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Reduced Hippocampal Long-Term Potentiation and Context-Specific Deficit in Associative Learning in mGluR1 Mutant Mice

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Summary

We generated a novel strain of mutant mouse with a deletion in the gene encoding metabotropic glutamate receptor 1 (mGluR1). Gross anatomy of the hippocampus, excitatory synaptic transmission, long-term depression, and short-term potentiation in the hippocampal CA1 region are all apparently normal in the mutant mice. In contrast, long-term potentiation (LTP) is substantially reduced, and a moderate level of impairment is observed in context-specific associative learning. We propose that mGluR1 is not "in line" in LTP production, but rather modulates the plasticity process, and hence affects context-specific associative learning.

Introduction

Long-term potentiation (LTP) has been studied widely as a candidate cellular mechanism for learning and memory. Calcium influx through N-methyl-D-aspartate (NMDA) receptor channels is generally agreed to be necessary but not sufficient for the induction of LTP in the CA1 region of hippocampus. Several observations have implicated metabotropic glutamate receptors (mGluRs) in the production of LTP (Zheng and Gallagher, 1992; Bashir et al., 1993a; Musgrave et al., 1993; Riedel et al., 1994), as well as in synaptic function (Forsythe and Clements, 1990; Charpak and Gähwiler, 1991; Baskys and Malenka, 1991; Hayashi et al., 1993) and in long-term depression (LTD) (Bashir et al., 1993b; Bolshakov and Siegelbaum, 1994). A recent report has proposed that activation of an mGluR throws a biochemical switch that needs to be in the "on" position if LTP induction is to occur (Bortolotto et al., 1994). We have produced a mutant mouse that is lacking mGluR1, a particular subtype of mGluR (Masu et al., 1991), in order to investigate the role of this receptor in synaptic plasticity and to assess the link between synaptic plasticity and learning and memory.

Seven subtypes of mGluRs are currently recognized, called mGluR1–mGluR7 (Nakanishi, 1992; Westbrook, 1994). They are distributed widely through various brain regions, are G protein coupled, and are linked to various second messenger cascades such as phosphoinositol turnover or cAMP formation (Nakanishi, 1992; Schoepp and Conn, 1993). Anatomical studies on the subtype mGluR1 showed pre- and postsynaptic expression patterns in various regions of the central nervous system (Masu et al., 1991; Shigemoto et al., 1992; Martin et al., 1992; Fotuhi et al., 1993; Baude et al., 1993); this subtype is richly represented in hippocampus, especially in the subregion of dentate gyrus and in the CA3, but its expression in the CA1 region appears to be limited to occasional nonpyramidal neurons (Martin et al., 1992; Baude et al., 1993). Thus, effects on synaptic plasticity involving Schaffer collateral synapses in CA1 would involve presynaptic signaling. Because of its prevalence in hippocampus and the limitation to the presynaptic side of the Schaffer collateral CA1 synapses, we chose mGluR1 among the multiple mGluRs as the gene to be mutated.

We find that, in the mGluR1 mutant mice, gross anatomy of the hippocampus is apparently normal, as are excitatory synaptic transmission, LTD, and short-term potentiation (STP) in the CA1 region. LTP is, however, substantially reduced but not completely eliminated in hippocampal slices taken from the mutant animals. We also find that the mGluR1 mutant mice exhibit a moderate level of impairment in the acquisition or retention of the memory that allows the mice to perform a context-dependent fear conditioning task.

Results

Generation of mGluR1 Mutant Mice

The mGluR1 targeting vector (Figure 1A) was constructed from genomic DNA clones that contain exon 1 of the mouse mGluR1 gene and its 5′ upstream region. In this vector, the 2.1 kb SalI–EcoRI fragment containing the 3′ part of exon 1 was replaced with a 1.8 kb SalI–EcoRI fragment containing a *neo* gene under the control of the *pgk1* promoter (see Experimental Procedures for the details of the vector construction).

The targeting vector DNA was transfected into embryonic stem (ES) cells of the D3 line (derived from the strain 129/Sv), and the transfectants were subjected to G418 selection. Resistant clones were screened for the desired homologous recombinants by Southern blotting. Blots were hybridized with a probe 3′ to exon 1 (Figure 1A). Of 240 clones analyzed, 9 contained the desired homologous recombination events. Two mutant clones were separately injected into C57BL/6 blastocysts to produce chimeric animals. Chimeric males were mated with C57BL/6 females, and six chimeric animals transmitted the mutation through the germline. Heterozygous offspring were intercrossed to produce homozygous mutants, and the offspring were typed for the mGluR1 mutation by Southern blot analysis. Wild-type and mutant alleles are indicated by the presence of a 9.7 kb XbaI–XbaI fragment and a 3.8 kb BamHI–XbaI fragment, respectively (Figures 1A and 1B). Homozygous

