

Presynaptic m1 muscarinic receptors are necessary for mGluR long-term depression in the hippocampus

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To investigate the role of M1 muscarinic acetylcholine receptors (m1 receptors) in metabotropic glutamate receptor (mGluR)-mediated long-term depression (LTD), we produced mouse lines in which deletion of the m1 gene is restricted to the forebrain (FB–m1KO) or hippocampal CA3 pyramidal neurons (CA3–m1KO). Stimulation in FB–m1KO hippocampal slices resulted in excitatory postsynaptic potentials and long-term synaptic plasticity (long-term potentiation and LTD) similar to controls. The mice were deficient in (S)-3,5-dihydroxyphenylglycine hydrate (DHPG)-induced mGluR LTD, which correlated with a presynaptic increase in the release of neurotransmitters. Protein kinase C (PKC) activity, which is downstream from both mGluRs and m1 receptors, was reduced in CA3 but not in CA1. The presynaptic requirement of m1 receptors was confirmed by the lack of DHPG-induced mGluR LTD in the CA1 of slices from CA3–m1KO mice. mGluR LTD was rescued by stimulating PKC activity pharmacologically in CA3–m1KO mice. These data confirm a role for PKC activation in presynaptic induction of mGluR LTD and distinguish between the roles of mGluRs and m1 receptors.

m1 receptor | neuromodulation | synaptic plasticity | protein kinase C | fragile X

M1 muscarinic acetylcholine receptors (m1 receptors) and group I metabotropic glutamate receptors (mGluRs) are expressed in hippocampal pyramidal neurons, activate protein kinase C (PKC) via a “q” type G protein (Gq)-coupled cascade (1–3), and have been implicated in inducing and modulating plasticity in CA3–CA1 synapses. The nature of the interaction between these two overlapping signal pathways is unknown. Although ionotropic *N*-methyl-D-aspartate (NMDA) receptors have been most prominently implicated in the phenomena of long-term potentiation (LTP) and long-term depression (LTD), changes in synaptic efficacy can also be mediated by metabotropic-receptor-mediated processes. G-protein-coupled receptors (such as m1 receptors and mGluRs) can change synaptic efficacy when stimulated and can also act as neuromodulators by changing synaptic responses to other parallel stimuli (1).

Stimulating mGluRs by perfusing the agonist (S)-3,5-dihydroxyphenylglycine hydrate (DHPG) causes a long-lasting decrease in synaptic efficacy (mGluR LTD) (4–6). Similarly, muscarinic agonists also produce transient or long-lasting depression in synaptic efficacy (6, 7). These muscarinic effects vary with the concentration of the agonists used and may be the result of the simultaneous activation of different kinds of muscarinic receptors at distinct synaptic locations (8). It is still not clear which cholinergic receptors mediate cholinergic LTD and whether they act presynaptically or postsynaptically. It is also not clear what the relationship is between mGluR LTD and cholinergic LTD. Recently, Volk et al. (6) have shown that mGluR LTD can be blocked by applying a muscarinic antagonist and that LTD produced by a muscarinic agonist occludes mGluR LTD. They concluded that mGluR LTD and muscarinic LTD are the same process and that mGluRs are required for LTD only when m1 muscarinic receptors are blocked.

The mechanism of mGluR LTD has been extensively studied, it can be induced while NMDA receptor activity is blocked, it does

not require calcium (9), and it is occluded by LTD induced by a train of paired pulses (10). There is no agreement on the site of induction or maintenance of mGluR LTD, and conflicting results suggest both presynaptic (5, 11, 12) or postsynaptic (13, 14) mechanisms of expression, although participation of the presynaptic side in the induction phase of mGluR LTD can be consistent with all of the above studies. Although this interpretation of the literature is congruent with our findings, it is not the aim and scope of this work to study the presynaptic versus postsynaptic mechanisms for mGluR LTD exhaustively or extensively but to focus on the role of m1 in this type of synaptic plasticity.

There is also disagreement regarding the role of PKC in mGluR LTD, although several groups have shown that PKC activity is necessary for mGluR LTD in several brain regions, including CA1 (3, 15). It was also demonstrated that high-frequency-induced PKC activation inhibited synaptically induced mGluR LTD in the dentate gyrus (16, 17).

To study the specific role of m1 receptors in mGluR LTD, we generated two conditional KO mouse lines using the Cre/Lox system with Cre expression driven by the EMX1 promoter or the KA-1 promoter. The EMX1 promoter is active in excitatory neurons of the forebrain, including the hippocampus, but not in the striatum (18) whereas the KA-1 promoter is active in hippocampal CA3 but not in CA1 pyramidal cells. We found that these mice are deficient in mGluR LTD and were able to trace this deficiency to a lack of m1 receptors in presynaptic CA3.

Results

M1 Receptors Are Not Necessary for NMDA-Receptor-Dependent Synaptic Plasticity but Are Required for mGluR-Dependent LTD.

Mice carrying a forebrain-restricted deletion of the m1 receptor (FB–m1KO) were generated and assessed behaviorally (*SI Materials and Methods* and *Fig. S1*). To assess the role of m1 receptors on hippocampal physiology, we prepared hippocampal slices from FB–m1KO mice and recorded excitatory postsynaptic potentials (EPSPs) in CA1 stratum radiatum in response to Schaffer collateral stimulation. It has been reported previously that LTP is reduced in slices from global m1 KO mice (19). However, when we applied theta burst stimulation (TBS) to slices from FB–m1KO mice and controls, we found no effect in the KO mice: FB–m1KO EPSPs were 1.66 ± 0.13 of baseline, and controls were 1.66 ± 0.11 of baseline 60 min after stimulation ($n = 8$). The basal synaptic properties of the mutants and the controls were similar (*Fig. 1B*).

