

# Multiple Gq-Coupled Receptors Converge on a Common Protein Synthesis-Dependent Long-Term Depression That Is Affected in Fragile X Syndrome Mental Retardation

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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. Here, we 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. Our 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.

**Key words:** muscarinic acetylcholine; Gq coupled; metabotropic glutamate; hippocampus; Fragile X syndrome; long-term depression; protein synthesis

## 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 (Bolshakov and Siegelbaum, 1994; Oliet et al., 1997; Kemp and Bashir, 1999), 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

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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, we find that synaptic or pharmacological activation of M<sub>1</sub> 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 (AMPA) trafficking. To examine whether FMRP generally regulates Gq- and protein synthesis-dependent plasticity, we 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)-(+)- $\alpha$ -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<sup>-</sup> (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  $\mu$ m (300  $\mu$ m for intracellular recordings) in slushed (partially frozen) dissection buffer containing the following (in mM): 2.6 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 0.5 CaCl<sub>2</sub>, 5 MgCl<sub>2</sub>, 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<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, and 10 dextrose for field recordings or 119 NaCl, 2.5 KCl, 1 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 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<sub>2</sub>/5% CO<sub>2</sub>.

For intracellular recording, slices were transferred to a submerged recording chamber (Warner Instruments, Hamden, CT), maintained at 30°C and perfused continuously with ACSF at a rate of 2.5–3 ml/min. Picrotoxin (20  $\mu$ M) 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 M $\Omega$ ) 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  $\mu$ m from the recorded neurons with monophasic current pulses (5–25  $\mu$ A, 200  $\mu$ s). 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 M $\Omega$ . Initial input resistance values ranged from 126 to 282 M $\Omega$ . 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 M $\Omega$ ) filled with ACSF and placed in stratum radiatum of area CA1. Field EPSPs were evoked by monophasic stimulation (200  $\mu$ s 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  $\mu$ A) 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  $\mu$ M 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 ( $\theta$ ) 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 ( $\pm$ 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<sup>2</sup> 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  $\mu$ M TTX for 15 min, labeled with anti-GluR<sub>1</sub> N-terminal antibody (1:10; Calbiochem, La Jolla, CA) for 15 min, followed by treatment with 50  $\mu$ M CCh or water ( $\pm$ 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<sub>1</sub> 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<sub>1</sub>. For surface GluR<sub>1</sub> experiments, cells were pretreated with 1  $\mu$ M TTX ( $\pm$  anisomycin where indicated) for 20 min followed by treatment with 50  $\mu$ M 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<sub>1</sub> N-terminal antibody (1:50; 1 h, RT) washed, and then treated with Alexa Fluor 2° antibody. For M<sub>1</sub> mAChR staining, cultures were fixed as described above and treated with anti-M<sub>1</sub> mAChR antibody (1:100; Millipore, Billerica, MA).

Fluorescence was detected using a Nikon (Tokyo, Japan) TE2000 in-

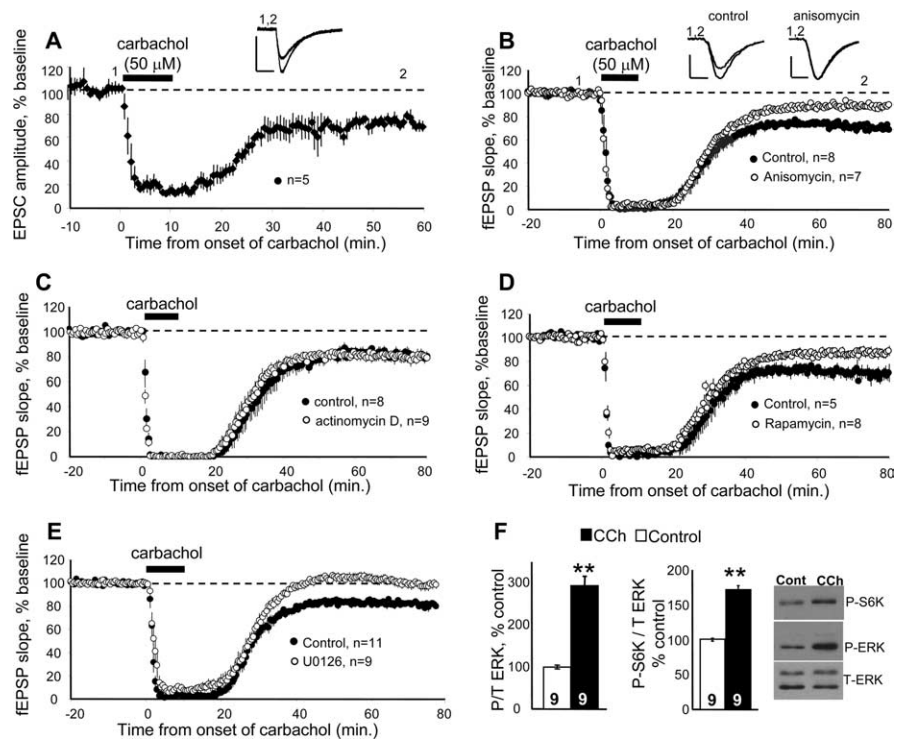
verted microscope equipped with a cooled CCD camera (Roper CoolSnapHQ). Images were analyzed and quantitated using MetaMorph software (Universal Imaging, West Chester, PA). For synaptic staining, immunoreactive puncta are defined as discrete points along a dendrite (within 50  $\mu\text{m}$  from the 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  $\pm$  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<sub>2</sub>/5% CO<sub>2</sub>. 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  $-80^{\circ}\text{C}$  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), EF1 $\alpha$  (1:10,000; Upstate Biotechnology, Lake Placid, NY),  $\alpha$ Ca<sup>2+</sup>/calmodulin-dependent protein kinase II ( $\alpha$ 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. 1F) or by a one-sample  $t$  test as percentage control (see Fig. 6E,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  $\mu\text{M}$ ; 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, we performed whole-cell voltage clamp (at  $-60$  mV) recordings of CA1 pyramidal neurons in rat acute hippocampal slices. After a stable baseline of EPSC amplitudes was established, CCh application (50  $\mu\text{M}$ ; 10 min) induced a robust, acute depression followed by a long-lasting depression of EPSCs