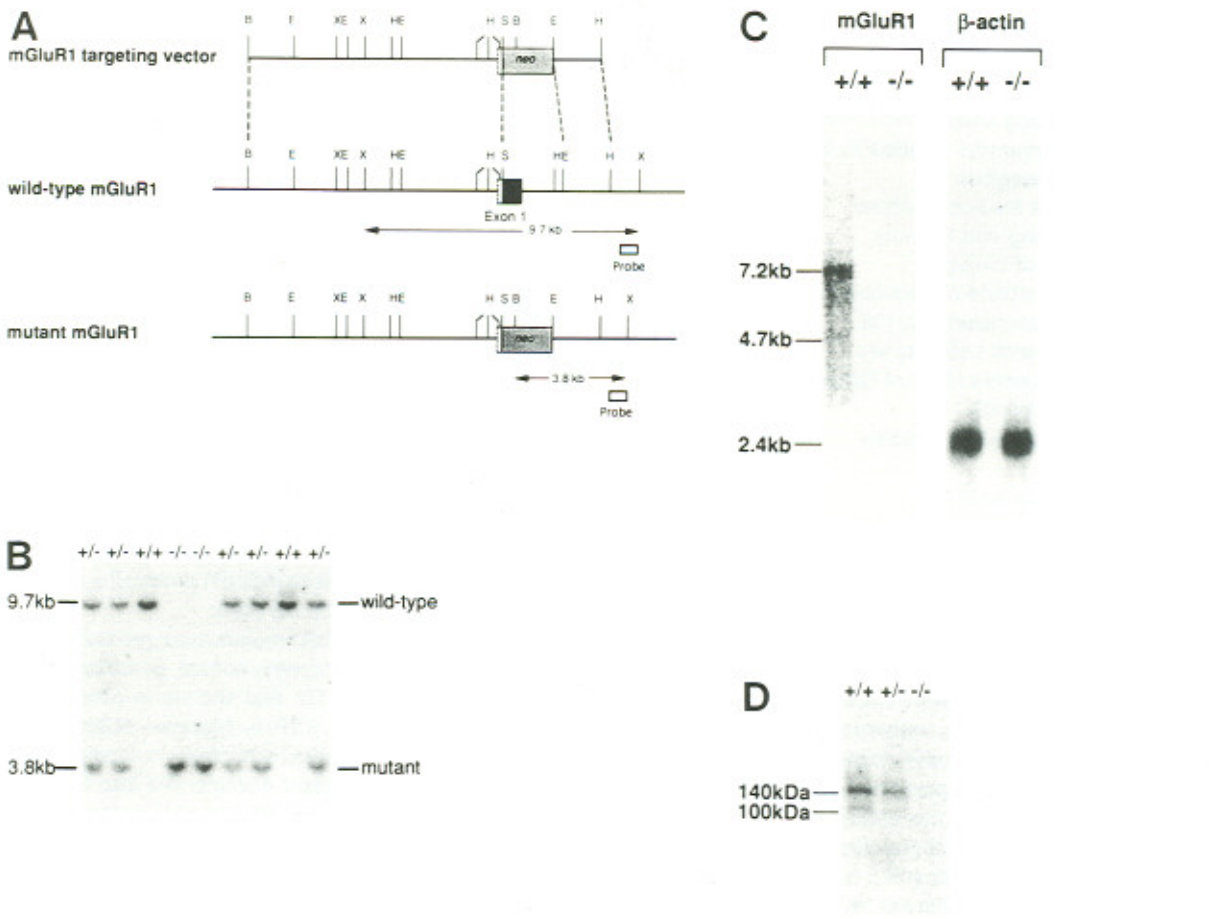

Figure 1. Production of mGluR1 Mutant Mice

(A) The mGluR1 locus and targeting construct. A schematic drawing of a region of the mGluR1 gene that contains exon 1 is shown. This exon contains 5′ untranslated sequence and encodes the first 233 amino acids (closed box) of the protein, including the initiation codon (Masu et al., 1991) and the putative ligand-binding domain (O'Hara et al., 1993). The precise location of 5′ end of exon 1 is not known. A 2.1 kb fragment from the mGluR1 gene, which includes 700 bp of the exon 1 coding region, was deleted and replaced with a *neo* gene. An mGluR1 probe flanking exon 1 used for screening of ES cell clones and mice is indicated as an open box, together with the expected sizes of hybridizing restriction fragments from wild-type and mutant mGluR1 alleles (see [B]). Abbreviations for restriction enzyme sites: B, BamHI; E, EcoRI; H, HindIII; S, SalI; X, XbaI.

(B) Southern blot analysis of representative tail biopsies. Genomic DNA was isolated from a litter of nine mice derived from a heterozygous intercross. DNA was digested with BamHI and XbaI and hybridized with the probe 3′ to exon 1 (see [A]). Wild-type and mutant alleles are indicated. Two mice in the litter are homozygous for the mutation.

(C) Northern blot analysis of mGluR1 expression in wild-type and mGluR1 mutant mice. Total RNA was isolated from wild-type mice (+/+) and homozygous mutant mice (−/−). The blot was hybridized with an 870 bp PCR product, which corresponds to amino acids 579–869 of the mGluR1 protein. No mGluR1 transcript is detected in mutant mice. As a control for RNA quantity, the same blot was reprobed with a human β-actin cDNA fragment.

(D) Western blot analysis of mGluR1 expression in wild-type, heterozygous, and mGluR1 mutant mice. The blot was probed with affinity-purified mGluR1 antibody. No mGluR1 protein is detected in mutant mice (−/−).

mutants are not sterile, but are poor breeders. Therefore, in vitro fertilization was employed to produce homozygous litters. (Hereafter, homozygous mutants are referred to as mutants.)

Northern blot analysis of RNA from wild-type and mutant mice (Figure 1C) demonstrated that the mutant mice do not express mGluR1 transcripts. Western blot analysis with an affinity-purified mGluR1 antibody demonstrated an absence of mGluR1 protein in brains of the mutant mice (Figure 1D).

General Appearance of Mutant Mice

The mGluR1 mutant mice appear healthy and well groomed and are fully capable of caring for themselves. However, by the end of postnatal week 2, the mutant mice can be easily identified on the basis of their motor behaviors (Aiba et al., 1994 [this issue of *Cell*]). The mutant mice display a mild ataxia. They can sit still, but upon initiation of movement, they exhibit a whole-body tremor of significant amplitude that persists for the duration of the movement.

Adult brains were perfused with fixative and examined

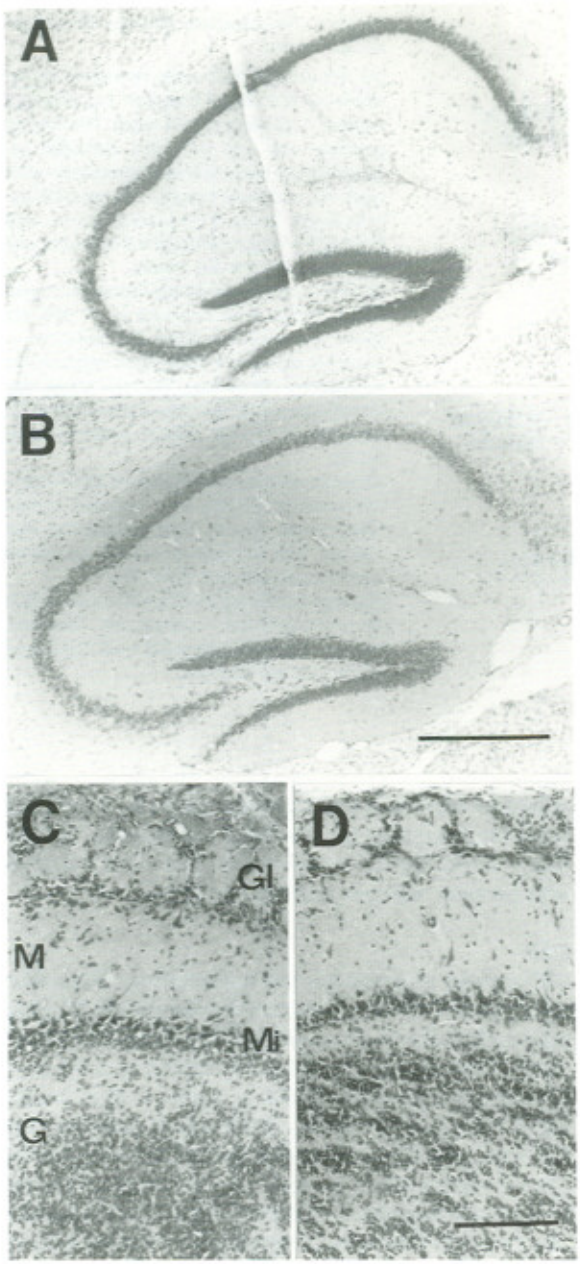

Figure 2. Anatomy of the mGluR1 Mutant Brain

(A and B) The hippocampal formation of a wild type (A) and a mutant (B).