LTP and LTD in response to different frequencies of single-pulse stimulation are known to be NMDA receptor dependent (1). Low-frequency stimulation ranging from 1 to 5 Hz induces

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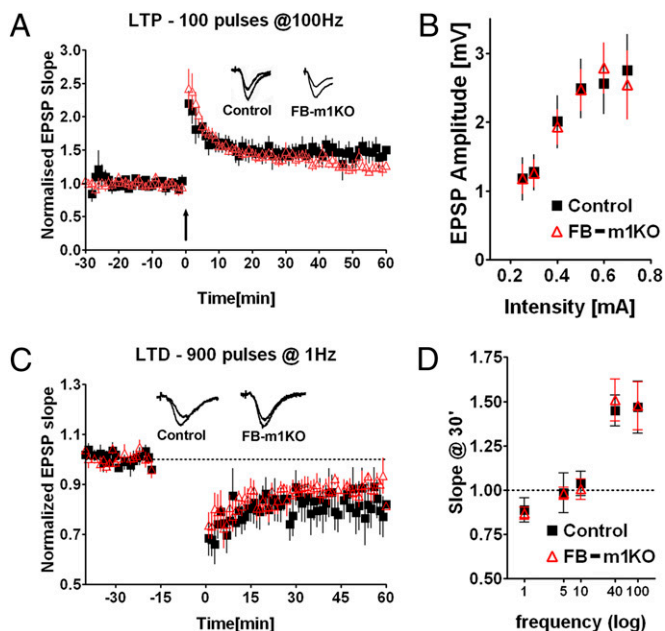


Fig. 1. Hippocampal slice physiology. (A) LTP induced by 100 Hz. (Inset) Sample traces from FB-m1KO and control mice at baseline and 60 min after tetanization. (B) Input-output relationship for SC-stimulation-induced EPSPs measured in CA1 stratum radiatum. (C) Responses of slices from FB-m1KO and control mice to low-frequency stimulation consisting of 900 pulses delivered at 1 Hz. (D) Bienenstock-Cooper-Munro (BCM) curve. Degree of change in EPSP slope 30 min after stimulation at different frequencies.

LTD, whereas frequencies above 10 Hz induce LTP. The responses of slices from FB-m1KO mice were similar to controls in all frequencies tested, including LTP to 100 Hz stimulation (Fig. 1, A and D) and LTD to 1 Hz stimulation (Fig. 1, C and D).

We next examined a possible effect of the m1 receptor KO on mGluR-dependent LTD. To this end, we perfused the mGluR agonist DHPG on hippocampal slices (Fig. 2A) and found that DHPG produced LTD in slices from control but not from FB-m1KO mice. EPSPs in control slices were 0.72 ± 0.05 of baseline 60 min after DHPG perfusion and FB-m1KO were 0.94 ± 0.05 ($n = 10$, $P < 0.05$).

The lack of m1 receptor activity can produce both acute and long-lasting changes. Therefore, we perfused DHPG in the presence of the muscarinic antagonist pirenzepine (6). EPSPs 60 min after DHPG perfusion with pirenzepine were 0.9180 ± 0.067 of baseline ($n = 20$) compared to 0.7015 ± 0.069 ($n = 9$) in interleaved slices perfused with DHPG alone ($P < 0.05$, Fig. 2B).

To determine whether a presynaptic component was affecting responses to DHPG, we applied paired pulses at varying intervals and monitored the degree of facilitation of the second response, which was lower for FB-m1KO mice ($n = 10$, $P < 0.05$; two-way ANOVA indicated genotype as a significant source of variation; $F = 9.821$). Lower paired-pulse facilitation could indicate that synaptic vesicles are released at a higher probability, making the additive effect of the second pulse on presynaptic calcium concentrations less effective in increasing the amount of released neurotransmitter. To verify this interpretation, we applied paired pulses in the presence of a higher calcium concentration (Fig. 3A, $n = 3$). We found that increasing the concentration of extracellular calcium from 2.5 to 5 mM affected only control slices. Taken together, these results suggested that a lack of m1 receptors in the hippocampus resulted in an increase in release probability in the Schaffer collaterals, which changed the synaptic response to mGluR stimulation in such a way that LTD did not occur.

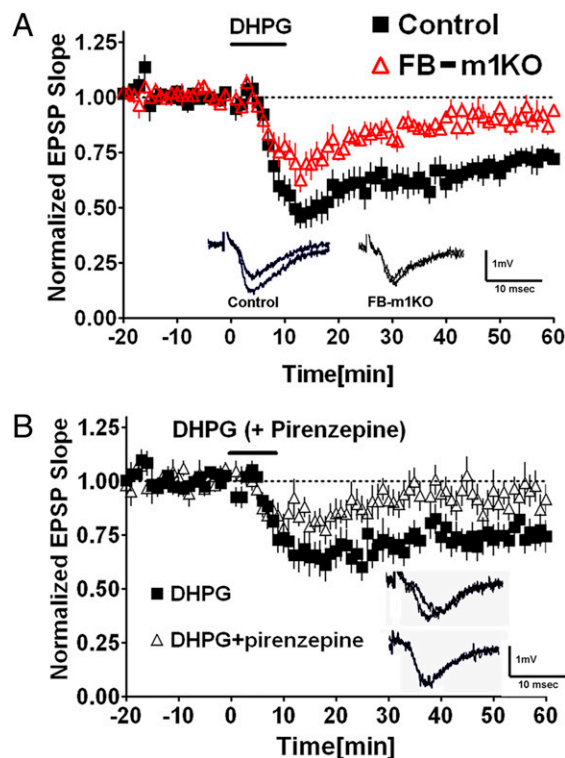


Fig. 2. mGluR LTD requires m1 receptors. (A) Slices from FB-m1KO and control mice were perfused for 10 min (horizontal black bar) with DHPG at a concentration of 100 μ M. (B) Slices from control mice were perfused with either DHPG alone (solid squares) or DHPG together with the muscarinic antagonist pirenzepine (10 μ M, open triangles).

