; U0126, 99 ± 3; control vs U1026, $p < 0.001$; U0126 1 h after CCh washout vs U0126 baseline, $p = 0.7$].
F, The mTOR antagonist rapamycin (20 nM) reduces CCh-induced LTD [control (0.1% DMSO), 69 ± 5%; rapamycin, 87 ± 3%; $p < 0.01$).
G, Acute rat hippocampal slices (CA3 removed) were treated ±10 min with 50 μM CCh. After treatment, CA1 was microdissected on ice, and samples were immediately frozen on dry ice and processed for Western blotting. Right panel, Representative Western blots. P-S6K, phospho-p70 S6 kinase; P-ERK, phospho ERK; T-ERK, total ERK. Middle panel, Quantification of CCh-induced activation of p70 S6 kinase at the rapamycin-sensitive site (Thr389) (P-S6K/T-ERK, % control) in nine slices (control, 100 ± 2%; CCh, 171 ± 6%; $^{**}p < 0.001$). Left panel, Quantification of CCh-induced ERK activation (P-ERK/T-ERK, % control) in nine slices (control, 100 ± 5%; CCh, 293 ± 23%; $^{**}p < 0.001$).
*** Note that Brad Pfeiffer and Jay Gibson performed experiments in panel A.

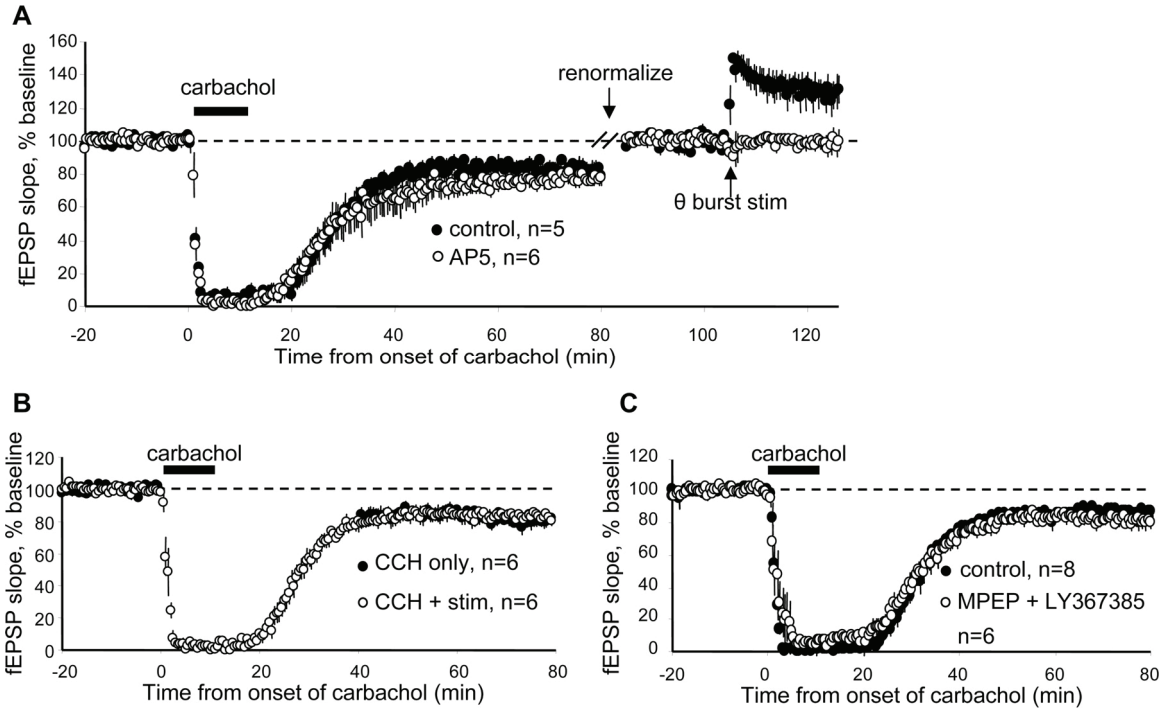


Figure 3.2, Protein synthesis-dependent CCh-induced LTD does not require activity of NMDARs, group 1 mGluRs, or presynaptic stimulation.

A, Preincubation of slices in the NMDA receptor antagonist, D,L-AP-5 (100 μM) before CCh had no effect on LTD (control, 82 ± 5%; AP-5, 77 ± 2%; $p = 0.3$). AP-5 was effective in blocking LTP induction with a theta-burst stimulation (θ) delivered after CCh-LTD induction (measured 25 min after theta burst; control, 129 ± 7%; AP-5 = 99 ± 1%; θ in AP-5, $p = 0.77$ vs baseline; θ in control, $p = 0.01$ vs baseline). fEPSP slope values are renormalized to pre-theta baseline.
B, Cessation of presynaptic stimulation immediately before and for 30 min after CCh application (CCh only) had no effect on LTD magnitude compared with slices in the same recording chamber, which received continuous stimulation during CCh (CCh + stim) (CCh + stim, 83 ± 2%; CCh no stim, 80 ± 3%; $p = 0.4$.).
C, Blockade of mGluR5 and $mGluR_1$ with MPEP (10 μM) and LY367385 (100 μM), respectively, had no effect on LTD induced with CCh (control, 87 ± 1; MPEP + LY, 81 ± 4%; $p = 0.2$).

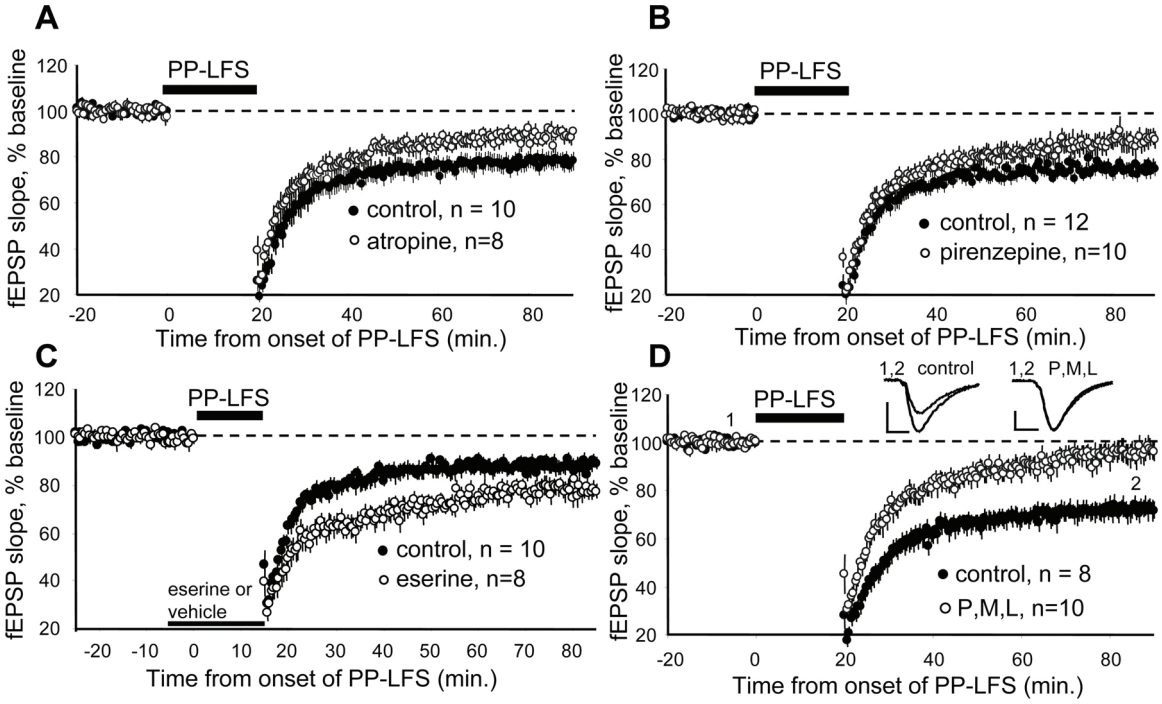


Figure 3.3, Synaptically induced protein synthesis-dependent LTD requires M1 mAChR activation.