(C and D) The olfactory bulb of a wild type (C) and a mutant (D). Abbreviations: G, granule cell layer; Gl, glomeruli; M, molecular layer; Mi, mitral cell layer.

Scale bars in (A) and (B), 500 μm; in (C) and (D), 100 μm.

for possible histological abnormalities. In light of the reported distribution of the mGluR1 mRNA and protein in adult mice (Martin et al., 1992; Shigemoto et al., 1992), we focused our attention on the hippocampus, cerebellum, and olfactory bulb. At 4 months of age, the exterior appearance and size of the mutant brains were indistinguishable from wild-type brains, and the gross anatomy of the various brain regions appeared normal as revealed by cresyl violet staining. For example, the size and overall structure of the hippocampal formation and olfactory bulbs of the mutant mice were normal as compared with wild-type controls (Figure 2). Similar observations were made for the cerebellum (Aiba et al., 1994) and for inferior olive and red nucleus (data not shown). In the absence of a complete quantitative study, it is impossible to rule out the loss of a fraction of the neurons in one or more of these nuclei, but the size and density of the neuronal populations seemed comparable to controls.

Synaptic Transmission

Transmission at the CA1 Schaffer collateral synapses is indistinguishable from those in wild-type slices, as we detail below. Specifically, amplitude and the time course of synaptic currents at various voltages, obtained with whole-cell recording, are not detectably different between mutant and wild-type neurons (Figure 3A). Furthermore, the current–voltage relations for the non-NMDA and NMDA components of the synaptic currents are not different ($n = 8$; Figure 3B).

Because the amplitude of macroscopic synaptic currents depends on the stimulus intensity and because stimulus intensities are not precisely comparable from slice

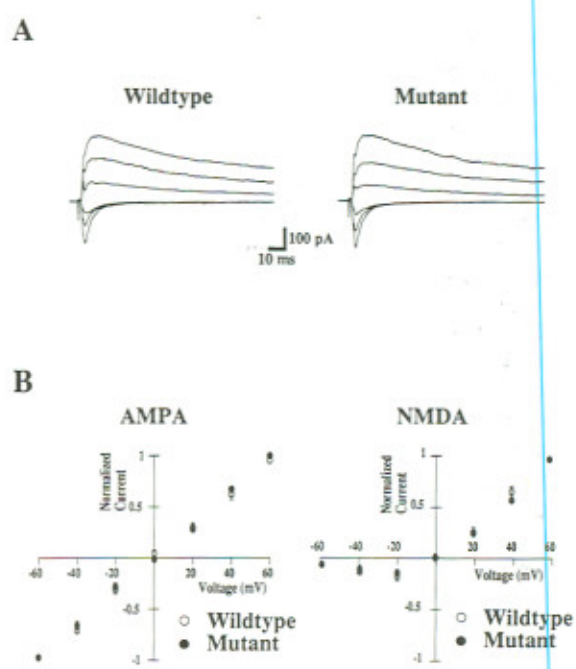

Figure 3. The Voltage Dependence of Glutamate-Mediated EPSCs in Slices

(A) Example traces of a wild-type (left panel) and a mutant CA1 neuron (right panel) of dual-component EPSCs recorded in whole-cell voltage clamp. Holding potentials were −60, −40, −20, +20, +40, and +60 mV.

(B) The graph shows the averaged amplitudes of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)- and NMDA-mediated responses. The AMPA-mediated component was measured at the first 5 ms of the EPSC; the NMDA component was determined from the averaged response at the region 50–100 ms after the stimulus. The medium contained 1 μM glycine and 1.3 mM Mg^{2+}.

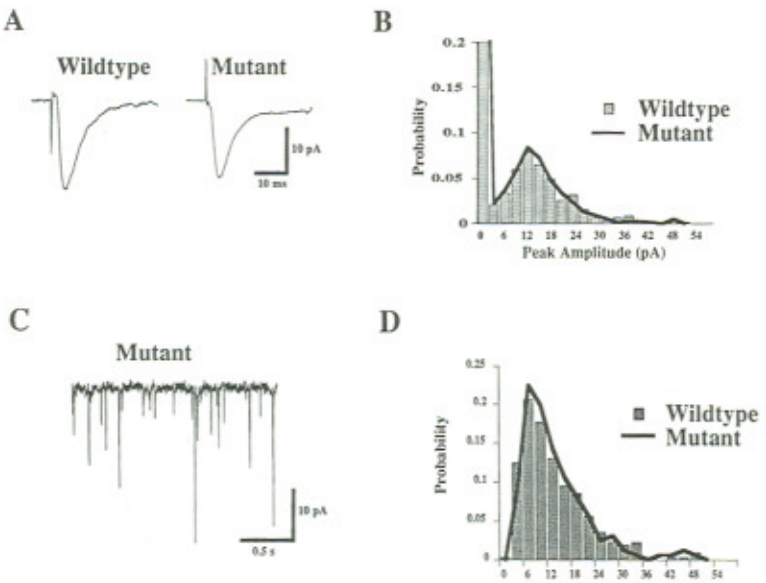

Figure 4. Responses to Minimal Stimulation Appear Normal

(A) Miniature EPSCs were measured during whole-cell recordings from CA1 hippocampal neurons from slices (14–21 days). The holding potential was −80 mV. The averaged responses from traces without failures from wild-type (left) and mutant (right) mice were similar in its shape. Decay of the synaptic current was fitted with a time constant of 4.8 ms in the wild type and 5.3 ms in the mutant.