To further establish the importance of m1 receptors in maintaining a normal level of synaptic release in Schaffer collaterals, we used patch-clamp technology to record miniature excitatory postsynaptic currents (minis) from CA1 pyramidal neurons. The amplitude of the minis from FB-m1KO neurons was similar to controls (37 ± 4 and 44 ± 2 pA, respectively; Fig. 3C), demonstrating similar levels of postsynaptic receptors. Whereas the frequency of events was significantly higher in FB-m1KO, 26.8 ± 8 minis/5-min recording ($n = 10$ cells, 8 mice) compared to 14.6 ± 1.5 in controls ($n = 23$ cells, 14 mice, $P < 0.05$; Fig. 3D). These results confirm that deletion of m1 receptors from the hippocampus results in an increase in release probability in Schaffer collaterals.

PKC Activity Is Lower in CA3 of Mutant Mice. Both m1 receptors and mGluRs are coupled to transduction elements that activate PKC (6, 20). We hypothesized that the lack of mGluR LTD observed in FB-m1KO mice may be due to a shift in PKC activity caused by the deficiency in m1 signaling. Additionally, although m1 receptors are expressed equally in all CA regions of the hippocampus, it is not known whether cholinergic stimulation of receptors results in equal PKC activation throughout. To answer these questions, we determined the level of PKC activity in mini slices cut from hippocampal slices (Fig. 4A). Under basal conditions, we found a significant decrease in PKC activity in CA3 of the FB-m1KO mice (t test; $P < 0.05$, $n =$ nine slices, three each from three mice of either genotype; Fig. 4B). Reduced PKC activity in CA3 but not in CA1 of FB-m1KO mice suggests that m1 receptors are necessary for maintaining activity of PKC in CA3 under baseline conditions. We then examined whether PKC activity is raised when hippocampal slices are incubated with DHPG under conditions that induce mGluR LTD. Treatment with DHPG resulted in a dramatic increase in PKC activity in CA3 for both genotypes (Fig. 4B,

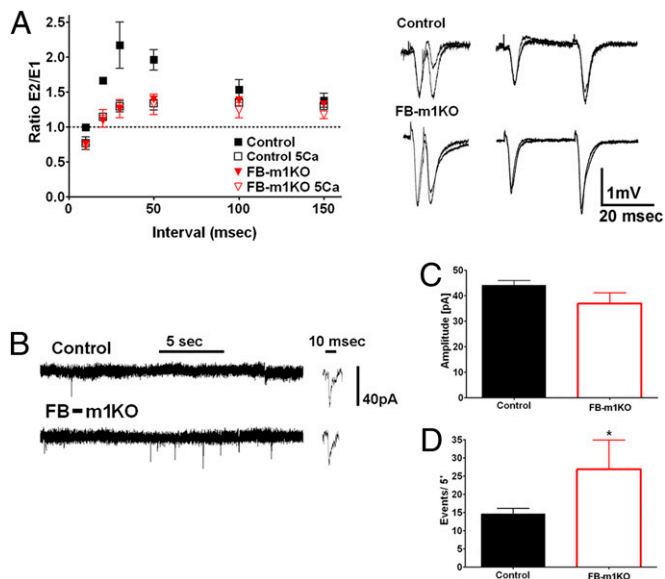


Fig. 3. Lack of m1 receptors modifies presynaptic release. (A) Paired-pulse facilitation in slices from FB-m1KO and control mice. (B) Sample traces of continuous patch-clamp recordings made from CA1 pyramidal cells in slices from control and FB-m1KO mice aged p12–15. (C) Average amplitude of minis. (D) Average frequency of measured events. Frequency is expressed as the number of minis counted per 5 min recording.

$P < 0.05$). Thus, the PKC cascade was activated in response to mGluR stimulation in CA3 in the absence of m1 receptors. The data also indicate that the larger response to mGluR activation in the hippocampus was in presynaptic CA3. This would not be expected in light of the postsynaptic change in AMPA receptor expression following mGluR LTD (4, 6). The reactions for each lysate were carried out under two conditions: first, with only the lysate as a source for PKC-activating cofactors and, second, with the addition of phospholipids and diacylglycerol to determine whether differences in activity level result from a decrease in viable PKC or from a lower level of messenger molecules in the cell produced by activation of the signaling cascade at the membrane surface. Exogenously added phospholipids increased PKC activities for all tissues. This difference was more pronounced (but not statistically significant) in the CA3 region of FB-m1KO mice that were not treated with DHPG as compared to in the CA3 region of controls (Fig. 4C), suggesting a lower intracellular level of upstream signal molecules in the mutants. Upstream signal molecules are produced by receptor activation; therefore, CA3 m1 receptors may be necessary for proper basal levels of PKC activity, which, in turn, is necessary for a correct response to mGluR activation that would promote LTD expression.

M1 Receptors Act Presynaptically in Modulating mGluR LTD. Having shown indirectly by electrophysiological and biochemical methods that m1 receptors have a presynaptic effect on mGluR LTD, we sought to demonstrate this effect directly by generating conditional m1 KO mice restricted to CA3 (CA3-m1KO).