All experiments are in the presence of the NMDA receptor antagonist D,L-AP-5 (100 μM). ***A***, Blockade of mAChRs with the nonselective muscarinic antagonist atropine (5 μM) reduces LTD induced synaptically with paired-pulse low-frequency stimulation (PP-LFS, 2 pulses with 50 ms interstimulus interval delivered at 1 Hz; 20 min) (measured 1 h after PP-LFS, control, 78 ± 3%; atropine, 90 ± 2%; $p = 0.01$).
B, Blockade of the M_1 subtype of mAChRs with the M_1-selective antagonist pirenzepine (75 nM) reduces PP-LFS induced LTD (measured 1 h after PP-LFS; control, 75 ± 3%; pirenzepine, 88 ± 4%; $p < 0.01$).
C, Brief application of the acetylcholine esterase inhibitor eserine (2 μM; 5 min before and during PP-LFS) facilitated LTD induced with PP-LFS (15 min) (measured 70 min after PP-LFS; control, 88 ± 2%; eserine, 78 ± 3%; $p = 0.02$).
D, PP-LFS-induced LTD is blocked by combined antagonism of M_1 mAChRs (75 nM pirenzepine) and group I mGluRs (mGluR5-specific antagonist MPEP at 10 μM plus $mGluR_1$-specific antagonist, LY367385 at 100 μM), "P,M,L" (measured 1 h after PP-LFS control, 72 ± 4%; "P,M,L", 96 ± 4%; control vs "P,M,L", $p < 0.001$; "P,M,L" at 85–90 min vs baseline, $p = 0.37$).

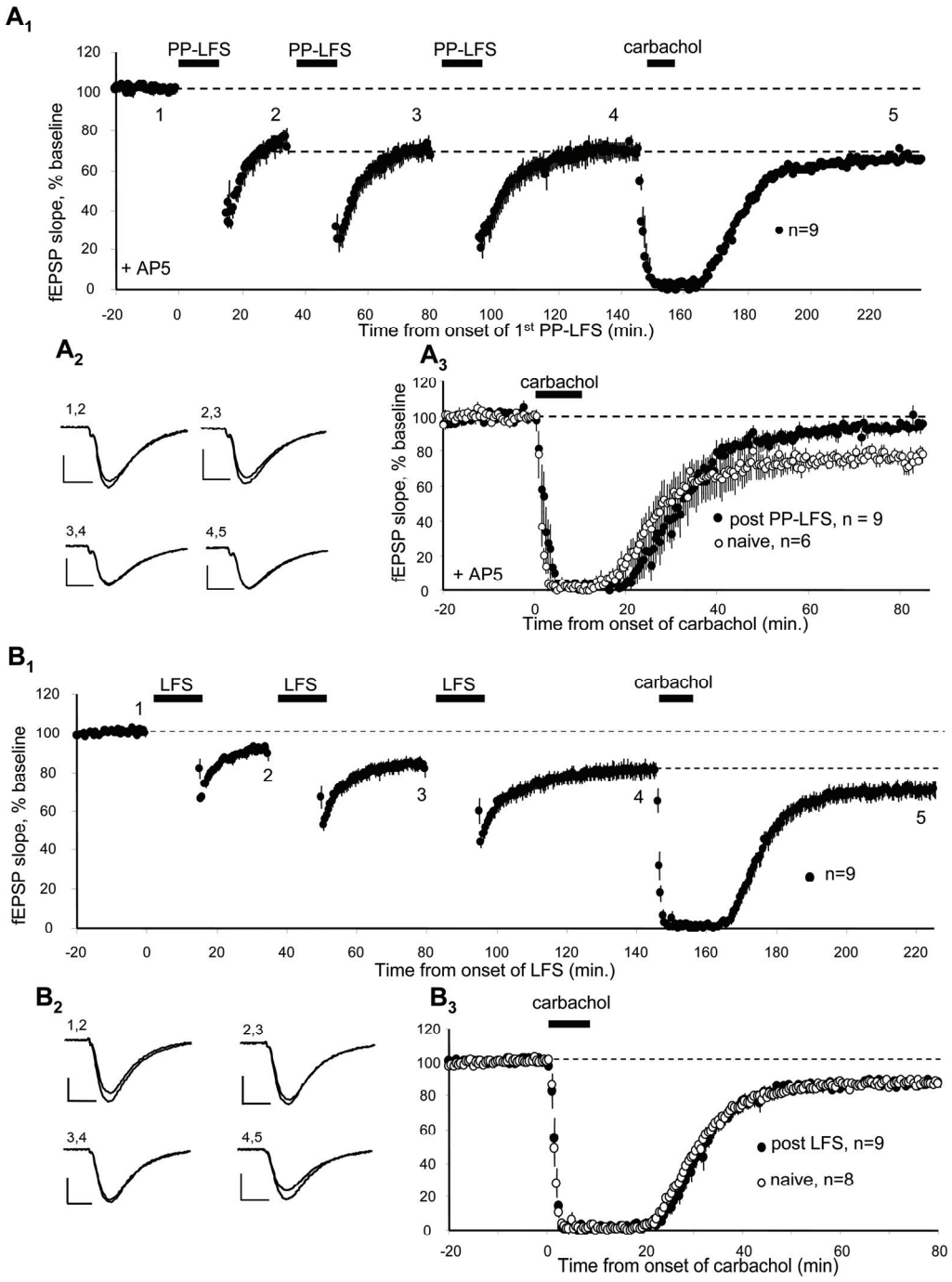


Figure 3.4, Saturation of synaptically induced, Gq-dependent LTD occludes CCh-induced LTD.