(B) The combined amplitude histogram of miniature EPSCs from wild type (n = 988, six slices, four mice; thin trace) and mutant (n = 1343, six slices, four mice; thick trace) shows no apparent differences. The total failure rate for wild type was 0.58 and for mutant 0.59. Data were binned in 3 pA. Stimuli were evoked at 0.1 Hz.

(C) Example trace of spontaneous AMPA channel–mediated EPSCs from cultured mutant hippocampal neuron. Holding potential was −60 mV. The extracellular solution contained 1 μM tetrodotoxin and 5 mM Mg^{2+}.

(D) Amplitude histogram of spontaneous EPSCs for a wild-type and a mutant cell. Threshold for peak detection was set at 4 pA. Data were binned in 3 pA intervals.

to slice, the data presented above may provide only a crude estimate of synaptic effectiveness. We therefore have used minimal stimulation to activate only one or a few synapses, a method that provides much more nearly comparable postsynaptic responses from cell to cell (Raastad et al., 1992). The average minimal synaptic current at a holding potential of −80 mV was 16.8 pA in wild-type synapses as compared with 17.8 pA at mutant synapses. Furthermore, the amplitude distribution of wild-type and mutant synapses are not significantly different (Figure 4). The failure rate for synaptic transmission was also comparable ($p = 0.58$ in wild type, $n = 988$, six experiments; compare $p = 0.59$ in mutant, $n = 1343$, six experiments) at wild-type and mutant synapses. We also tested synaptic function of mutant neurons in primary hippocampal cultures. Synaptic transmission appeared normal ($n = 5$; data not shown), and the analysis of spontaneous miniature excitatory postsynaptic currents (EPSCs) showed similar amplitude distribution (Figures 4C and 4D) and similar decay time constants (wild type, 4.8 ms ± 0.3, $n = 201$; mutant, 4.5 ± 0.4, $n = 156$).

We also employed paired-pulse facilitation to test for abnormal presynaptic function. When two stimuli are presented in rapid succession, the response to the second stimulus is usually enhanced by an extent that depends on the interpulse interval. In whole-cell recording from mutant and wild-type slices, no significant difference was seen in the magnitude of paired-pulse facilitation over an interpulse interval range of 20–500 ms (Figure 5).

We conclude that, for the mutant animals, Schaffer collateral fibers are present in comparable numbers, that release is normal, and that postsynaptic receptors are present at their usual densities. Both NMDA and non-NMDA receptors are present and operate normally, and NMDA receptors have their usual voltage dependence. In summary, we can detect no differences in synaptic transmission between wild-type and mutant slices.

LTD

LTD appears to be intact in the mutant slices. When averaged across slices, an initial transient depression following the standard low frequency stimulation (Dudek and Bear, 1992) gave way to a maintained LTD of 15.8 ± 5.5% ($n = 9$) in wild-type slices and 13.9 ± 5.3 ($n = 9$) in mutant slices (Figure 6A); however, the onset of recovery from low frequency stimulation was accelerated during the first 3 min in mutant slices. As usual, the amount of LTD varied from slice to slice; the cumulative probability distributions for LTD magnitude were not significantly different between mutant and wild-type slices (Figure 6B).

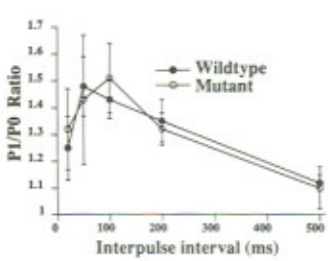

Figure 5. Paired Pulse Facilitation Appears Normal

The plot shows the summarized (wild type, five slices, three animals; mutant, six slices, three animals) facilitation of the second EPSC (P1) compared with the first EPSC (P0) as a function of the interpulse interval as measured in whole-cell recordings. Holding potential was −80 mV. Error bars show SEM.

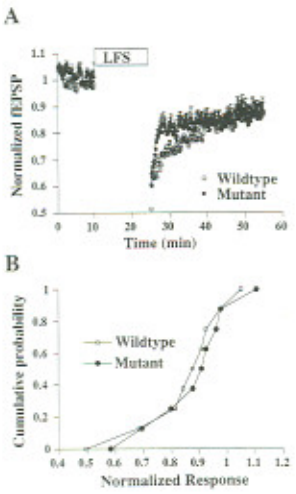

Figure 6. LTD Appears to Be Normal

(A) Summarized field potential recording (fEPSP) from nine wild-type slices (six animals) and nine mutant slices (five animals). The box labeled LFS represents the 15 min low frequency stimulation (1 Hz) period. Error bars show SEM.

(B) Cumulative histogram of LTD in wild type (open squares) and mutant (closed circles).

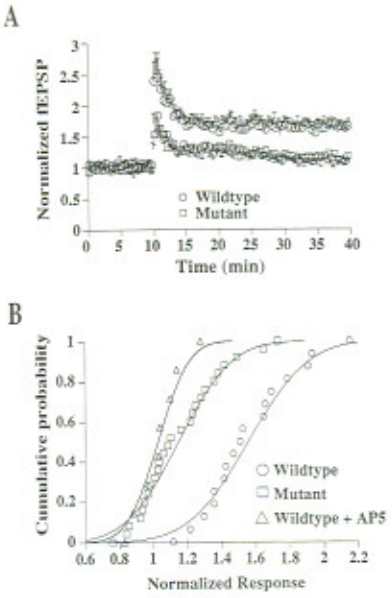

Figure 7. LTP in Hippocampus Is Reduced in Mutant Slices

(A) Summary of field potential recordings from wild-type (n = 17), and mutant (n = 25) slices in the stratum radiatum region of CA1. The initial slope of field excitatory postsynaptic potential (fEPSP) is normalized to the baseline value preceding the induction of LTP. Tetanus (t = 10 min; 100 Hz) was given in five sets of 200 ms duration 10 s apart. Error bars represent SEM. Recordings were done at room temperature.

(B) Cumulative probability histogram of the same set of slices including wild type plus 50 μM AP5 (n = 8). The lines represent the normal distribution of each data set.