As shown in Fig. 5, C–F, at 5 months of age, there was a significant reduction of the m1 RNA signal ($P < 0.0001$), specifically in CA3, whereas there was no indication of such reduction in CA1 (SI Materials and Methods). Slices from CA3-m1KO mice expressed normal NMDA-receptor-dependent LTD in response to 1 Hz stimulation (Fig. 6A). Thus, the postsynaptic machinery responsible for AMPA receptor distribution necessary for LTD expression was normal. Baseline activity and input/output ratios for CA3-m1KO slices were also similar to controls (Fig. 6B). mGluR LTD in response to DHPG perfusion was absent in CA3-m1KO mice, and

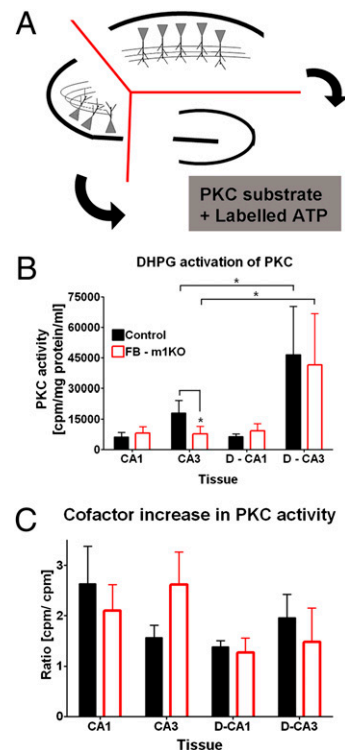


Fig. 4. PKC activity in hippocampal slices. PKC activity was assessed as described in *Materials and Methods*. (A) Schematic of the assay after incubation slices were dissected and mini slices of the CA1 and CA3 regions were homogenated and reacted with a substrate peptide in the presence of labeled ATP. (B) Under basal conditions, mini slices of the CA3 region of the hippocampi of FB-m1KO had a reduced level of PKC activity. DHPG treatment of the hippocampal slices resulted in an increase in PKC activity in the CA3 region of both genotypes. (C) PKC activity was measured in the presence of phospholipids, and the ratio of the increase in activity as compared to the control reaction is shown.

EPSPs of CA3-m1KO mice were 1.1 ± 0.09 of baseline 60 min after DHPG, which is significantly higher than controls ($P < 0.05$, Fig. 6C). Interestingly, as in FB-m1KO mice, paired-pulse facilitation in CA3-m1KO mice was also lower than in controls (Fig. 6D).

In light of our finding that the basal PKC activity is lower in CA3 of FB-m1KO mice, we tested whether DHPG-induced mGluR LTD could be rescued in slices from CA3-m1KO mice if we increased PKC activity. To this end, we perfused a PKC activator 12-*O*-tetradecanoylphorbol 13-acetate (PMA) onto CA3-m1KO slices before perfusing DHPG. We found that perfusing $10 \mu\text{M}$ PMA for 20 min immediately before perfusing DHPG resulted in LTD (Fig. 6E). Interestingly, the same pretreatment with PMA reduced DHPG-induced mGluR LTD in control slices (Fig. 6E).

Discussion

Acetylcholine and glutamate activate transduction cascades that increase the activity of PKC via Gq proteins coupled to m1 receptors and mGluRs, respectively (1). Both receptor types are present in pyramidal neurons in both CA1 and CA3 of the hippocampus, and stimulating either of these receptors can induce LTD (2, 3). Until recently (6), research has focused on the effects of stimulating only one of these pathways without considering the downstream convergence of cholinergic and glutamatergic signals on PKC. Although several groups have shown that PKC activity is necessary for mGluR LTD in several brain regions, including CA1 (3, 15), it was also demonstrated that high-frequency-induced PKC activation inhibited synaptically induced mGluR LTD in the dentate gyrus (16, 17), demonstrating the dependence of mGluR LTD metaplasticity on PKC.

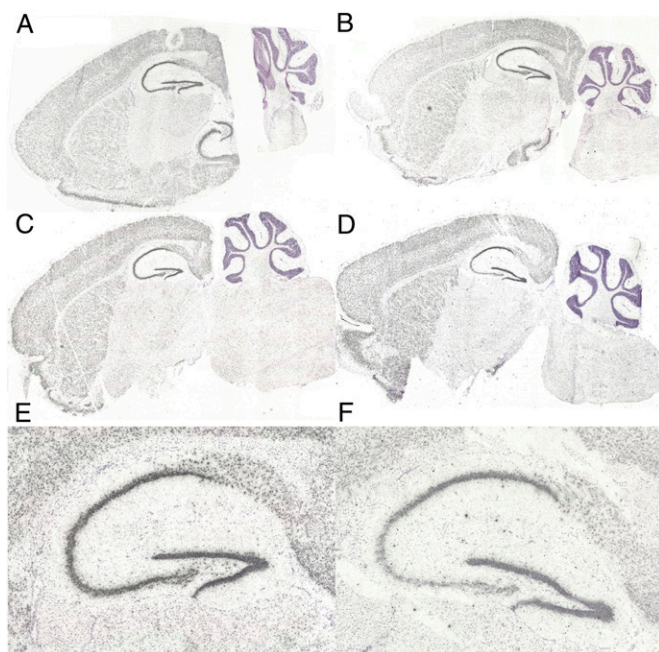


Fig. 5. CA3-specific reduction of m1 RNA. Sagittal sections of brains from CA3–m1KO and control littermates blotted by in situ hybridization with probes against m1 muscarinic receptors and counterstained with hematoxylin. (A and B) Sections taken from 3-month-old mice (A) Control. (B) CA3–m1KO. (C and D) Sections from 5-month-old mice showing a marked reduction in m1 expression (C) Control. (D) CA3–m1KO. (E and F) Magnification of the hippocampal region.