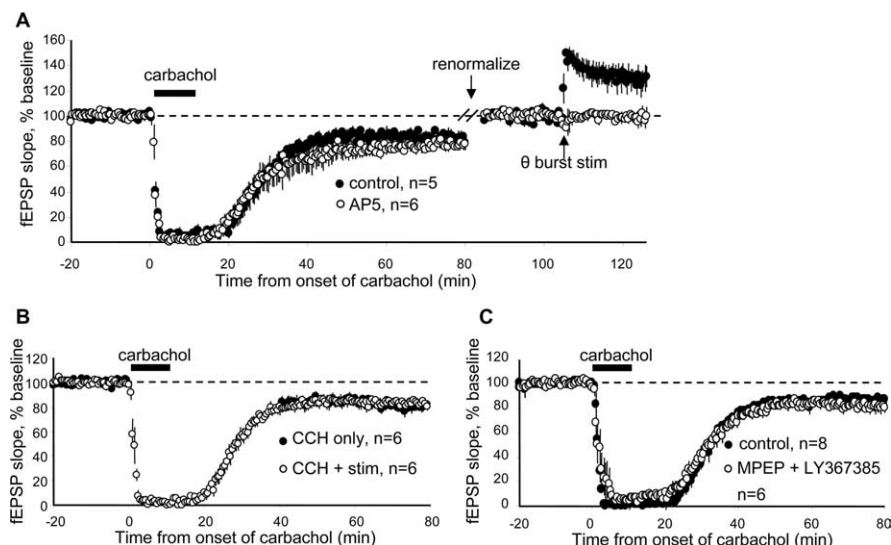


**Figure 1.** Muscarinic acetylcholine receptor-induced long-term synaptic depression requires protein synthesis, ERK, and mTOR activity. **A–E**, Application of the muscarinic acetylcholine receptor agonist CCh (50  $\mu\text{M}$ , 10 min) to acute hippocampal slices from mature rats (P25–P41) induces a long-term depression of EPSCs (**A**) and fEPSPs (**B–E**). Plotted are average ( $\pm$  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  $\pm$  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  $\mu\text{M}$ ) significantly reduces CCh-induced LTD (control, 70  $\pm$  2%; anisomycin, 87  $\pm$  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 transcription inhibitor actinomycin D (25  $\mu\text{M}$ ) has no effect on CCh-LTD [control (0.1% DMSO), 81  $\pm$  4%; actinomycin D, 80  $\pm$  3%;  $p = 0.8$ ]. **D**, The mTOR antagonist rapamycin (20 nM) reduces CCh-induced LTD [control (0.1% DMSO), 69  $\pm$  5%; rapamycin, 87  $\pm$  3%;  $p < 0.01$ ]. **E**, The MEK inhibitor U0126 (20  $\mu\text{M}$ ) blocks CCh-induced LTD [control (0.1% DMSO), 82  $\pm$  2%; U0126, 99  $\pm$  3%; control vs U0126,  $p < 0.001$ ; U0126 1 h after CCh washout vs U0126 baseline,  $p = 0.7$ ]. **F**, Acute rat hippocampal slices (CA3 removed) were treated  $\pm$  10 min with 50  $\mu\text{M}$  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  $\pm$  2%; CCh, 171  $\pm$  6%;  $**p < 0.001$ ). Left panel, Quantification of CCh-induced ERK activation (P-ERK/T-ERK, % control) in nine slices (control, 100  $\pm$  5%; CCh, 293  $\pm$  23%;  $**p < 0.001$ ).