***A*$_1$**, Three episodes of PP-LFS (15 min) delivered to Schaffer collateral axons in the presence of D,L-AP-5 (100 μM) saturated LTD (LTD after second, 70 ± 4%, and third PP-LFS, 69 ± 4%, episode were not different; $p = 0.86$). LTD induced by CCh after saturation of PP-LFS-induced LTD is dramatically reduced.
***A*$_2$**, fEPSP waveforms (average of 4–6 traces) from a representative experiment are taken at the time points indicated on the graph in ***A*$_1$**.
***A*$_3$**, Previous saturation of PP-LFS-induced LTD reduced subsequent CCh-induced LTD compared with interleaved naive control slices, which received only baseline stimulation ("post PP-LFS" replotted from 130–230 min of ***A*$_1$**; measured 1 h after CCh, naive, 77 ± 2%; CCh LTD after PP-LFS saturation, 93 ± 2%; $p < 0.001$).
***B*$_1$**, Three episodes of LFS (15 min) delivered to Schaffer collateral axons saturated LTD (LTD after second, 84 ± 2%, and third PP-LFS, 81 ± 3%, episodes were not different; $p = 0.1$). Stimulation of mAChRs with CCh-induced normal levels of LTD after LFS saturation.
***B*$_2$**, fEPSP waveforms (average of 4–6 traces) from a representative experiment are taken at the time points indicated on the graph in ***B*$_1$**.
***B*$_3$**, Previous saturation of LFS-induced LTD has no effect on subsequent CCh-induced LTD compared with interleaved naive control slices, which received only baseline stimulation ("post LFS" replotted from 130–230 min of ***B*$_1$**; naive, 87 ± 1%; CCh LTD after LFS saturation, 86 ± 1%; $p = 0.1$)

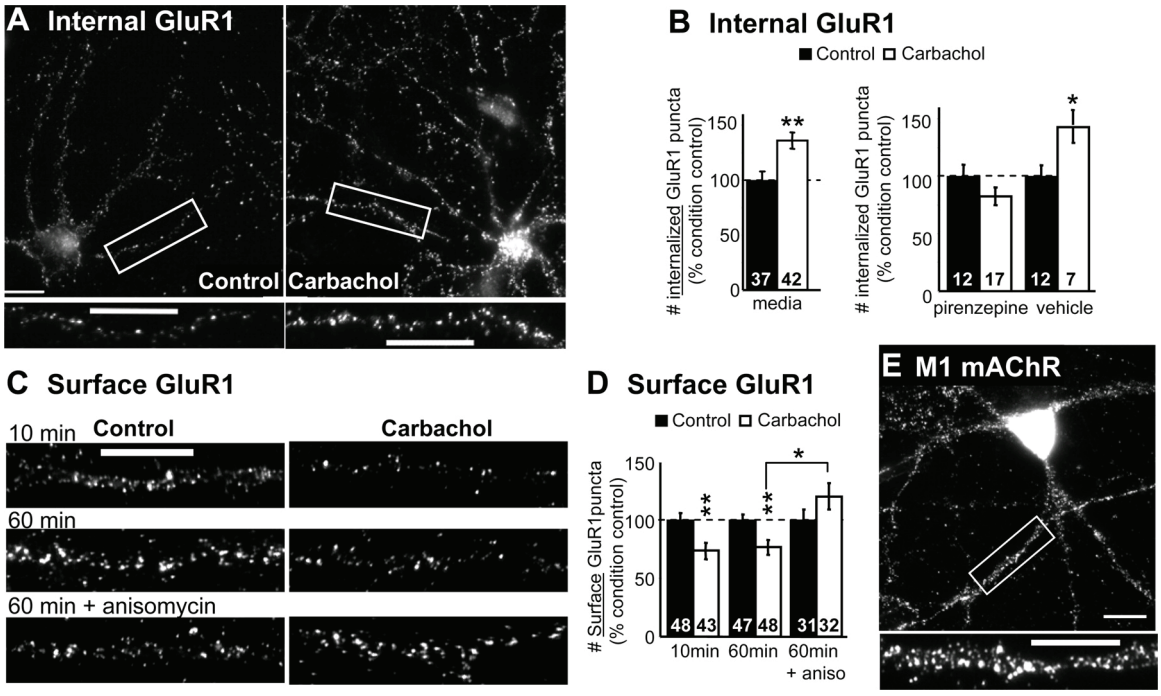


Figure 3.5, Activation of M1 muscarinic acetylcholine receptors induces endocytosis and long-term, protein synthesis-dependent decreases of surface GluR1.

A, Representative experiment in which internalized $GluR_1$ was labeled on live dissociated cultured hippocampal neurons using N-terminal $GluR_1$ antibody (see Materials and Methods). CCh (50 μM; 10 min) treatment increases internalized $GluR_1$. Scale bar, 15 and 10 μm.

B, CCh increases the number of internalized $GluR_1$ puncta per 50 μm of proximal dendrite measured 15 min after CCh washout (left panel; control, 100 ± 8%; CCh, 137 ± 8%), which is blocked by the M_1 mAChR selective antagonist pirenzepine (75 nM) (right panel; control, 100 ± 10%; CCh, 143 ± 13%; pirenzepine, 100 ± 10%; pirenzepine plus CCh, 83 ± 10%; $p = 0.2$; pirenzepine vs pirenzepine + CCh). Pirenzepine had no effect on basal surface AMPAR expression ($p = 0.4$). The intensity and area of internalized receptor puncta were unchanged. *n* (number of cells) is indicated on each bar. Data from two to four cultures per condition.

C, Representative experiment in which surface $GluR_1$ was labeled on fixed neurons 10 or 60 min after CCh (50 μM; 10 min) or media (control) treatment (± anisomycin; 25 μM). Scale bar, 10 μm.

D, Quantification of surface $GluR_1$ puncta reveals that CCh induces decreases in surface $GluR_1$ at 10 and 60 min after CCh application (10 min: control, 100 ± 6%; CCh, 74 ± 7%; 60 min: control, 100 ± 5%; CCh, 77 ± 6%). Anisomycin blocks the surface $GluR_1$ decreases at 60 min (anisomycin, 100 ± 9%; anisomycin plus CCh, 121 ± 11%; $p = 0.2$).

n (number of cells) indicated on each bar. Data from two to four cultures per condition. **p* < 0.05; ***p* < 0.01.
E, M_1 mAChRs are expressed and punctate on cultured hippocampal pyramidal neurons. Scale bar, 15 and 10 μm.