The location of mGluR LTD activation and maintenance has also been the subject of debate with some studies implicating a presynaptic expression mechanism and others implicating a postsynaptic one (5, 13, 21). To study the dynamics and location of this possible interaction, it was necessary to isolate the effects of m1-mediated signaling on the response to stimulating mGluRs by removing m1 receptors.

We report here on the generation of mice in which the m1 muscarinic receptor was knocked out specifically in excitatory neurons of the forebrain. In hippocampal slices derived from FB–m1KO mice, we found that although basal transmission and LTP at the Schaffer collateral-CA1 synapses were intact, mGluR LTD was lacking. Our finding of reduced paired-pulse facilitation in the FB–m1KO slices, which was similar to control slices under high-calcium concentrations, suggested a presynaptic mechanism for the mGluR LTD deficiency due to a change in release probability (22, 23).

We verified this in two ways. First, we showed, by patching onto CA1 pyramidal cells, that the frequency of minis was higher in FB–m1KO mice than in control mice. Second, we created a line of mice in which the m1 gene is knocked out in presynaptic CA3 pyramidal cells but is intact in postsynaptic CA1 pyramidal cells. We then demonstrated that mGluR LTD was deficient in these mice. mGluR LTD can be induced pharmacologically or synaptically (1, 3, 10), and it is still unclear how glutamate activates mGluRs, which can be both synaptic and extrasynaptic. Expression of mGluR LTD may depend on the combined effects of glutamate on mGluRs and other glutamate receptors. Thus, increased release probability would change the overall stimulation of both mGluRs and other glutamate receptors necessary for LTD expression.

To investigate the effects of m1 receptor elimination on PKC activity, we measured it in hippocampal slices. We found a reduction in PKC activity in region CA3 of the FB–m1KO mice, the region containing the cell bodies of the Schaffer collaterals.

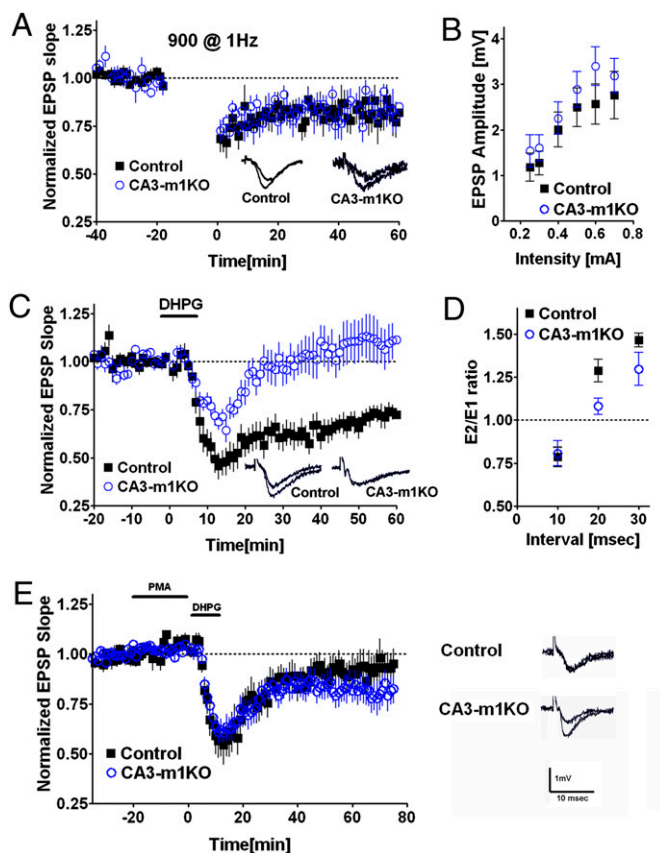


Fig. 6. Hippocampal slice physiology in CA3–m1KO mice. (A) Normal LTD in response to 1 Hz stimulation. (B) Normal input–output relationship of EPSPs. (C) CA3–m1KO slices are deficient in LTD induced by the mGluR agonist DHPG. (D) CA3–m1KO slices show decreased paired-pulse facilitation in response to Schaffer collateral stimulation. (E) Slices from CA3–m1KO mice and littermate controls were perfused with the PKC-activating agent PMA at a concentration of 10 μ M for 20 min before 10 min of perfusion of the mGluR agonist DHPG. This pretreatment resulted in rescue of the LTD phenotype in CA3–m1KO mice. In contrast, control slices did not express long-lasting depression under these conditions.

This reduced activity under basal conditions was contrasted by a normal increase in activity in response to DHPG as in the controls. On the basis of these results we hypothesized that the lack of m1 receptors in CA3 resulted in lower PKC activity, which in turn causes an increase in the probability of glutamate release from these synapses (22). This hypothesis is supported by the reversal of the LTD deficiency by a pharmacological stimulation of PKC activity before mGluR stimulation. Interestingly, control slices pretreated with a PKC activator had decreased LTD, which may have been caused by a desensitization of the mGluRs by PKC (24) in a similar manner to responses seen in the dentate gyrus (16, 17).