STP and LTP

When averaged across 42 slices studied with field potential recording, LTP of 115.7 ± 6.6% (n = 25) was found in the mutant slices as compared with 155.7 ± 4.8% (n = 17) in wild-type controls (Figure 7A); this difference was statistically significant (Student's t-test, $P < 0.01$). The cumulative probability distributions for the magnitude of LTP reveals that the mutant slices exhibited significantly less LTP than did the wild-type slices, but significantly more LTP than seen with wild-type slices that have been treated with 50 μM D-(−)-2-amino-5-phosphonovaleric acid (AP5) (103.4 ± 2.1; n = 8; $p < 0.05$; Figure 7B). Thus, LTP is definitely present in the mutant animals, but it is markedly reduced as compared with wild-type slices. Note that the cumulative histograms for all conditions are S shaped: what varies from one condition to another is the mean and variance of the distributions. The histogram for wild-type slices treated with AP5 gives a measure of the variance obtained when little or no LTP is present. Note that the mutant slices also exhibit an apparently continuous distribution of LTP magnitudes.

In protein kinase Cγ (PKCγ) mutant mice that lack normal LTP, rescue of LTP occurred when the Schaffer collaterals were primed with low frequency (1 Hz) stimulation prior to a tetanic stimulation (Abeliovich et al., 1993a). In contrast, LTP induction in mGluR1 mutant slices after low frequency stimulation did not result in an significantly larger LTP than in "unprimed" slices (121.4 ± 5.4%; n = 6; three animals). Thus, the priming effect seen in PKCγ mutants is absent in our mutants.

Because no methods are available for producing LTP without STP, the magnitude of STP is difficult to estimate when LTP is also present; one does not know how rapidly LTP develops, so the initial magnitude of the STP is unclear. From Figure 7A, however, one can conclude that STP is definitely present. If magnitude of STP is taken as the difference between the initial posttetanus synaptic strength and the final level of LTP, then STP is very nearly normal in the mutant animals. The STP peak amplitude was 78% with an average decay time constant of 108 s in wild-type slices and was 71% and 84 s for the mutant slices.

Consequences of mGluR Activation

Several different effects have been attributed to activation of mGluRs. The mGluR agonist such as (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid ((1S,3R)-ACPD) has been reported to inhibit calcium currents (Lester and Jahr, 1990; Sayer et al., 1992; Swartz and Bean, 1992; Sahara and Westbrook, 1993) and to produce a transient depression of synaptic transmission (Baskys and Malenka, 1991). (1S,3R)-ACPD has also been reported to produce an LTP-like increase in synaptic strength at longer times (Bashir et al., 1993a). The mGluR antagonist (+)-α-methyl-4-carboxyphenylglycine (MCPG; Birse et al., 1993, Hayashi et al., 1994) was found to block LTP and LTD (Bashir et al., 1993a, 1993b; Bolshakov and Siegelbaum, 1994). Which mGluR subtypes are responsible for these various effects? Since our mice lack mGluR1, we can potentially identify which of the actions are mediated through mGluR1 and

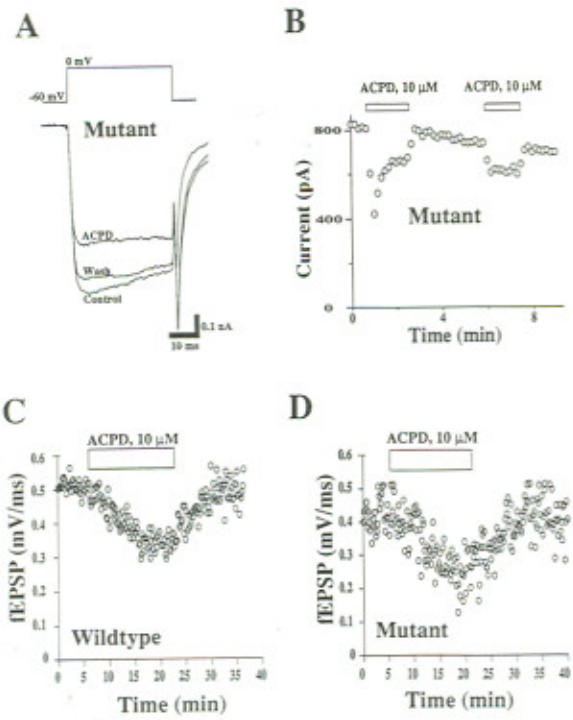

Figure 8. Inhibition of Calcium Currents and Inhibition of Field Potentials in Hippocampus Are Not Mediated through mGluR1 Receptors

(A) Example current traces from a neuron derived from a mutant hippocampal culture before, during, and after application of (1S,3R)-ACPD (10 μM). The neuron was voltage clamped at −60 mV and stepped to 0 mV to record the high threshold calcium current. This current was blocked by 100 μM Cd^{2+} (data not shown). The bath contained additional 1 μM tetradotoxin, 10 mM tetraethylammonium–HCl, and 5 mM $BaCl_2$.

(B) Amplitude time course of calcium currents from the same neuron as in (A). Responses were evoked at 10 s interval.

(C) A typical field potential response (fEPSP) in CA1 region to 10 μM (1S,3R)-ACPD recorded from a wild-type slice. The open bar indicates the time of (1S,3R)-ACPD perfusion.

(D) Same as (C), but from a mutant slice. Mice 3–4 weeks old were used.

which are not. The strategy is to examine the effects of agonists and antagonists in the mutant slices: if mGluR1 is responsible, neither agonists nor antagonists should be effective, whereas if other receptor subtypes mediate an effect, agonists and antagonists should work in mutant slices as they do in wild-type slices.

Calcium currents are reversibly decreased by (1S,3R)-ACPD (10–30 μM) in neurons cultured from both wild-type (31.5 ± 7.4%, n = 5) and mutant hippocampus (27.6 ± 8.1%, n = 5; Figures 8A and 8B). Thus, at least a part of the neuromodulatory effects of mGluRs are mediated by isoforms other than subtype 1. When (1S,3R)-ACPD (10 μM) is applied to slices, the field excitatory postsynaptic potential (fEPSP) is transiently decreased in both to approximately the same extent and with the same time course (wild-type, 43.0 ± 4.2% depression after 10 min, n = 7; mutant, 39.1 ± 6.4%, n = 5; Figures 8C and 8D). Thus, neuromodulatory effects on calcium channel and on synaptic transmission in CA1 region of the hippocampus appear not to depend on mGluR1.

The role of mGluR1 on LTP with respect to the actions produced by mGluRs is more difficult to assess. We have been unable to produce the LTP-like effects of (1S,3R)-ACPD reported to occur in wild-type slices (Desai and Conn, 1991; Bashir et al., 1993a), so we could not evaluate the participation of mGluR1 in that effect.