(Fig. 1A). CCh also induced a trend toward a long-term increase in input resistance (baseline  $R_m$ , 190  $\pm$  28 M $\Omega$ , 60 min after CCh;  $R_m$ , 244  $\pm$  39 M $\Omega$ ,  $p = 0.09$ ) consistent with the findings that M<sub>1</sub> 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  $\mu\text{M}$ ). Consistent with previous studies, LTD was induced in interleaved control slices with the general cholinergic agonist CCh (50  $\mu\text{M}$ ; 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. 1B). Similarly, preincubation of slices with a mecha-





**Figure 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  $\mu$ M) before CCh had no effect on LTD (control,  $82 \pm 5\%$ ; AP-5,  $77 \pm 2\%$ ;  $p = 0.3$ ). AP-5 was effective in blocking LTP induction with a theta-burst stimulation ( $\theta$ ;  $\uparrow$ ) delivered after CCh-LTD induction (measured 25 min after theta burst; control,  $129 \pm 7\%$ ; AP-5 =  $99 \pm 1\%$ ;  $\theta$  in AP-5,  $p = 0.77$  vs baseline;  $\theta$  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,  $83 \pm 2\%$ ; CCh no stim,  $80 \pm 3\%$ ;  $p = 0.4$ ). **C**, Blockade of mGluR5 and mGluR1, with MPEP (10  $\mu$ M) and LY367385 (100  $\mu$ M), respectively, had no effect on LTD induced with CCh (control,  $87 \pm 1\%$ ; MPEP + LY,  $81 \pm 4\%$ ;  $p = 0.2$ ).

nistically different protein synthesis inhibitor, cycloheximide (60  $\mu$ M), blocked CCh-LTD (LTD measured 75 min after CCh washout; control,  $78 \pm 3\%$ ,  $n = 6$ ; cycloheximide,  $94 \pm 4\%$ ;  $p = 0.001$ , control vs cycloheximide;  $p = 0.1$ , cycloheximide baseline vs 75 min after CCh washout). These results reveal that newly synthesized proteins are required for mAChRs to establish LTD. To determine whether CCh-LTD requires transcription of new mRNAs, we pretreated slices in the transcription inhibitor actinomycin D (25  $\mu$ M). Interleaved control slices were treated with vehicle (0.1% DMSO). CCh-LTD is unaffected by actinomycin D (Fig. 1C), 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 Dufner and Thomas, 1999; Kelleher et al., 2004a; 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  $\mu$ M U0126). Interleaved control slices were incubated in vehicle (0.1% DMSO). Rapamycin reduced the magnitude of CCh-LTD, similar to anisomycin (Fig. 1D), and U0126 completely blocked CCh-LTD (Fig. 1E). Consistent with a role for ERK and mTOR in CCh-LTD, treatment of hippocampal slices with CCh (50  $\mu$ M; 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. 1F) (Dufner and Thomas, 1999; 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  $\mu$ M). Normal levels of CCh-LTD were observed in AP-5 compared with interleaved control slices (Fig. 2A). AP-5 was active in the slice preparation, because long-term potentiation (LTP) was blocked in all slices subsequent to mAChR-LTD (Fig. 2A,  $\theta$ -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. 2B, 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. 2B). 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).

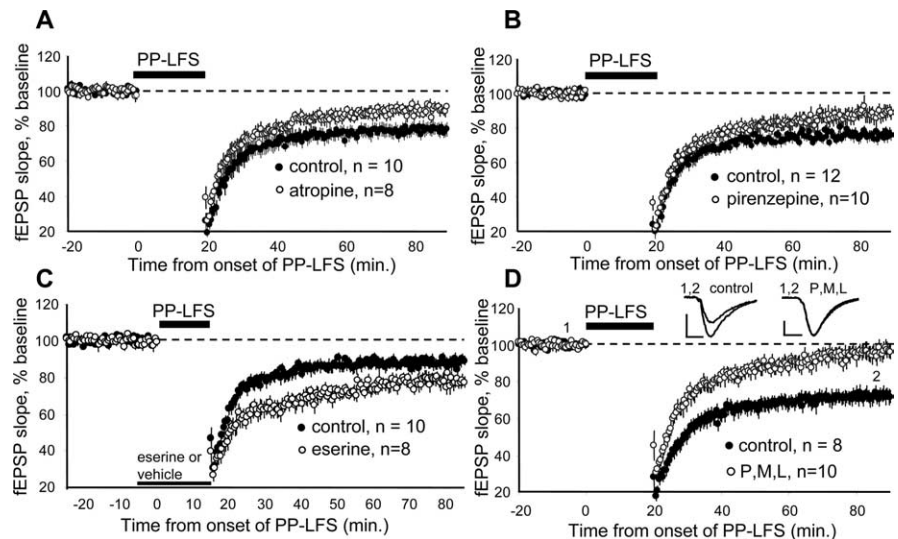
The Gq-coupled group 1 mGluRs induce LTD in CA1, which relies on rapid protein synthesis (Huber et al., 2000). Therefore, mAChRs may induce protein synthesis-dependent LTD indirectly by enhancing neuronal excitability, glutamate release, and activation of group 1 mGluRs (Benardo and Prince, 1982). To test whether group 1 mGluR activity was required for CCh-LTD, slices were preincubated in mGluR1 and mGluR5 antagonists, LY367385 (100  $\mu$ M) and MPEP (10  $\mu$ M), 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. 2C). 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 $M_1$ mAChRs induces protein synthesis-dependent LTD