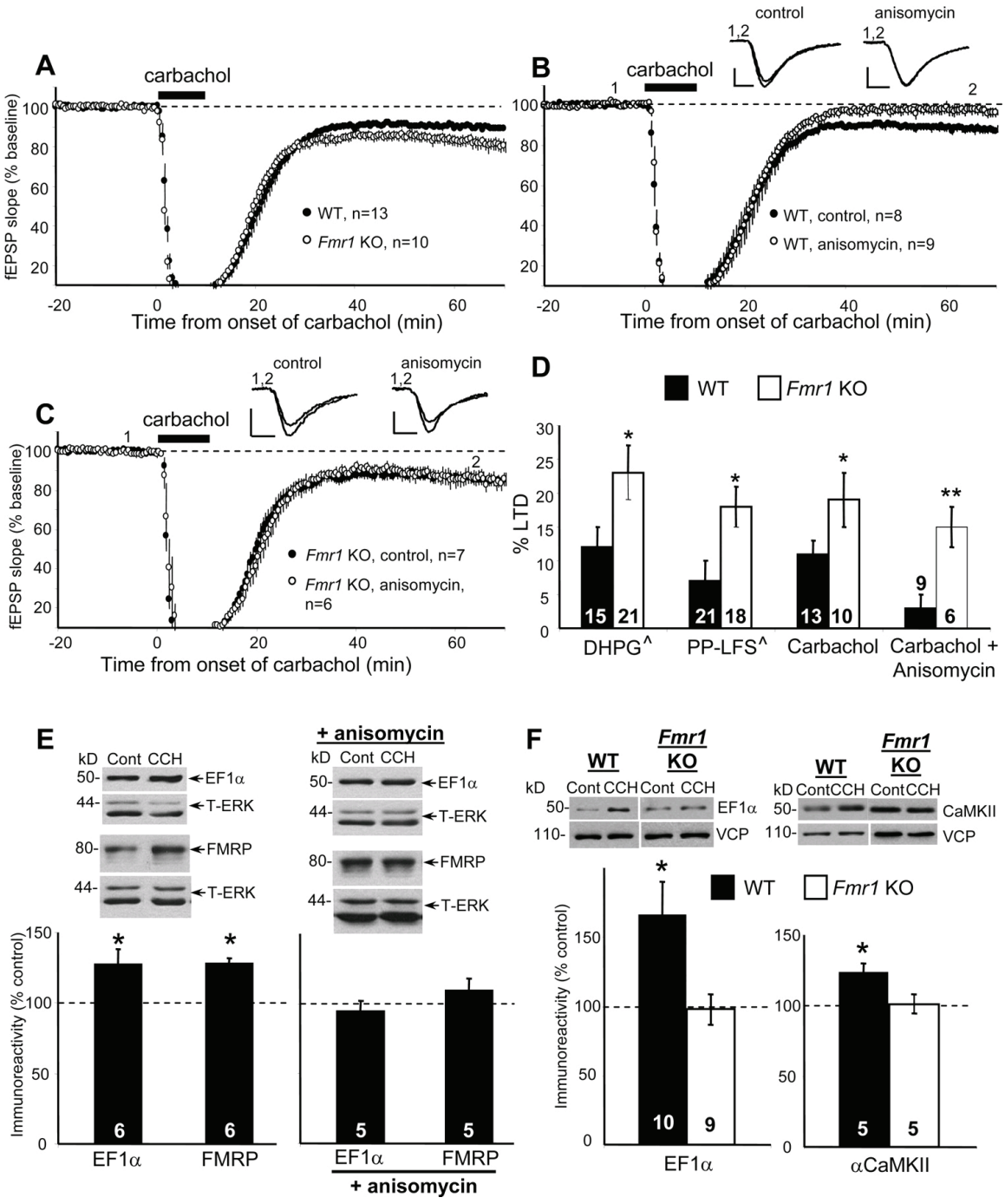


Figure 3.6, Muscarinic acetylcholine receptor-stimulated LTD and protein synthesis are altered in Fmr1 KO mice.

A, CCh (50 μM; 10 min) induced LTD is larger in magnitude in *Fmr1* KO mice compared with interleaved experiments in WT mice (WT, 89 ± 2%; *Fmr1* KO, 81 ± 3%; $p = 0.01$).
B, As in rats, CCh-LTD is blocked by anisomycin in WT mice (WT control, 88 ± 2%; WT + anisomycin, 97 ± 2%; $p < 0.01$).
C, In contrast, CCh-LTD persists in anisomycin in *Fmr1* KO mice (*Fmr1* KO control, 85 ± 3%; *Fmr1* KO plus anisomycin, 85 ± 2%; $p = 0.1$).
D, Comparison and summary of LTD induced by chemical stimulation of group I mGluRs (DHPG) or muscarinic acetylcholine receptors (carbachol) or synaptic stimulation (PP-LFS) in WT and *Fmr1* KO mice. *n* (number of slices) is indicated on each bar. Data are replotted from the study by Huber et al. (2002).
E, Top, Representative Western blots demonstrating that CCh treatment (50 μM; 10 min) induces a rapid and anisomycin-sensitive increase in protein levels for EF1-α and FMRP in acute hippocampal slices from WT mice. Total ERK (T-ERK) levels were used as a loading control and were not changed in response to CCh. Bottom, Quantified group data (percentage control) of Western blots (EF1-α, 131 ± 10%; FMRP, 128 ± 4%; EF1-α + aniso, 93 ± 8%; FMRP + aniso, 109 ± 9%). *n* (number of mice) is indicated on each bar. $*p < 0.05$, one-sample *t* test, percentage control.
F, Top, Representative Western blots demonstrating that CCh treatment (50 μM; 10 min) induces an increase in EF1-α and α-CaMKII protein levels in acute hippocampal slices (isolated CA1) from WT but not *Fmr1* KO littermates. VCP levels were used as a loading control and were not changed in response to CCh. Bottom left, Quantified group data (percentage control) of Western blots for EF1-α (WT: 167 ± 24%; $*p < 0.05$; KO, 98 ± 11%; $p = 0.5$; WT vs KO: $p < 0.05$). *n* (number of slices) is indicated on each bar. Bottom right, Quantified group data (percentage control) of Western blots for α–CaMKII (WT: 123 ± 6%, $*p < 0.05$; KO: 101 ± 6%, $p = 0.9$; WT vs KO: $p < 0.05$).

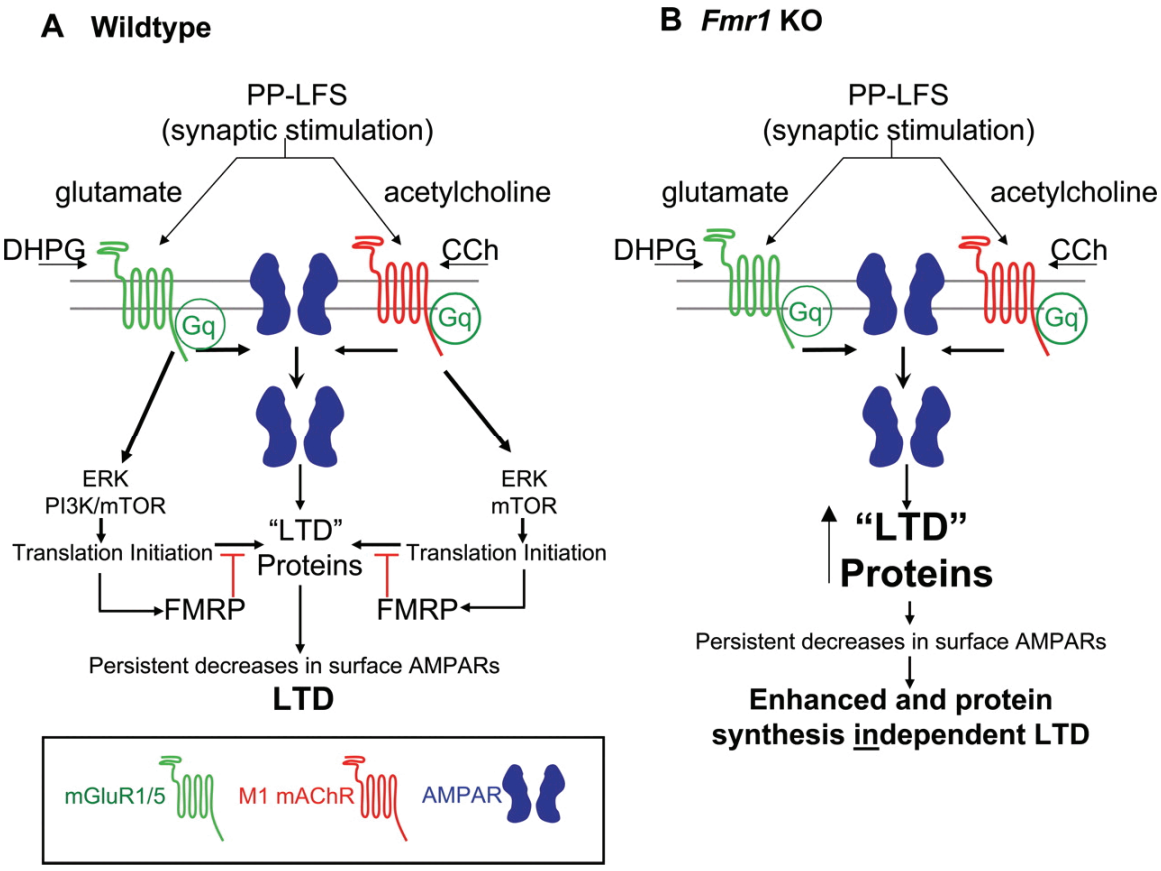


Figure 3.7, Working model of Gq-dependent LTD in wild-type rodents and Fmr1 KO mice.