Our interpretation of the data are summarized schematically in Fig. 7. When m1 receptors are absent from CA3 cells (Fig. 7A), spontaneous release of vesicles is high and a transient increase in PKC activity resulting from mGluR stimulation is sufficient only for transient LTD. In contrast, under normal conditions (Fig. 7B), basal activation of m1 receptors induces a higher level of PKC activity and less spontaneous release of vesicles. Subsequent stimulation of mGluRs produces a higher level of activity, which results in longer-lasting depression that is also expressed postsynaptically by reducing the permeability of AMPA receptors (1, 6). Interestingly, acute blockade of m1 receptors with pirenzepine also blocked mGluR LTD. This bath application over the entire slices may indicate that the participation of m1 receptors in in-

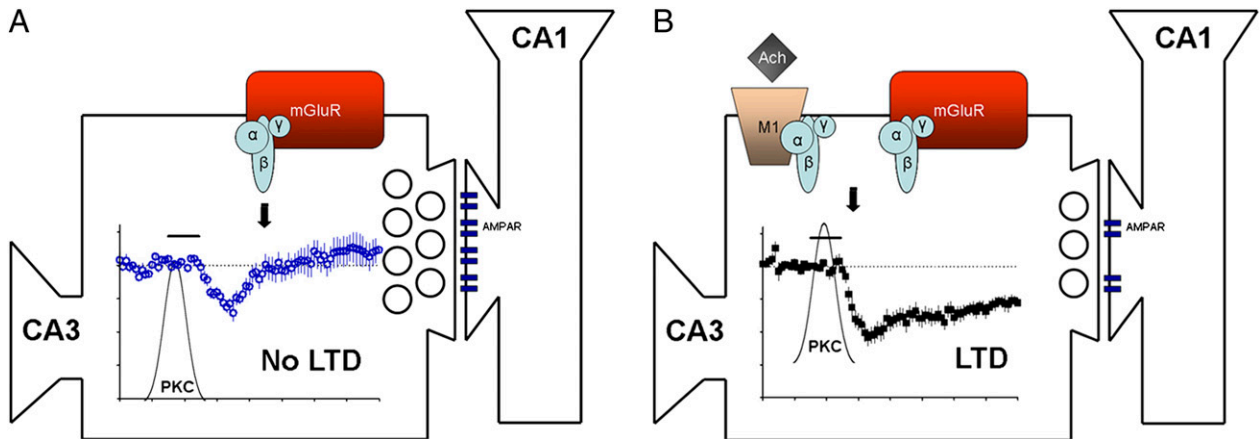


Fig. 7. Schematic of the role of m1 receptors in mGluR LTD. (A) In the mutant, perfusion of DHPG (horizontal black bar) does not induce PKC activity that is sufficient for LTD expression. (B) M1 cholinergic receptors provide basal activation of PKC, which is further activated by DHPG (see Discussion).

ducing LTD is necessary, but it may also indicate the effects of stimulation of the synapses that are not active in the experiments conducted with synapse-specific mutations.

Although our findings point to a presynaptic induction mechanism for mGluR LTD, there is an ongoing debate as to the locus of expression of mGluR LTD. And, although Zakarenko et al. (5) found a reduction in the content of released glutamate from presynaptic vesicles following mGluR LTD, others (6, 14) have found expression to be a postsynaptic reduction in AMPA receptor expression although Rammes et al. (12) have found no change in AMPA receptor activity. Notwithstanding this debate, the aim and scope of this work is not to study the presynaptic versus postsynaptic mechanisms for mGluR LTD exhaustively or extensively but instead to focus on the role of m1 in this type of synaptic plasticity, a presynaptic mechanism of LTD induction can be consistent with most studies, especially because LTD is usually measured by following the long-term effects on EPSPs well after the induction phase (14). Additionally, studies of mGluR LTD have used mice and rats of different, mostly juvenile ages (4, 5, 14) whereas here we report on findings from mice aged 3–6 months.

mGluR LTD has recently become the focus of interest due to the finding that it is enhanced in hippocampal slices from *fmr1*-deficient mice (25). These mice lack the gene that has suppressed expression in the human mental retardation syndrome “fragile X.” This phenotype is the opposite of what we find here when m1 receptors are deleted and thus suggests a mode of dampening mGluR LTD by inhibiting m1-receptor-mediated transmission as a possible therapy. Interestingly, in both *fmr1* KO and FB-m1KO mice, NMDA-receptor-dependent LTD is not changed. Our findings may relate to human cognition in other ways, too. Neuromodulation by acetylcholine has been known to facilitate processes of learning and memory (2). In fact, a cholinergic deficit is one of the hallmarks of Alzheimer’s disease and may underlie much of the cognitive dysfunction associated with the disease (26). Because cholinergic receptors are expressed throughout the brain and in different isoforms that could sometimes have opposite effects, it is of major importance to uncover the specific activities, modes of action, and location of the different receptor types. Moreover, our ability to synthesize ever-specific pharmacological agents that interact with the different subtypes (27) makes it imperative that we understand the contribution of each receptor to cognitive function.

In summary, by using electrophysiology, biochemistry, and region-specific gene manipulation, we demonstrate that mGluR LTD in hippocampus Schaffer collaterals depends on basal PKC activity levels that are controlled by m1 muscarinic acetylcholine receptors. This modulation and the acute change in PKC activity in

response to mGluR stimulation are restricted to CA3, providing evidence for the presynaptic location of mGluR LTD induction. Thus, the two seemingly parallel ligand-controlled activation cascades for Gq-coupled receptors have different roles. Acetylcholine, which is present in tonic, fluctuating concentrations in the hippocampus acts to poise CA3 for acute glutamate stimulation of mGluRs.