We 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 acetyl-

choline 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  $\mu$ M) to prevent induction of NMDA receptor-dependent LTD and isolate G $\alpha$ q-dependent LTD. Both the broad range mAChR antagonist atropine (5  $\mu$ M) (Fig. 3A) and the M<sub>1</sub>-selective antagonist pirenzepine (Dorje et al., 1991; Scheiderer et al., 2006) (75 nM) (Fig. 3B) significantly reduced LTD induced with PP-LFS. To implicate endogenous release of acetylcholine in LTD induction, we applied the acetylcholine esterase inhibitor eserine (2  $\mu$ M) during PP-LFS to enhance acetylcholine concentrations in the slice (Fig. 3C). 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;  $98 \pm 2\%$ ;  $p = 0.54$  compared with baseline). Antagonism of M<sub>1</sub> 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 G $\alpha$ q 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, we observe that combined application of an M<sub>1</sub> mAChR selective antagonist (75 nM pirenzepine; “P”) and group I mGluR antagonists (mGluR<sub>5</sub>-specific antagonist MPEP; “M”; 10  $\mu$ M and mGluR<sub>1</sub>-specific antagonist LY367385; “L”; 100  $\mu$ M) blocks PP-LFS induced LTD (P,M,L) (Fig. 3D). Because PP-LFS induced LTD is unaffected by group I mGluR antagonists alone (Volk et al., 2006), but completely blocked by the addition of pirenzepine (Fig. 3D), this indicates that synaptic activation of M<sub>1</sub> mAChRs is sufficient to induce the full level of protein synthesis-dependent LTD. It is only when M<sub>1</sub> mAChRs are blocked that group I 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, we performed an occlusion experiment (Fig. 4A). 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. 4A). 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