A, In wild-type rodents, paired pulses of low frequency electrical stimulation (PP-LFS) to Schaffer collateral axons activates both group 1 mGluRs ($mGluR_1$ and $mGluR_5$) and mAChRs to induce LTD. Pharmacological stimulation of group 1 mGluRs (with DHPG) or M_1 mAChRs (with CCh) activates each of these pathways individually. Activation of either group 1 mGluRs or mAChRs induces endocytosis of AMPARs and stimulates mRNA translation initiation through the ERK and mTOR pathways (Huber et al., 2000 ; Banko et al., 2006). Synthesis of proteins, which maintain a reduction in surface AMPARs and LTD are called LTD proteins. mGluRs and mAChRs also stimulate the synthesis of FMRP, which, based on its known function as a translational suppressor, may feedback to inhibit synthesis of LTD proteins.
B, In *Fmr1* KO mice, mGluRs and mAChRs stimulate endocytosis of AMPARs to induce LTD (Nosyreva and Huber, 2006). In the absence of FMRP, there is a loss of translational suppression and increased steady state level of LTD proteins, which leads to enhanced LTD and AMPAR surface decreases that does not require protein synthesis.

CHAPTER FOUR
Discussion and Future Directions

RELATIVE CONTRIBUTIONS OF MGLUR1 AND MGLUR5 TO HIPPOCAMPAL LTD

mGluR1Plays a Role in Hippocampal LTD

Data presented in Chapter Two of this dissertation demonstrate for the first time a clear role for mGluR1 in LTD at SC-CA1 synapses. Recent findings that blockade of mGluR1 or mGluR5 in the CA1 region of the hippocampus inhibits consolidation of inhibitory avoidance memory while mGluR1 but not mGluR5 is required for extinction of IA memory support a role for mGluR1 in hippocampal-dependent memory (Simonyi et al., 2007).

While I find an unambiguous role for mGluR1 in induction and expression of hippocampal LTD, other groups report no effect of mGluR1 antagonists on mGluR LTD in the hippocampus (Fitzjohn et al., 1999; Huang and Hsu, 2006). The recent findings of excitotoxicity-induced cleavage of mGluR1 and facilitation of mGluR1-dependent LTD by acute stress demonstrate that numerous factors can affect the responsiveness of mGluRs, and suggest that different slice preparation conditions can profoundly affect physiology (Chaouloff et al., 2007; Xu et al., 2007). Brain tissue is vulnerable to excitotoxic damage during slice preparation, and excessive NMDAR activation is implicated in this process. Fitzjohn et al. prepare their slices in ACSF, which contains roughly physiological levels of NaCl, Ca^{2+}, and Mg^{2+}, whereas our lab and other labs that see an effect of mGluR1 inhibition on LTD in the hippocampus use dissection buffer

containing high Mg^{2+}, low Ca^{2+}, and substitution of NaCl with sucrose to minimize the excitotoxic effects of slicing (Faas et al., 2002; Volk et al., 2006). Xu et al find that NMDAR-mediated excitotoxicity results in cleavage of mGluR1 but not mGLuR5, which results in an inability of mGluR1 to activate the Akt/mTOR pathway (though mGluR1-induced increase in $[Ca^{2+}]_I$ is intact) and a redistribution of truncated mGluR1 to axons, whereas full length mGluR1 is mostly excluded from axons (Xu et al., 2007). Thus, it is possible that brain slices prepared under conditions that do not minimize the excitotoxic effects of slicing have less functional mGluR1 expressed at synapses and therefore do not see a contribution of this receptor to plasticity. It would be useful to perform a study of the effect of different slice preparation conditions (as highlighted above) on the receptor requirements for DHPG-induced LTD in the hippocampus. In addition, acute restraint stress lowers the threshold for DHPG-induced LTD, and LTD induced by sub threshold DHPG stimulation in stressed rats is mGluR1-dependent. These data highlight the importance of careful slice preparation and animal handling in achieving consistent, physiologically meaningful results from acute slices.

Speculation on the Physiological Meaning of Transient LTD Reversal by mGluR1 antagonism

An interesting property of LTD induced with DHPG is the ability of mGluR antagonists to transiently "reverse" LTD (Fig.2.2). This effect was first demonstrated ten years ago with a broad range mGluR antagonist (Palmer et al., 1997). However, the mechanism and physiological relevance of this effect remain unclear (but see Huang and Hsu, 2006).

One concern is that DHPG is simply not washing out of the slice, thus receptors are persistently activated by agonist and the reversal experiments reveal DHPG LTD to be a pharmacological artifact. Multiple pieces of data presented in Chapter Two suggest that this is not the case. Transient application of mGluR1 + mGluR5 antagonists during DHPG stimulation completely blocks DHPG LTD. If DHPG did not wash out of the slice it could reactivate receptors after the antagonists were washed out, but this does not occur. In addition, application of the mGluR1 antagonist LY367385 reverses LTD in mature rats, but not in young rats, suggesting that LY367385 is specifically affecting a postsynaptic mechanism distinct from mGluRs to induce reversal.

Preliminary data presented in chapter two suggest that mGluR1 becomes constitutively active after stimulation with DHPG, and that this constitutive activity is partly responsible for maintaining LTD. DHPG LTD is transiently reversed by the mGluR1 inverse agonist LY367385, but the mGluR1 antagonist CPCCOEt has very little effect when applied after LTD is induced, supporting the idea that constitutive mGluR1 activity maintains DHPG LTD. Recall that an inverse agonist blocks agonist independent, i.e. constitutive, receptor activity in addition to agonist-dependent activity, whereas an antagonist only blocks agonist-dependent receptor activity. In addition, LY367385 has no significant effect on baseline synaptic transmission, suggesting that there is in increase in constitutively active mGluR1 after DHPG application. mGluRs bind to a long form of the scaffolding protein Homer, which serves to couple mGluRs to various signaling molecules (Duncan et al., 2005). Evidence that activity can induce a shift in mGluR conformation to the constitutively active state comes from data demonstrating that the activity-inducible short form of Homer, Homer1a, uncouples

mGluRs from long forms Homer and results in constitutive receptor activity (Ango et al., 2001; Bottai et al., 2002). However, there is no direct demonstration that DHPG induces an increase in mGluR1 that is in the constitutively active form, and is a necessary future study to support this hypothesis.