Our results with MCPG are also somewhat different from what has been recently reported: rather than two clear categories of responses, corresponding to the switch on and switch off (Bortolotto et al., 1994), we find that LTP is significantly decreased with an apparently continuous distribution of LTP magnitudes for slices treated with 200 μM MCPG throughout the experiment (129.7% ± 4.6% SEM, n = 18; compare with 157.7% ± 4.8% SEM, n = 17 wild-type control; Figure 9). The cumulative histogram for the wild-type mice with and without MCPG treatment are significantly different (Kolmogorov–Smirnov test, p = 0.05). When compared with the cumulative probability distributions for LTP amplitude in mutant slices, we find that 200 μM MCPG decreases LTP approximately as much as the lack of mGluR1 (Figure 9). In the cumulative histogram (Figure 9B), the difference between the mutant and the wild type with MCPG is not statistically significant (p = 0.11), although the median for the wild-type with MCPG

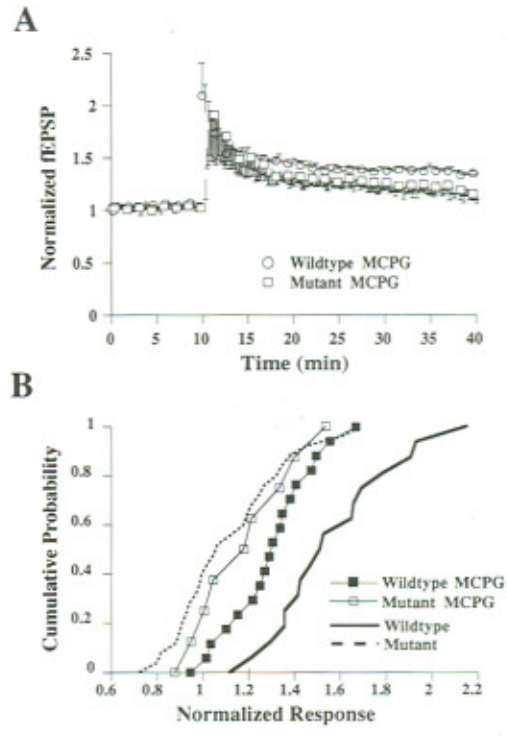

Figure 9. MCPG Reduces LTP in Wild-Type but Not in Mutant Slices

(A) Average synaptic strength as a function of time during the experiment for 18 wild-type slices (five animals) and nine mutant slices (five animals) bathed in 200 μM MCPG.

(B) Cumulative histogram of LTP in presence of 200 μM MCPG. The thick line for the wild type and the dotted line for the mutant represent the LTP distribution as shown in Figure 7B.

appears to be slightly larger. If the nonsignifiant difference is real, this can be interpreted in two ways. First, the MCPG effect may not involve mGluR1, so the failure exactly to mimic the defect in the mutant animals might be expected with this interpretation. Second, the effect of MCPG on LTP might be mediated through mGluR1, but the MCPG could have been less effective than the complete absence of the receptor produced by the mutation, even though we used a relatively high dose of MCPG (200 μM).

To distinguish between these two alternatives, we have applied MCPG to mutant slices. If the drug mechanism of action does not involve mGluR1, we would usually expect to have some effect on the magnitude of LTP, whereas if the second alternative is correct, MCPG would have no effect in the mutant slices. We find that the magnitude of LTP, measured by the cumulative probability distributions, is not different in mutant slices with and without MCPG (Kolmogorov–Smirnof test, $p > 0.1$; Figure 9B). Also, in the presence of 200 μM MCPG, 10 μM (1S,3R)-ACPD blocks field potentials by 27.1 ± 2.9% ($n = 5$) in wild-type slices (compared with 43.0 ± 5.0 in the absence of MCPG; $p < 0.05$). Thus, the MCPG was adequately inhibiting other mGluR subtype effects. The simplest interpretation of these observations is that MCPG actions are mediated by mGluR1.

Moderate Deficit in Context-Specific Associative Learning

We tested mGluR1 mutant mice for their learning ability by associative fear conditioning. In the paradigm employed, an initially neutral stimulus (an experimental chamber or tone) is paired with an aversive, unconditioned stimulus (an electric shock). A conditioned freezing response, characterized by an immobile, crouching posture, is observed upon subsequent presentation of the conditioned stimuli. Hippocampal lesions in rodents serve to define two forms of fear conditioning: one to nonspecific cues, such as the context of an experimental chamber that is sensitive to hippocampal lesions, and the other to specific cues, such as a tone that is insensitive to hippocampal lesions (Kim and Fanselow, 1992; Phillips and LeDoux, 1992). Furthermore, the context-specific fear conditioning has been shown to depend on NMDA receptor function in rodents (Kim et al., 1991).

In the training phase of contextual fear conditioning, mice were placed in a shocking chamber and given three foot shocks. Mice were then removed and placed back to home cages. They were returned to the shocking chamber 1 day later and monitored for freezing behavior. Wild-type ($n = 12$) and mGluR1 mutant ($n = 12$) mice displayed comparable freezing during the conditioning phase of the experiment ($p = 0.2781$; Figure 10A). Thus, the mGluR1 mutant mice, like their wild-type counterparts, do not harbor any performance deficit in this task and are able to acquire the conditioned response during training. However, when returned the next day to the conditioning chamber, mutants froze significantly less than wild-type mice ($p < 0.0001$; Figure 10B), indicating a moderate impairment in the formation or retention of fear memory.

To evaluate the specificity of the fear memory impairment observed, we tested mutant and wild-type mice in the tone-dependent fear conditioning paradigm. During the training phase, three tones, each 20 s long, were presented along with three foot shocks. Both mutant and wild-type animals demonstrated significant conditioning over trials (Figure 10C), and the degree of conditioning did not differ between two groups ($p = 0.2957$). When a tone was presented 1 day later in a new context, both wild-type and mutant animals displayed similar degree of conditioned freezing to the tone ($p = 0.4567$; Figure 10D). Thus, the memory deficit in mGluR1 mutants is specific to hippocampus-dependent, as opposed to hippocampus-independent fear conditioning.