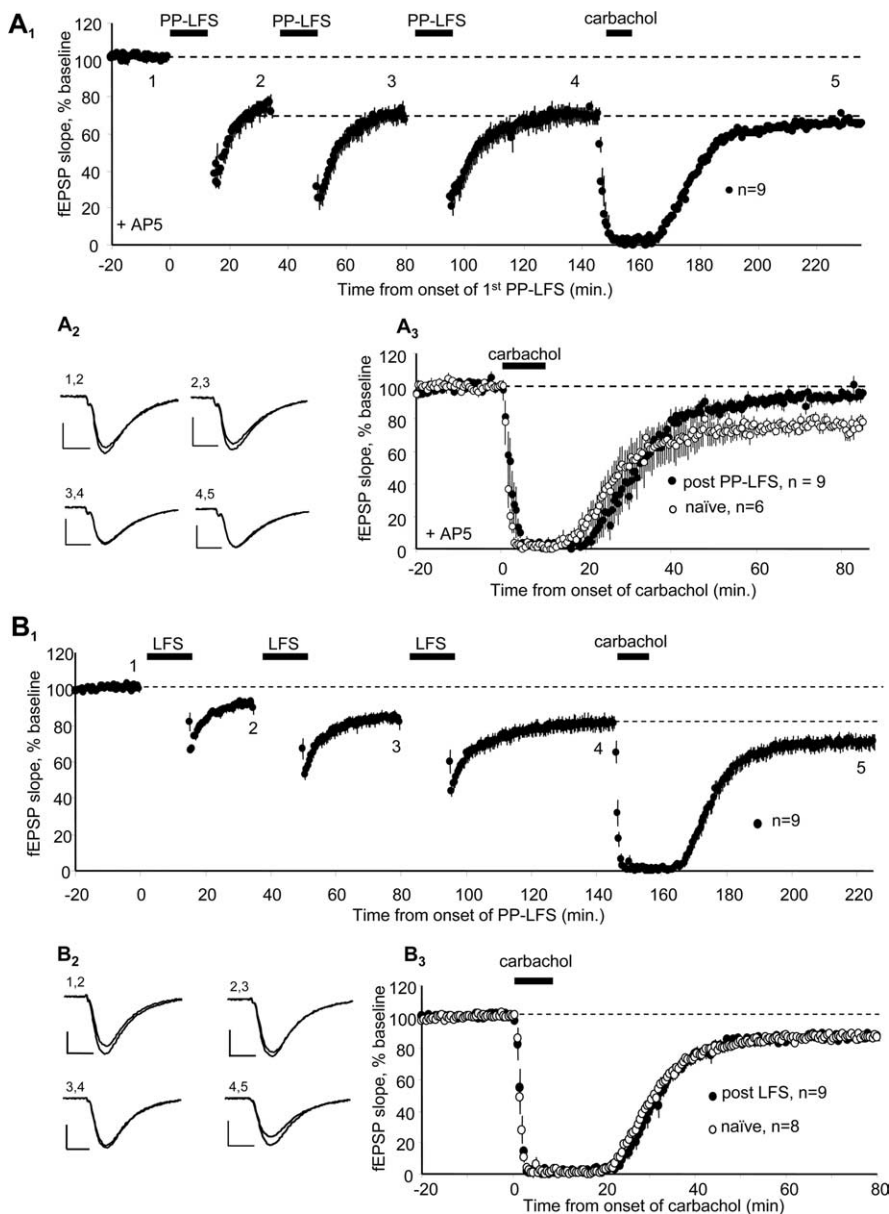


**Figure 3.** Synaptically induced protein synthesis-dependent LTD requires M<sub>1</sub> mAChR activation. All experiments are in the presence of the NMDA receptor antagonist D,L-AP-5 (100  $\mu$ M). **A**, Blockade of mAChRs with the nonselective muscarinic antagonist atropine (5  $\mu$ M) reduces 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,  $78 \pm 3\%$ ; atropine,  $90 \pm 2\%$ ;  $p = 0.01$ ). **B**, Blockade of the M<sub>1</sub> subtype of mAChRs with the M<sub>1</sub>-selective antagonist pirenzepine (75 nM) reduces PP-LFS induced LTD (measured 1 h after PP-LFS; control,  $75 \pm 3\%$ ; pirenzepine,  $88 \pm 4\%$ ;  $p < 0.01$ ). **C**, Brief application of the acetylcholine esterase inhibitor eserine (2  $\mu$ M; 5 min before and during PP-LFS) facilitated LTD induced with PP-LFS (15 min) (measured 70 min after PP-LFS; control,  $88 \pm 2\%$ ; eserine,  $78 \pm 3\%$ ;  $p = 0.02$ ). **D**, PP-LFS-induced LTD is blocked by combined antagonism of M<sub>1</sub> mAChRs (75 nM pirenzepine) and group I mGluRs (mGluR<sub>5</sub>-specific antagonist MPEP at 10  $\mu$ M plus mGluR<sub>1</sub>-specific antagonist, LY367385 at 100  $\mu$ M), “P,M,L” (measured 1 h after PP-LFS control,  $72 \pm 4\%$ ; “P,M,L”,  $96 \pm 4\%$ ; control vs “P,M,L”,  $p < 0.001$ ; “P,M,L” at 85–90 min vs baseline,  $p = 0.37$ ).

CCh (Fig. 4B) 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 our assertion that M<sub>1</sub> mAChRs and group I mGluRs converge on a common, protein synthesis-dependent LTD expression mechanism, which is distinct from LTD induced by activation of NMDARs.

### M<sub>1</sub> 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? We 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. 5A–D). Because our cultures do not contain the medial septum, the major cholinergic projection to the hippocampus, we first confirmed that the cultured neurons expressed M<sub>1</sub> muscarinic receptors (Fig. 5E). Cultures were treated with CCh (50  $\mu$ M; 10 min) in the presence of TTX (1  $\mu$ M) to prevent action potential-induced glutamate release. CCh application to hippocampal neurons in culture caused an increase in endocytosis of the AMPAR subunit GluR<sub>1</sub>, which was blocked by the M<sub>1</sub> mAChR selective antagonist, pirenzepine (75 nM) (Fig. 5A,B). The endocytosis of GluR<sub>1</sub> in response to CCh resulted in decreases in GluR<sub>1</sub> surface expression at 10 and 60 min after CCh application (Fig. 5C,D). Like CCh-LTD, the persistent decrease in GluR<sub>1</sub> surface expression required protein synthesis, because it was blocked by anisomycin (25  $\mu$ M) (Fig. 5C,D). These results suggest that the newly synthesized proteins maintain LTD through a persistent decrease in AMPAR surface expression and