Materials and Methods

In Vitro Hippocampal Physiology. Hippocampal slices from 3- to 5-month-old FB-m1KO or control mice and from 5- to 6-month-old CA3-m1KO or control mice were prepared as follows. After decapitation, the hippocampus was removed, and 400- μ m slices were made with a McIlwain tissue slicer. The slices were placed in an interface-type chamber for all experiments that did not include perfusion of pharmacological agents. Slices that were subsequently perfused with pharmacological agents were first incubated for 2 h in artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl, 124; KCl, 2; NaHCO₃, 26; KH₂PO₄, 1.24; CaCl₂, 2.5; MgSO₄, 2; glucose, 10; at pH 7.4 at room temperature saturated with 95% O₂/5% CO₂ gas mixture. Recordings were made with a glass pipette containing ACSF (4 M Ω) placed in stratum radiatum. Stimulation was delivered through bipolar Platinum/Iridium electrodes placed in stratum radiatum. LTP was induced by TBS of the Schaffer collaterals (10 trains of four pulses at 100 Hz separated by 200-msec intertrain intervals at the same intensity as the test stimulation). Or by stimulation protocols described in the text. Stimulation and data acquisition were performed using the most recent WinLTP Program (www.winLTP.com).

Pharmaceuticals Were Added into the Perfusion Medium with Special Care to Prevent Changes in Temperature, pH, Flow Rate, or Degree of Oxygenation of the ACSF.

For intracellular patch-clamp experiments, young mice 12–15 days old (p12–15) homozygous for the floxed m1 allele, either expressing EMX1-driven Cre or littermates not expressing Cre were used. The mice were decapitated and the brains were removed and placed in ice-cold ACSF similar to that used for extracellular recordings. Horizontal brain slices 400 μ m thick containing the hippocampus were cut with a vibratome and incubated for 2 h at room temperature (22°C) in bubbled ACSF before recording. The slices were bath perfused with ACSF at a rate of 1 mL/min and visualized under an Axioscope 2 FS plus microscope (Zeiss) at \times 40 magnification using differential infrared contrast optics. CA1 pyramidal cells were recorded using the whole-cell patch-clamp technique. The internal pipette solution was composed of (in mM) 115 potassium gluconate, 20 KCl, 10 sodium phosphocreatine, 10 HEPES, 2 MgATP, and 0.3 NaGTP, and the pH was raised to 7.25 with KOH. The internal solution had an osmolarity of 290. Patch-clamp electrodes were pulled with a Sutter instruments P-97 puller to yield electrodes with resistances of 3–5 M Ω . Cells recorded with serial resistance higher than 30 M Ω in whole-cell configuration were discarded. Signals were acquired using an axopatch 200B amplifier (Molecular Devices). Miniature EPSCs (minis) were isolated in voltage-clamp mode at a holding potential of -60 mV in the presence of 1 μ M tetrodotoxin to block action potentials and 50 μ M picrotoxin to block GABA_A currents. Minis were recorded using pClamp10 for 5 min and then again after a 20-min recovery period. Only recordings made in

cells that were stable for 30 min were included in the final analysis. The data were analyzed with Clampfit10 (Molecular Devices) using a threshold search and verified manually. The genotypes of the mice used in the experiment were ascertained by tail DNA PCR after the minis were quantified.

Biochemical Analyses. Sample preparation. Mice of similar genetic background and age to those used for electrophysiology (three of each genotype) were used for biochemical analyses. After decapitation, the brains were placed in ice-cold ACSF saturated with 95% O₂/5% CO₂ gas mixture. Hippocampal slices similar to those used for electrophysiology were incubated in a holding chamber containing bubbled ACSF. After 2 h, three slices were removed from the holding chamber and mini slices consisting of CA1 or CA3 were prepared, frozen in liquid nitrogen, and stored at -80°C. Next, DHPG at a final concentration of 100 μM was added to the holding chamber. After 10 min exposure to DHPG, three slices were removed and mini slices were prepared, frozen, and stored at -80°C.

PKC activity assay. PKC activity was assayed using the Promega PKC assay kit in a similar way to that used by Siarey and colleagues (28). Autonomous and cofactor-dependent PKC activity was assessed using the synthetic peptide (biotinylated) AAKIQASFRGHMARKK derived from amino acids of the phosphorylation domain of neurogranin. The assay was carried out as follows. Mini slices were crushed on dry ice with a plastic pestle and motor (Kontes) in 50 μl extraction buffer (25 mM Tris, 5 mM EGTA, 0.1%

Triton-X, and Roche complete mini protease inhibitor mixture). The homogenate was spun for 5 min at 14,000 × g, and the supernatant was quickly removed.

The reaction was initiated by adding 5 μl of the extracted homogenate to 20 μl reaction buffer (100 μM ATP and [³²P]ATP; 20 mM Tris-HCl, pH 7.5; 10 mM MgCl₂; 250 μM EGTA; 400 μM CaCl₂; 0.1 mg/mL BSA; 100 μM biotinylated peptide substrate) in the absence or presence of phosphatidylerine and diacylglycerol. After 10 min of incubation at 30°C, the reaction was terminated with 7.5 M guanidine hydrochloride, and 10 μl was spotted onto a SAM2 membrane square and washed with 2 M NaCl and 1% H₃PO₄. The squares were air-dried for 30 min and placed in a scintillation vial for measurement of radioactivity. As a measure of background radioactivity, samples that did not contain enzyme homogenate were measured as well. The remaining homogenate was used for protein quantification using a Bio-Rad protein assay. PKC activity was measured as the total cpm on the membrane minus the background radioactivity divided by the protein concentration of the sample.

Statistical Analysis. Results are presented as mean ± SEM, depending on the experimental condition differences between treatments and were assessed using *t* tests and one- or two-way ANOVAs. A confidence level of *P* < 0.05 was considered significant.