How does constitutive mGluR1 activity maintain LTD? The fact that LY367385 reverses LTD in mature but not young rats suggests that constitutively active mGluR1 may be regulating AMPAR endocytosis. mGluR LTD in mature rodents is maintained by a persistent increase in AMPAR endocytosis rate (Waung et al., 2007), so determining if transient application of LY367385 reverses this effect is a key experiment in understanding how constitutively active mGluRs maintain LTD.

Surprisingly, I find that the mGluR5 inverse agonist MPEP does not reverse DHPG LTD, even though mGluR5 can also exist in a constitutively active state that is regulated by binding to Homer (Pagano et al., 2000; Ango et al., 2001). While both LY367385 and MPEP are classified as inverse agonists (compounds that block agonist independent in addition to agonist-dependent effects of a receptor), there is an important difference in the actions of these compounds that may shed some light on this issue. mGluR1 and mGluR5 exhibit agonist independent phosphoinositide hydrolysis (see signaling section in introduction) as well as agonist independent internalization. LY367385 blocks both of these effects, but MPEP only blocks agonist independent phosphoinositide hydrolysis, it has no effect on constitutive mGluR5 internalization (Pagano et al., 2000; Fourgeaud et al., 2003; Pula et al., 2004). Taken together with my data, this suggests that constitutive mGluR internalization may play a role in maintaining an increase in AMPAR internalization rate, and that MPEP has no effect on expression of

LTD because MPEP does not affect constitutive mGluR5 internalization. How might mGluR internalization affect AMPAR internalization? One possibility is that AMPARs get endocytosed in a complex with mGluRs. Though synaptic AMPARs are not co-localized with perisynaptic mGluRs, AMPARs are trafficked in and out of synapses through lateral movement, and endo/exoocytosis is thought to occur at perisynaptic sites (Zhou et al., 2001; Adesnik et al., 2005). Both AMPAR and constitutive mGluR1 endocytosis are clathrin dependent (Pula et al., 2004; Rial Verde et al., 2006), and Preliminary data indicate that synthesis of the protein Arc, which interacts with clathrin-dependent endocytosis machinery, is required for DHPG-induced persistent increases in AMPAR internalization rates (Waung et al., 2007). Interestingly, constitutive mGluR5 internalization is clathrin independent, suggesting that is internalized through a different mechanism. It would be informative to determine if DHPG induces an increase in internalization rate of mGluRs in addition to AMPARs, and to determine if these internalized receptors are colocalized.

Implications for the mGluR Theory of Fragile X Mental Retardation

Surprisingly, though mGluR LTD is enhanced in *Fmr1* KO mice, no one has examined the dose response of DHPG for inducing LTD in these mice. Acute restraint stress lowers the threshold for mGluR LTD induced with DHPG, and the facilitatory effect of stress on DHPG LTD is mediated by glucocorticoid receptors (GRs) (Chaouloff et al., 2007). FMRP binds to the mRNA for GRs. In addition, the response to acute stress is elevated in children with FXS, and acute restraint stress results in a prolonged elevation of serum glucocorticoid levels in *Fmr1* KO compared to WT mice (Nagai et al.,

2007). These data raise the possibility that enhanced GR signaling mediates the enhancement in mGluR LTD observed in *Fmr1* KO mice. Consistent with this idea, Chaouloff et al observe that 100μM DHPG (the concentration standardly used to induce LTD) induces LTD in control and stressed rats, but LTD in stressed rats was 11% bigger. Though this is a small increase, it is similar in magnitude to the difference we see between LTD in *Fmr1* KO and WT mice. Interestingly, multiple members of our lab, including myself, have observed that enhanced mGluR (and mAChR) LTD in *Fmr1* KO mice is a less robust phenotype than the loss of protein synthesis-dependence of mGluR (and mAChR) LTD, suggesting that these two phenotypes may be mediated by separate processes. Considering that acute stress prior to slicing could vary greatly from animal to animal, this may explain the variability of LTD enhancement seen in *Fmr1* KO mice. This idea predicts that: 1. GR antagonists would abolish the enhancement of LTD in *Fmr1* KO mice without affecting the loss of protein synthesis-dependence, and conversely, exposing WT but not KO mice to acute stress should enhance LTD in WT mice to the level of *Fmr1* KOs, 2. Fmr1 KO mice should be more sensitive to acute stress than WT littermates, and 3. LTD in *Fmr1* KO and WT mice should be the same magnitude in mGluR1 antagonists.

Because initial studies suggested that mGluR5 was the primary mediator of protein synthesis-dependent mGluR LTD in the hippocampus, and because the mGluR theory of fragile X mental retardation proposes that exaggerated protein synthesis-dependent mGluR processes can account for many of the cognitive and behavioral phenotypes of FXS, the mGluR5 antagonist MPEP has gained great popularity as a potential treatment for FXS. Studies in FXS model systems validate mGluR5 antagonism

as a possible treatment for FXS. However, data presented here support an important role for mGluR1 in protein synthesis-dependent LTD that is affected in *Fmr1* KO mice, and previous reports demonstrate a role for mGluR1 in protein synthesis-dependent prolongation of epileptiform bursts in the CA3 region of the hippocampus, which are also affected in *Fmr1* KO mice, suggesting that mGluR1 antagonism may also be effective in ameliorating the symptoms of FXS. While the MPEP studies are promising, it may be that combined antagonism of mGluR1 and 5 would be more effective, or that lower concentrations of each antagonist could be used in order to minimize side effects and maximize therapeutic benefit. It is not known if the relative contributions of mGluR1 and 5 to mGluR LTD are altered in *Fmr1* KO mice, and this may be informative in understanding how FMRP regulates synaptic plasticity (see previous paragraph).

mAChRs: Role in Hippocampal LTD and FXS

Alzheimer's disease is characterized by a reduction in cholinergic innervation of the hippocampus and cortex, and loss of hippocampal mAChR function may contribute to the cognitive deficits observed in AD patients (Clader and Wang, 2005; Mikiciuk-Olasik et al., 2007). In addition, age-related decline in spatial learning and memory in rats is associated with a selective decrease in hippocampal mAChR-dependent LTD (Lee et al., 2005b) and with uncoupling of M1 mAChRs from Gq proteins (Rossi et al., 2005). Blockade of mAChRs inhibits recognition memory, inhibitory avoidance learning, and spatial memory (Massey et al., 2001; Warburton et al., 2003; Hasselmo, 2006). Also, I find that mAChR-dependent LTD is aberrant in the mouse model of fragile X syndrome mental retardation (Volk et al., 2007). All of these data point to a critical role for

mAChRs and mAChR-dependent plasticity in learning and memory. However, prior to publication of the work in this dissertation, little was known about the signaling mechanisms by which mAChR activation induces LTD.

mAChRs Induce LTD Through a Conserved Gq- and Protein Synthesis-Dependent Mechanism

I find that LTD induced by activation of Gq-coupled mAChRs shares a common expression mechanism with mGluR LTD. LTD induced by both of these receptors is protein synthesis dependent and requires activation of the ERK and mTOR signaling pathways, which regulate translation in neurons. New proteins maintain LTD through a persistent and protein synthesis-dependent decrease in surface AMPAR expression. Importantly, LTD induced by either of these Gq-coupled receptors is enhanced and protein synthesis-independent in the mouse model of fragile X syndrome mental retardation. These findings suggest the existence of a conserved mechanism for induction of protein synthesis-dependent LTD by Gq-coupled receptors. Additionally, the aberrant mAChR LTD observed in *Fmr1* KO mice suggests that the *mGluR* theory of fragile X mental retardation is too specific. Instead, my data suggest that protein synthesis-dependent plasticity induced by multiple neurotransmitter systems is affected in FXS and that the deficit lies in regulation of protein synthesis-dependent plasticity. Considering the promising results seen with mGluR5 antagonism in treating symptoms of FXS in model organisms, mAChR antagonists may also be effective in treating FXS, and mGluR antagonists such as scopolamine are already approved for use in humans for treatment of other conditions.