**Figure 4.** Saturation of synaptically induced, Gq-dependent LTD occludes CCh-induced LTD. **A<sub>1</sub>**, Three episodes of PP-LFS (15 min) delivered to Schaffer collateral axons in the presence of D,L-AP-5 (100  $\mu$ M) saturated LTD (LTD after second,  $70 \pm 4\%$ , and third PP-LFS,  $69 \pm 4\%$ , episode were not different;  $p = 0.86$ ). LTD induced by CCh after saturation of PP-LFS-induced LTD is dramatically reduced. **A<sub>2</sub>**, fEPSP waveforms (average of 4–6 traces) from a representative experiment are taken at the time points indicated on the graph in **A<sub>1</sub>**. **A<sub>3</sub>**, 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<sub>1</sub>**; measured 1 h after CCh, naive,  $77 \pm 2\%$ ; CCh LTD after PP-LFS saturation,  $93 \pm 2\%$ ;  $p < 0.001$ ). **B<sub>1</sub>**, Three episodes of LFS (15 min) delivered to Schaffer collateral axons saturated LTD (LTD after second,  $84 \pm 2\%$ , and third PP-LFS,  $81 \pm 3\%$ , episodes were not different;  $p = 0.1$ ). Stimulation of mAChRs with CCh induced normal levels of LTD after LFS saturation. **B<sub>2</sub>**, fEPSP waveforms (average of 4–6 traces) from a representative experiment are taken at the time points indicated on the graph in **B<sub>1</sub>**. **B<sub>3</sub>**, 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<sub>1</sub>**; naive,  $87 \pm 1\%$ ; CCh LTD after LFS saturation,  $86 \pm 1\%$ ;  $p = 0.1$ ).

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, we evaluated CCh-LTD in the mouse model of FXS, *Fmr1* KO mice. We observed that CCh-LTD is enhanced in *Fmr1* KO mice compared with wild-type (WT) mice (Fig. 6A). Although the magnitude of CCh-induced LTD is smaller in WT mice compared with rats (Fig. 1), it is still blocked by anisomycin (Fig. 6B). In contrast, CCh-LTD no longer requires protein synthesis in *Fmr1* KO mice (Fig. 6C,D). Gq-dependent LTD induced by chemical activation of group 1 mGluRs or PP-LFS is similarly affected in *Fmr1* KO mice (Fig. 6D) (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, we investigated whether mAChRs stimulate the rapid synthesis of FMRP as well as FMRP target mRNA, elongation factor 1 $\alpha$  (EF1 $\alpha$ ) (Sung et al., 2003). Acute hippocampal slices from WT mice were treated with CCh (50  $\mu$ M; 10 min) and quantitative Western blotting was performed on homogenates of CA1 for FMRP and EF1 $\alpha$ . CCh stimulates a rapid (10 min) increase in FMRP and EF1 $\alpha$  levels, which was blocked by anisomycin (Fig. 6E). To determine whether FMRP is required for mAChR-dependent protein synthesis, we measured CCh-induced synthesis of EF1 $\alpha$  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 EF1 $\alpha$  and CaMKII protein in WT mice, which was not observed in *Fmr1* KO littermates (Hou et al., 2006) (Fig. 6F). In support of previous studies, we observed a trend toward an increase in EF1 $\alpha$  and  $\alpha$ CaMKII levels in isolated CA1 slices of *Fmr1* KO mice (EF1 $\alpha$ ,  $124 \pm 20\%$  of WT;  $p = 0.27$ ;  $\alpha$ CaMKII,  $136 \pm 23\%$  of WT;  $p = 0.14$ ) (Sung et al., 2003; Zalfa et al., 2003; Hou et al., 2006). These