- Malenka RC, Bear MF (2004) LTP and LTD: an embarrassment of riches. *Neuron* 44: 5–21.
- Hasselmo ME (1999) Neuromodulation: Acetylcholine and memory consolidation. *Trends Cogn Sci* 3:351–359.
- Oliet SH, Malenka RC, Nicoll RA (1997) Two distinct forms of long-term depression coexist in CA1 hippocampal pyramidal cells. *Neuron* 18:969–982.
- Bolshakov VY, Siegelbaum SA (1994) Postsynaptic induction and presynaptic expression of hippocampal long-term depression. *Science* 264:1148–1152.
- Zakharenko SS, Zablow L, Siegelbaum SA (2002) Altered presynaptic vesicle release and cycling during mGluR-dependent LTD. *Neuron* 35:1099–1110.
- Volk LJ, Pfeiffer BE, Gibson JR, Huber KM (2007) Multiple Gq-coupled receptors converge on a common protein synthesis-dependent long-term depression that is affected in fragile X syndrome mental retardation. *J Neurosci* 27:11624–11634.
- Auerbach JM, Segal M (1994) A novel cholinergic induction of long-term potentiation in rat hippocampus. *J Neurophysiol* 72:2034–2040.
- Seeger T, et al. (2004) M2 muscarinic acetylcholine receptor knock-out mice show deficits in behavioral flexibility, working memory, and hippocampal plasticity. *J Neurosci* 24:10117–10127.
- Fitzjohn SM, et al. (2001) A characterisation of long-term depression induced by metabotropic glutamate receptor activation in the rat hippocampus in vitro. *J Physiol* 537:421–430.
- Huber KM, Roder JC, Bear MF (2001) Chemical induction of mGluR5- and protein synthesis-dependent long-term depression in hippocampal area CA1. *J Neurophysiol* 86:321–325.
- Faas GC, Adwanikar H, Gereau RW, IV, Saggau P (2002) Modulation of presynaptic calcium transients by metabotropic glutamate receptor activation: A differential role in acute depression of synaptic transmission and long-term depression. *J Neurosci* 22: 6885–6890.
- Rammes G, et al. (2003) Activation of mGlu receptors induces LTD without affecting postsynaptic sensitivity of CA1 neurons in rat hippocampal slices. *J Physiol* 546: 455–460.
- Watabe AM, Carlisle HJ, O'Dell TJ (2002) Postsynaptic induction and presynaptic expression of group 1 mGluR-dependent LTD in the hippocampal CA1 region. *J Neurophysiol* 87:1395–1403.
- Zhang XL, Zhou ZY, Winterer J, Müller W, Stanton PK (2006) NMDA-dependent, but not group I metabotropic glutamate receptor-dependent, long-term depression at Schaffer collateral-CA1 synapses is associated with long-term reduction of release from the rapidly recycling presynaptic vesicle pool. *J Neurosci* 26:10270–10280.
- Chung HJ, Steinberg JP, Huganir RL, Linden DJ (2003) Requirement of AMPA receptor GluR2 phosphorylation for cerebellar long-term depression. *Science* 300:1751–1755.
- Rush AM, Wu J, Rowan MJ, Anwyl R (2002) Group I metabotropic glutamate receptor (mGluR)-dependent long-term depression mediated via p38 mitogen-activated protein kinase is inhibited by previous high-frequency stimulation and activation of mGluRs and protein kinase C in the rat dentate gyrus in vitro. *J Neurosci* 22: 6121–6128.
- Wu J, Rowan MJ, Anwyl R (2004) Synaptically stimulated induction of group I metabotropic glutamate receptor-dependent long-term depression and depotentiation is inhibited by prior activation of metabotropic glutamate receptors and protein kinase C. *Neuroscience* 123:507–514.
- Iwasato T, et al. (2004) Dorsal telencephalon-specific expression of Cre recombinase in PAC transgenic mice. *Genesis* 38:130–138.
- Anagnostaras SG, et al. (2003) Selective cognitive dysfunction in acetylcholine M1 muscarinic receptor mutant mice. *Nat Neurosci* 6:51–58.
- Heinke B, Sandkühler J (2005) Signal transduction pathways of group I metabotropic glutamate receptor-induced long-term depression at sensory spinal synapses. *Pain* 118:145–154.
- Kemp N, McQueen J, Faulkes S, Bashir ZI (2000) Different forms of LTD in the CA1 region of the hippocampus: Role of age and stimulus protocol. *Eur J Neurosci* 12: 360–366.
- Chen G, Harata NC, Tsien RW (2004) Paired-pulse depression of unitary quantal amplitude at single hippocampal synapses. *Proc Natl Acad Sci USA* 101:1063–1068.
- Kremen T, et al. (2006) Muscarinic suppression in stratum radiatum of CA1 shows dependence on presynaptic M1 receptors and is not dependent on effects at GABA(B) receptors. *Neurobiol Learn Mem* 85:153–163.
- Gereau RW, IV, Heinemann SF (1998) Role of protein kinase C phosphorylation in rapid desensitization of metabotropic glutamate receptor 5. *Neuron* 20:143–151.
- Bear MF, Huber KM, Warren ST (2004) The mGluR theory of fragile X mental retardation. *Trends Neurosci* 27:370–377.
- Wilkinson DG, Francis PT, Schwam E, Payne-Parrish J (2004) Cholinesterase inhibitors used in the treatment of Alzheimer's disease: The relationship between pharmacological effects and clinical efficacy. *Drugs Aging* 21:453–478.
- Caccamo A, et al. (2006) M1 receptors play a central role in modulating AD-like pathology in transgenic mice. *Neuron* 49:671–682.
- Siarey RJ, et al. (2006) Altered signaling pathways underlying abnormal hippocampal synaptic plasticity in the Ts65Dn mouse model of Down syndrome. *J Neurochem* 98: 1266–1277.