Protein synthesis-dependent mAChR and mGluR LTD require activation of ERK and mTOR, and both ERK and mTOR signaling pathways facilitate translation in neurons (Hou and Klann, 2004; Banko et al., 2006; Volk et al., 2007). Surprisingly, no studies exist showing that ERK and/or mTOR activation are necessary for mGluR or mAChR-induced protein synthesis, thus it is possible that one or both of these signaling pathways mediate effects other than protein synthesis in mGluR and mAChR LTD. While maintaining a persistent decrease in AMPAR surface expression requires new protein synthesis, the initial AMPAR internalization step is independent of protein synthesis, and it is not known what signaling mechanisms are employed in the initial internalization step. Candidates include ERK, mTOR or tyrosine phosphatase activity. In addition, mGluR and mAChR LTD are protein synthesis independent in *Fmr1* KO mice, but mGluR activation still induces internalization of AMPARs in these mice, suggesting that mGluR and mAChR LTD in *Fmr1* KO mice rely only on the signaling pathway responsible for initial AMPAR internalization. Determining the signaling mechanisms necessary for the initial protein synthesis-independent internalization of AMPARs is critical in order to fully understand how mGluRs and mAChRs induce LTD, and how LTD signaling processes are affected in FXS. Hou et al find that DHPG-induced ERK activation is absent in *Fmr1* KO mice, and MEK inhibitors fail to block LTD in these mice (Hou et al., 2006). However, I see that mAChR stimulation induces robust ERK activation in *Fmr1* knockout mice (P/T-ERK, 50□M CCH, 10min = 732 ± 319 % of control, $p < 0.001$ compared to untreated control), and others in our lab see DHPG-induced ERK activation in *Fmr1* knockout mice (unpublished observations). Preliminary data suggest that mTOR signaling is disrupted in *Fmr1* KO mice, and that

LTD is insensitive to an mTOR antagonist (Ronesi and Huber, 2007; Sharma et al., 2007). These data suggest that mTOR activation is not required for mGluR-induced AMPAR internalization in *Fmr1* KO, and perhaps that ERK activation is also unnecessary is mGluR-induced AMPAR internalization. However, more studies are needed to determine the signaling mechanisms responsible for mGluR and mAChR-induced AMPAR endocytosis in WT and *Fmr1* KO mice.

Different levels of mGluR and mAChR stimulation (high vs. low concentrations of agonist) result in dramatically different effects on plasticity. For both receptors, stimulation with low concentrations of agonist results in facilitation of NMDAR-dependent LTP, while high concentrations of agonist induce LTD (Cohen and Abraham, 1996; Segal and Auerbach, 1997; Huber et al., 2001b; Massey et al., 2001; Shinoe et al., 2005). GPCRs are highly regulated by multiple mechanisms including desensitization, internalization, and activity-dependent alterations in coupling to intracellular signaling molecules. For example, α-adrenergic receptors activate ERK via a G protein – dependent mechanism, which results in rapid but transient ERK activation and by a G protein-independent mechanism that requires receptor internalization and results in slow onset but persistent ERK activation (Shenoy et al., 2006). mGluR and mAChR function are regulated by multiple interactions, including phosphorylation by PKC and G protein-coupled receptor kinase 2 (GRK2). The physiological consequences of receptor desensitization, internalization and alternate coupling to intracellular signaling molecules is not clear, but it is likely that this plays a role in the different responses of these receptors to strong vs. weak stimulation.

Why do multiple receptors activate the same plasticity mechanism?

A simple explanation may be that LTD processes mediated by different neurotransmitter systems are tapping into the "best" mechanism for modulating synaptic efficacy in a long-term manner. Alternatively, while pharmacological activation of receptors is a valuable tool for studying the mechanisms of plasticity, stimuli this intense are unlikely to occur under physiological conditions, so co-activation of mGluRs and mAChRs may be required to induce LTD under physiological conditions. This conserved LTD mechanism may serve as a "relevance detector" to couple sensory stimuli (mGluR activation) with emotional context (mAChR activation, others?). Factors such as novelty, motivation, concentration, stress, and reward can profoundly affect learning and memory, and acetylcholine release is associated with novelty and arousal (Pepeu and Giovannini, 2004). Gq-coupled receptors activated by other neurotransmitter systems, such as α-adrenergic receptors, 5HT2A seratonergic receptors and even Gq-coupled D1/5 dopamine receptors, may use this same mechanism to induce protein synthesis-dependent plasticity. Considering the importance of protein synthesis dependent LTD to normal cognitive function, it would be worth exploring this possibility. It would be particularly interesting to determine if subthreshold levels of mGluR and mAChR activation result in a synergistic induction of LTD.

Comments on the Role of Dendritic Protein Synthesis in Synaptic Plasticity

What is the physiological advantage of dendritic protein synthesis that warrants a neuron expending resources to make dendrites capable of protein synthesis? It is well established that maintaining long lasting changes in synaptic strength, on the order of

days to months and even years, requires macromolecular synthesis. Synthesizing proteins in dendrites, as opposed to the soma, could greatly decrease the time necessary to stabilize synaptic changes and might serve to increase the fidelity of this process by limiting interference from signals generated by many active synapses in the same neuron.

Though there is now little doubt that dendritic protein synthesis plays a critical role in maintaining long lasting changes in synaptic efficacy and function, the manner by which dendritically synthesized proteins contribute to plasticity and the synapse specificity of proteins synthesized by any particular stimulus is still unclear.