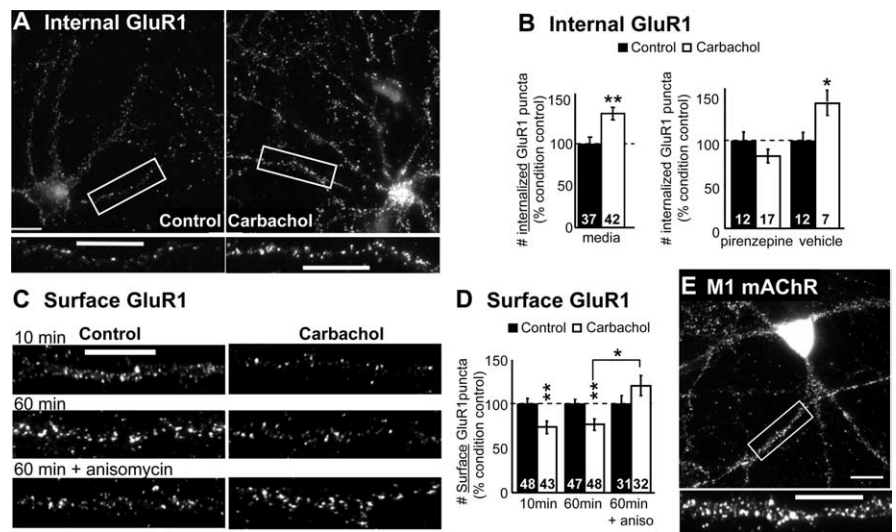


data demonstrate that mAChRs regulate translation of FMRP as well as FMRP target mRNAs. In turn, FMRP regulates mAChR induced protein synthesis and LTD. Our results suggest that Gq-coupled and protein synthesis-dependent plasticity is generally elevated and misregulated in FXS and may contribute to the cognitive deficits associated with the disease.

## Discussion

Here, we 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<sub>1</sub> mAChRs elicits protein synthesis-dependent LTD, and these forms of LTD occlude each other (Figs. 1, 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. 6) (Weiler et al., 1997, 2004; Huber et al., 2002; Koekkoek et al., 2005; Hou et al., 2006). We 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 <sup>3</sup>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, we demonstrate that pharmacological or synaptic activation of M<sub>1</sub> mAChRs (Fig. 1B) induces LTD, which relies on rapid (~20 min) protein synthesis from pre-existing mRNA (Figs. 1, 3) (Huber et al., 2000; Volk et al., 2006). Our 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). We 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. 1D–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.,

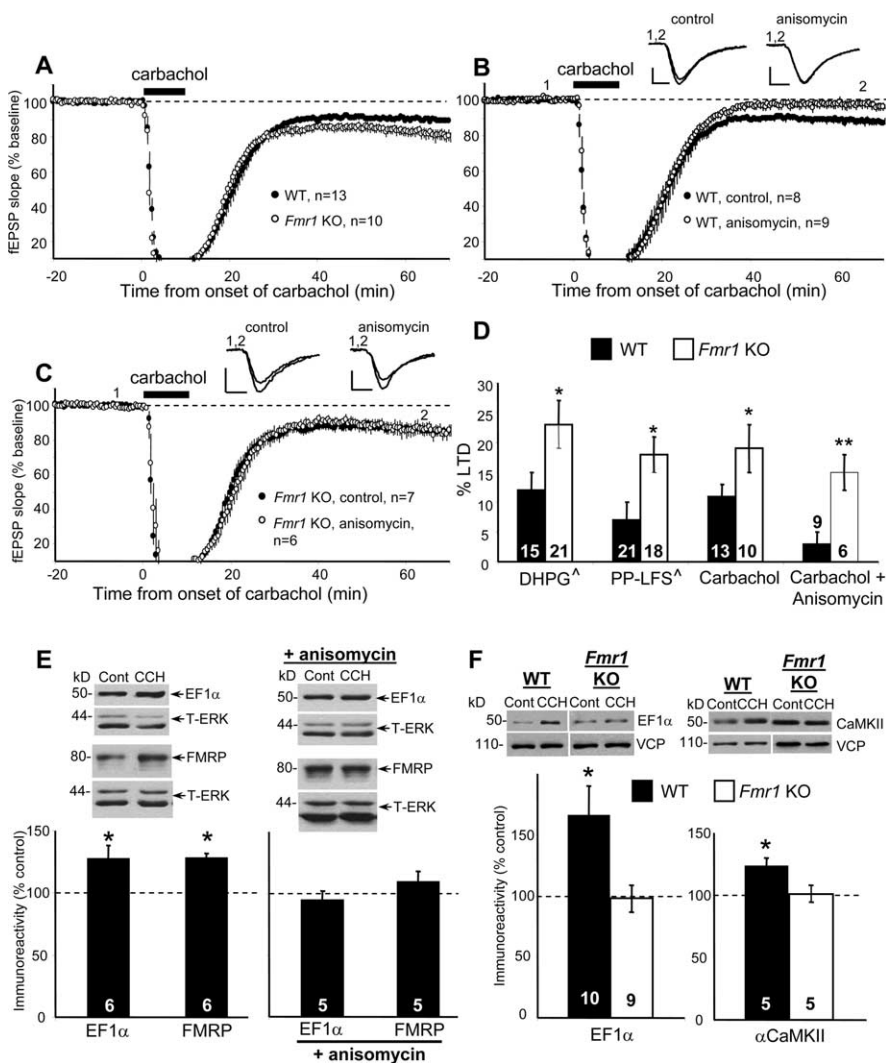


**Figure 5.** Activation of M<sub>1</sub> muscarinic acetylcholine receptors induces endocytosis and long-term, protein synthesis-dependent decreases of surface GluR<sub>1</sub>. **A**, Representative experiment in which internalized GluR<sub>1</sub> was labeled on live dissociated cultured hippocampal neurons using N-terminal GluR<sub>1</sub> antibody (see Materials and Methods). CCh (50 μM; 10 min) treatment increases internalized GluR<sub>1</sub>. Scale bar, 15 and 10 μm. **B**, CCh increases the number of internalized GluR<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> puncta reveals that CCh induces decreases in surface GluR<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> mAChRs are expressed and punctate on cultured hippocampal pyramidal neurons. Scale bar, 15 and 10 μm.