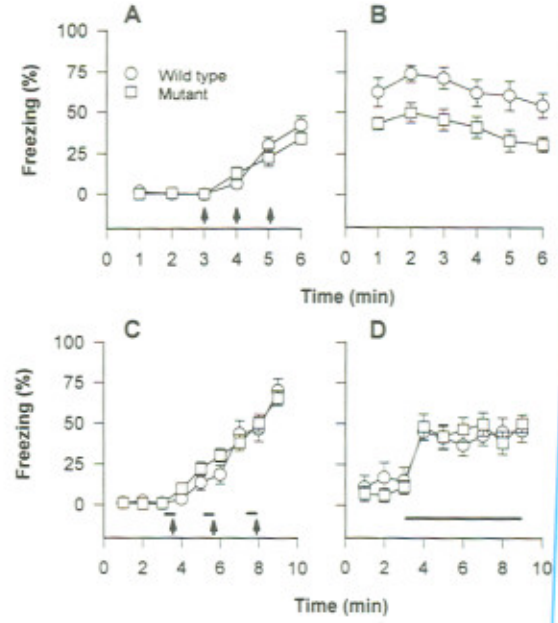

Figure 10. Context-Specific Fear Conditioning Is Impaired

(A) Context-dependent fear conditioning, training phase. Wild-type and mutant mice displayed a comparable degree of freezing ($F[1, 22] = 1.20$, $p = 0.2781$) immediately after the foot shocks (arrows). Both groups showed significant learning during training ($F[2, 66] = 21.36$, $p < 0.0001$).

(B) Context-dependent fear conditioning, testing phase. Mutant mice displayed significantly less freezing than wild-type mice did when returned to the shocking chamber the next day ($F[1, 22] = 23.44$, $p < 0.0001$).

(C) Tone fear conditioning, training phase. Wild-type and mutant animals displayed a comparable degree of freezing immediately after the foot shocks (arrows) at the offset of the tone (closed bars) ($F[1, 22] = 1.102$, $p = 0.2957$). All animals showed significant learning over tone training ($F[5, 132] = 34.716$, $p < 0.0001$).

(D) Tone fear conditioning, testing phase. Wild-type and mutant mice displayed a comparable degree of freezing when presented with the tone (solid line) in a novel context the next day ($F[1, 22] = 0.557$, $p = 0.4567$).

Discussion

The present work is relevant to our understanding of the role of mGluR1 in synaptic transmission and synaptic plasticity and of the role of the synaptic plasticity in learning and memory.

mGluR1 in Synaptic Transmission and Plasticity

We can conclude that mGluR1 is not required for normal formation and function of Schaffer collateral synapses on CA1 pyramidal cells. Nor is it necessary for the production of LTD or STP, although we cannot be certain that the magnitude of STP is completely unaffected. mGluR1 is not absolutely required for LTP, but the absence of this receptor quite significantly diminishes LTP magnitude. Thus, mGluR1 is not "in line" in LTP production, but rather modulates the plasticity process. Our observations add one more level of complexity to the biochemical regulatory network for LTP.

Our observation that mGluR1 mutant mice have apparently normal LTD in CA1 of hippocampus is in conflict with the recent findings of Bolshakov and Siegelbaum (1994), who reported that LTD in hippocampal slices of young rats was blocked by MCPG. The conflicting observations might arise from the fact that the two laboratories were studying somewhat different forms of LTD: the LTD of Bolshakov and Siegelbaum (1994) was not blocked by the application of AP5, whereas the form we study is.

The data of different laboratories regarding the effect of MCPG on synaptic transmission and LTP have resulted in conflicting conclusions (Bashir et al., 1993a; Bortolotto et al, 1994; Riedel et al., 1994; Bolshakov and Siegelbaum, 1994; Chinestra et al., 1993; Manzoni et al., 1994). Some of those differences may be accounted through methodological differences in the experiments. Our observations on the effects of MCPG in wild-type slices lead us to propose an alternative to the switch hypothesis of Bortolotto et al. (1994). Because high doses of MCPG decrease LTP to approximately 50% of its normal amplitude, it seems to act more like a volume control than a switch. Further, because MCPG has no effect of slices that lack mGluR1, the obvious (but not the only) interpretation is that the mGluR volume control is mediated through the subtype 1. If this interpretation is correct, it has interesting consequences because mGluR1 is expressed in CA3 pyramidal cells (Masu et al., 1991; Shigemoto et al., 1992) that give rise to Schaffer collateral synapses but not in the CA1 pyramidal cells (Martin et al., 1992; Fotuhi et al., 1993). These observations would place the mGluR1 volume control on the presynaptic side of the Schaffer collateral/CA1 synapses in which we study LTP.

If mGluR1 serves as a part of a presynaptic mechanism that regulates the magnitude of LTP, it could provide for an error-checking mechanism. Because calcium might enter spines by diffusion from the dendritic shafts (Kullmann et al., 1992; Wyllie et al., 1994) to produce the signal for LTP spuriously or because the postulated retrograde messenger might spread to synapses that have not met the requirements for LTP production (Bonhoeffer et al., 1990; Schuman and Madison, 1994), one can envision a need to ensure that both presynaptic activity and postsynaptic responses have indeed been present in a synapse that is to be strengthened. A presynaptic glutamate receptor would be most effectively activated by transmitter release at the very synapse bearing the receptor, so its proposed regulatory function could compare the past activity of that synapse with the magnitude of a retrograde signal and produce appropriate modification of synaptic strength. A volume control rather than a switch might be most useful because the brain might wish to strengthen synapses whose activity is less than perfectly correlated with the activity of other neighboring synapses.

Evidence from the Nicoll laboratory suggests that presynaptic activity is required for the production of LTP (Kullmann et al., 1992; Wyllie et al., 1994). At the same time, Cormier et al. (1993) have convincingly demonstrated that glutamate by itself is sufficient to produce LTP without presynaptic activity.

Our hypothesis for the function of mGluR1 may help to reconcile and explain these observations. The experiments performed in the Nicoll laboratory (Kullmann et al., 1992; Wyllie et al., 1994) produced STP (without LTP) by repeatedly depolarizing the neuron to cause calcium influx through dendritic calcium channels. When the postsynaptic depolarizations were paired with presynaptic activity (in the presence of AP5), LTP was produced. We would explain these findings by saying that the hypothetical retrograde signal produced by the increased spine calcium concentrations was not, without activation of the presynaptic mGluR1s, sufficient to produce appreciable LTP. But the presynaptic release of glutamate (in the presence of AP5) would activate the mGluR1s, so the retrograde signal arising from postsynaptic calcium concentration increases, caused by activation of postsynaptic calcium channels in dendrites, should induce LTP; the volume control would be turned up by the presynaptic activity, and the postsynaptic depolarizations would provide the retrograde signal necessary for LTP production. Cormier et al. (1993) found they could produce LTP by direct application of glutamate without presynaptic activity. Direct application of glutamate would activate both pre- and postsynaptic glutamate receptors, so the volume control would be turned up by glutamate action on the mGluR1s and the retrograde signal would be produced by the postsynaptic activation of NMDA receptors. The combination of both should produce LTP without activity of the presynaptic axons or synaptic glutamate release.