Synapse Specificity of Proteins Synthesized in Dendrites

When protein synthesis machinery was first discovered in dendrites, it was proposed that dendritic protein synthesis would specifically allow activated synapses to synthesize the proteins they need to consolidate or maintain plasticity (Bodian, 1965; Steward and Levy, 1982). In 1997 Frey and Morris proposed the "synaptic tagging" hypothesis to explain the interactions between early and late phases of NMDAR-dependent plasticity (see chapter 1, p.37). This differs from previous ideas regarding dendritic protein synthesis in its prediction that dendritic protein synthesis is not synapse specific. Unpublished data presented at this year's SfN meeting supports a different interpretation of synaptic tagging in which protein synthesis is synapse specific (Tsokas et al., 2007). Tsokas et al. suggest that the result of strong stimulation is not dendrite-wide protein synthesis, but a widespread and persistent elevation in components of the mTOR pathway, which facilitates initiation of translation. In this model, input specific

de-repression of mRNAs serves as the "tag" generated by a weak stimulus. Consistent with this idea, multiple forms of protein synthesis-dependent LTP *and* LTD require activation of the ERK and mTOR translation regulatory pathways. This model highlights the importance of RNA binding proteins in regulating input specific protein synthesis. Interestingly, dynamic FMRP-dependent regulation of mGluR and mAChR LTD (see p. 22) fits nicely into the model proposed by Tsokas et al. Stimulation of mGluRs and mAChRs activates mTOR and ERK to facilitate initiation of translation, and dephosphorylation of FMRP de-represses mRNAs bound to FMRP, which serves as the input-specific (LTD) tag. The hypothesis that FMRP dynamically regulates translation in an activity-dependent manner predicts the following: 1) FMRP is dephosphorylated by activity (e.g. stimulation of mGluRs or mACHRs), 2) *acute* knockdown of FMRP should prevent mGluR and mAChR-dependent protein synthesis, 3) expression of phospho-mimic FMRP (S500D) in FMRP knockout neurons should prevent the increase basal dendritic protein levels, but should not rescue the loss of mGluR and mAChR-dependent protein synthesis, 4) expression of dephospho-mimic FMRP (S500A) should maintain mGluR and mAChR-dependent protein synthesis but still result in an increase in basal protein levels. Testing these predictions would provide valuable insight into the mechanism by which FMRP regulates protein synthesis-dependent plasticity. Identifying the phosphatase responsible for dephosphorylating FMRP is also a priority.

The synapse specificity of dendritic protein synthesis remains to be clearly determined, including whether different forms of protein synthesis-dependent synaptic plasticity (e.g. NMDAR vs mGluR and mAChR) use the same mechanism for generating input specificity.

Interactions Between Gq-Receptors and NMDA Receptor Induced LTD

Protein synthesis-dependent mGluR, mAChR and NMDAR LTD are usually studied in isolation, though it is likely that they rarely occur in isolation *in vivo*, and little is known about how these forms of LTD are integrated at the synapse. Occlusion studies demonstrate that protein synthesis-independent NMDAR E-LTD (LFS = 900 pulses at 1Hz) uses a different expression mechanism than mGluR or mAChR LTD (Huber et al., 2001; Volk et al., 2007), and AMPAR endocytosis is differentially regulated by NMDA and DHPG (Waung et al., 2007), supporting the idea that these are distinct forms of plasticity. However, the more relevant question may be, does protein synthesis-*dependent* late phase NMDAR LTD share a common expression mechanism with protein synthesis-dependent mGluR and mAChR LTD? One way to test this is to determine if saturation of PP-LFS, mAChR or mGluR LTD occludes NMDAR L-LTD and visa versa.

Even if these different forms of LTD use different expression mechanisms (e.g. different mechanisms for regulating AMPAR endocytosis), they may use similar mechanisms to regulate dendritic protein synthesis. Protein synthesis-dependent NMDAR L-LTD in the hippocampus requires coactivation of dopamine D1/5 receptors (Sajikumar and Frey, 2004). These receptors canonically couple to Gs proteins, but they also signal through Gq proteins, and the D1/5 antagonist used by Sajikumar and Frey to block L-LTD prevents D1/5 receptor coupling to Gq proteins (Mannoury la Cour et al., 2007) In addition D1 receptors regulate protein synthesis-dependent recognition memory via ERK (Nagai et al., 2007). If PP-LFS (in the presence of a reversible NMDAR

antagonist) given in one pathway converts E-LTD in an independent pathway into L-LTD (and E-LTP into L-LTP), it would suggest LTD induced by activation of Gq-coupled receptors and LTD induced by activation of NMDARs use similar mechanisms to regulate dendritic protein synthesis. Loss of FMRP results in a general increase in dendritic protein synthesis (Brown V, 2001; Todd et al., 2003; Hou et al., 2006; Muddashetty et al., 2007), so the synaptic tagging hypothesis proposed by Frey and Morris predicts that weak E-LTD and E-LTP stimuli should induce late phase plasticity in *Fmr1* KO mice. If FMRP specifically binds to and regulates translation of proteins required for LTD, then only E-LTD and not E-LTP should be affected.

The Role of Protein Synthesis-Dependent LTD in Cognition

Evidence that LTD mediates learning and memory processes is not as strong as data supporting LTP as a mechanism underlying learning and memory. Does LTD function in learning and memory as a general method for reducing noise in order to isolate salient signals, or does it play a role in specific types of learning and memory tasks? Novelty recognition tasks reveal that fewer neurons in the perirhinal cortex are activated in response to familiar vs. novel objects, consistent with an LTD-like mechanism (Warburton et al., 2003). mAChR antagonists block novelty recognition memory and LTD in the perirhinal cortex, whereas LTP is unaffected. In addition mAChR LTD in the perirhinal cortex is protein synthesis-dependent. Mice lacking the retinoic acid receptor RXRα show a selective deficit in LTD but not LTP, and these mice demonstrate profound deficits in novel object recognition (Ming-Yi Chiang et al., 1998).

Retinoic acid induces protein synthesis in dendritic compartments, suggesting that the deficit in novel object recognition could be due to a loss of protein synthesis-dependent LTD (Poon et al., 2007). Extinction of fear conditioning in the lateral amygdala induces mGluR LTD, and protein synthesis-dependent extinction of IA memory requires mGluR1 activity (Power et al., 2006; Kim et al., 2007; Simonyi et al., 2007). These emerging roles for protein synthesis-dependent LTD in novelty acquisition and extinction learning support the idea that, perhaps in addition to a role in isolating salient stimuli, LTD may be important in specific types of learning and memory.

In conclusion, data presented in this dissertation demonstrate that multiple Gq-coupled receptors converge on a common, protein synthesis-dependent LTD mechanism that is aberrant in the mouse model of fragile X syndrome mental retardation. These findings emphasize the significance of protein synthesis-dependent plasticity to cognition, and reveal that general misregulation of a conserved protein synthesis-dependent plasticity mechanism, as opposed to a specific exaggeration of mGluR function, may contribute to the cognitive deficits in FXS. In addition, these data highlight the importance of understanding how multiple forms of plasticity interact and integrate at synapses.

APPENDIX
List of Pharmacological Reagents

Compound	Site of Action	Effect	Concentration
actinomycin D	transcription	antagonist	25μM
AP5	NMDA receptor	antagonist	100 μ M
anisomycin	translation	antagonist	25 μ M
atropine	muscarinic acetylcholine receptors	antagonist	5 μ M
carbachol	cholinergic receptors	agonist	50 μ M
cycloheximide	translation	antagonist	60 μ M
CPCCOEt	mGluR1	antagonist	50-100 μ M
DHPG	Group I mGluRs (mGluR1, mGluR5)	agonist	100 μ M
eserine	acetylcholine esterase	antagonist	2 μ M
MPEP	mGluR5	antagonist (inverse agonist	10 μ M
pirenzepine	M1 mAChRs	antagonist	75nM
LY367385	mGluR1	antagonist (inverse agonist)	100 μ M
LY341495 LY341495	Group II and II mGluRs Group I, II, and III mGluRs	antagonist antagonist	20 μ M 100 μ M
Rapamycin	mTOR	antagonist	20nM
U0126	MEK	antagonist	20 μ M

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