2007). Our data imply that similar translation initiation factors are regulated by mAChRs, which lead to LTD (Fig. 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).

Our findings 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<sub>1</sub> mAChRs also facilitate induction of NMDAR-dependent LTD by activation of phospholipase C and release of Ca<sup>2+</sup> 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 we describe 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 synapti-



**Figure 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 1 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 EF1α 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 (EF1α, 131 ± 10%; FMRP, 128 ± 4%; EF1α + 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 EF1α and αCaMKII 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 EF1α (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 αCaMKII (WT: 123 ± 6%, \**p* < 0.05; KO: 101 ± 6%, *p* = 0.9; WT vs KO: *p* < 0.05).

cally 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, we find that coapplication of M<sub>1</sub> mAChR and group I mGluR antagonists completely blocks PP-LFS-induced LTD, indicating that both receptor types contribute to LTD, and M<sub>1</sub> mAChRs fully induce LTD in group 1 mGluR blockade (Fig. 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. 4, 7A) (Huber et al., 2001).

Our data reveal that the newly synthesized proteins required for CCh-induced LTD function to maintain decreases in surface AMPAR expression (Fig. 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; Moulton 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. 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). We 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, we find that mAChR induced synthesis of EF1α and CaMKII are absent in *Fmr1* KO mice (Fig. 6E), 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). We hypothesize 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. 6, 7B) (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., 2004a,b; Elvander et al., 2004) (for review, see Hasselmo, 2006). Therefore, antagonism of M<sub>1</sub> mAChRs may actually facilitate cognitive function in FXS, alone or in combination with group 1 mGluR antagonists.

Here, we 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  $\alpha 1$  adrenergic and 5-HT<sub>2</sub> 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. 3A–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.

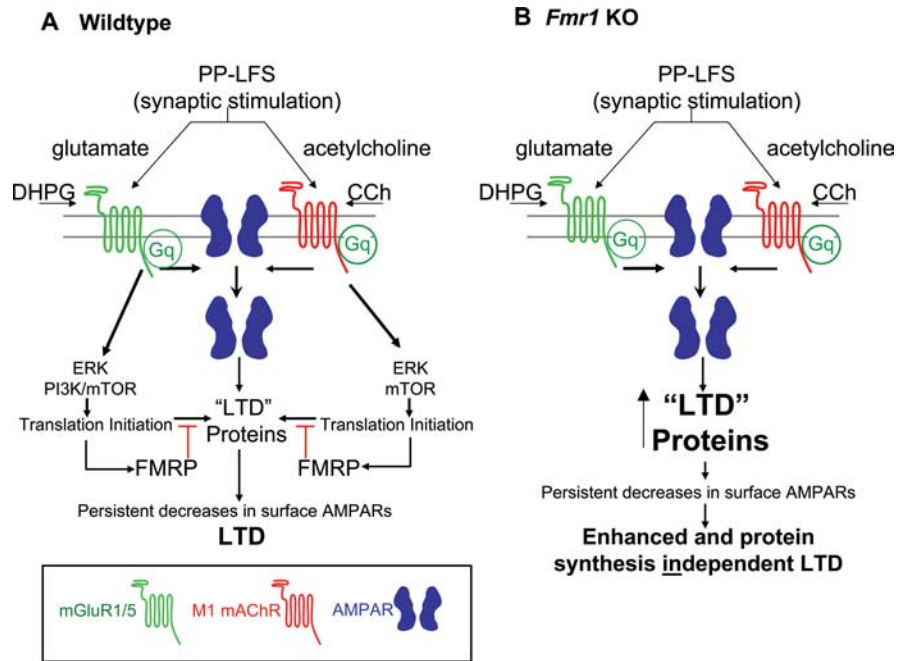
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**Figure 7.** Working model of Gq-dependent LTD in wild-type rodents and *Fmr1* KO mice. **A**, In wild-type rodents, paired pulses of low frequency electrical stimulation (PP-LFS) to Schaffer collateral axons activates both group 1 mGluRs (mGluR<sub>1</sub> and mGluR<sub>5</sub>) and mAChRs to induce LTD. Pharmacological stimulation of group 1 mGluRs (with DHPG) or M<sub>1</sub> 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.

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