The proposal put forth here explains various features of LTP and provides the basis for further experiments. Whether mGluRs act as switches or volume controls and related issues will have to await further investigations.

Synaptic Plasticity and Learning

The hippocampus is thought to play a key role in relational, contextual, spatial, or declarative learning and memory (for reviews see Squire, 1992; Eichenbaum et al., 1992). Further, spatial learning and contextual fear learning in rats could be disrupted by a specific antagonist for NMDA receptors that could also block hippocampal LTP in vivo (Morris et al., 1986; Kim et al., 1991). Thus, hippocampal LTP has been suggested to be critical for certain types of learning and memory. Recently, the gene targeting approach has been utilized to reexamine the relationship between hippocampal LTP and hippocampus-dependent learning tasks (Silva et al., 1992a, 1992b; Grant et al., 1992; Abeliovich et al., 1993a, 1993b). Studies on two mutant mice produced by the gene targeting technique,

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α-calcium–calmodulin kinase II (Silva et al., 1992a, 1992b) and *fyn* (Grant et al., 1992) mutants, provide further support for LTP as a learning mechanism. However, a more recent study with PKCγ mutant mice indicated that the synaptic plasticity represented by CA1 LTP induced in vitro by conventional tetanic stimulation may contribute to but is not required for the spatial and contextual learning; other forms of synaptic plasticity, such as the primed LTP and LTD, were suggested to correlate with the residual learning (Abeliovich et al., 1993a, 1993b).

The learning deficit in mGluR1 mutant mice appears to be similar to that in PKCγ mutants in that it is moderate and is observed in the contextual conditioning and not in the tone conditioning. The learning deficit observed in the mGluR1 mutant mice may again be attributed to the impaired LTP (both conventional and primed). The apparently normal LTD may contribute to residual part of contextual learning. Generation of mutant mice in which LTP is normal but LTD is impaired is much desired to confirm the role of the latter synaptic plasticity in learning and memory.

Experimental Procedures

Mapping and Cloning of Mouse mGluR1 Gene

A genomic fragment containing exon 1 of mGluR1 was isolated from a genomic EMBL3 phage DNA library prepared from D3 ES cells (Silva et al., 1992a) after screening with a probe generated by the polymerase chain reaction (PCR) that corresponds to amino acids 1–200 of the mouse mGluR1 protein. Exon 1 encodes amino acids 1–233 of the mouse mGluR1 protein. The amino acid sequence deduced from DNA sequence (our unpublished data) was identical to the published rat amino acid sequence in the corresponding region (Masu et al., 1991). The targeting construct was prepared in a quatrimolecular ligation reaction, using a 9.0 kb BamHI–SalI fragment located 5′ region of exon 1, a 1.8 kb SalI–EcoRI fragment containing a *neo* gene driven by the *pgk1* promoter (a gift from Dr. M. Rudnicki), a 1.7 kb EcoRI–HindIII fragment located 3′ of exon 1, and the plasmid pBluescript (Stratagene), digested with appropriate restriction enzymes. This construct was designed to delete a 2.1 kb fragment from the mGluR1 gene, including the 3′ part of exon 1 that encodes amino acids 1–233 of the mGluR1 protein.

Production of mGluR1 Mutant Mice

D3 ES cells (gift from Dr. R. Kemler) were transfected with 50 μg of linearized targeting vector by electroporation (Bio-Rad Gene Pulser set at 800V and 3 μF). G418 selection (150–175 μg/ml) was applied 24 hr after transfection, and G418-resistant colonies were isolated during days 7–10 of selection. Genomic DNA from these clones was digested with BamHI and XbaI and hybridized with a 600 bp DNA fragment 3′ to exon 1 (Figure 1). Chimeric mice were generated as described by Bradley (1987). The contribution of ES cells to the germline of chimeric mice was determined by breeding with C57BL/6 mice and screening for agouti offspring. Germline transmission was confirmed by Southern blotting of tail DNA, and mice heterozygous for the mutation were interbred to homozygosity. Homozygous females and males are fertile, but are poor breeders. To generate litters from homozygous intercrosses, we transferred in vitro fertilized eggs to a pseudopregnant mother as described elsewhere (Hogan et al., 1986). Initial screening of mice was done by Southern blotting of tail DNA; subsequently, mice were typed by PCR analysis with a set of *neo* primers (5′-GCTTGGGTGGAGAGGCTATTC-3′ and 5′-CAAGGTGAGATGACAGGAGATC-3′, 280 bp PCR fragment) and a set of primers from the deleted region of the mutant mGluR1 allele (5′-ATGGACGGAGATGTCATCATCGGAG-3′ and 5′-GATCACTCCAGCAATAGGCTTCTTA-3′, 360 bp PCR fragment).

Northern Blot Analysis

Total RNA was isolated from cerebellum by the acid guanidine thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987). RNA (20 μg) was electrophoresed on a 1.2% formaldehyde agarose gel. The gel was blotted onto Hybond-N nylon membrane (Amersham), probed with an 870 bp PCR product that corresponds to amino acids 579–869 of the rat mGluR1 protein, and reprobed with a 1.5 kb AvaI–BamHI fragment from the human β-actin gene.

Western Blot Analysis

Total extract (10 μg) from cerebellum was denatured in sodium dodecyl sulfate loading buffer and separated by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The gel was transferred onto a Hybond-ECL nitrocellulose membrane (Amersham), and the membrane was incubated with affinity-purified mGluR1 antibody (a gift from Dr. T. M. Dawson). The membrane was subsequently incubated with ^{125}I-labeled protein A (Amersham) to detect the polypeptides.

Nissl Staining

Animals were deeply anesthetized with Avertin and perfused through the heart with 4% paraformaldhyde in 0.1 M phosphate buffer. After perfusion, the brains were dissected free of the skull and immersed in fresh fixative for an additional 4–5 hr. The solution was changed to 18% sucrose in PBS for an additional 24 hr, after which the brains were embedded in OCT and frozen on dry ice. Cryostat sections (10 μm) were collected on gelatinized slides and stained with cresyl violet.

Electrophysiology

Hippocampal slices (400–500 μm) were cut from 2- to 12-week-old wild-type or mutant mice (strain C57BL/6 × 129/Sv). The LTP experiments were carried out on mice between P21 and P50, usually on mice between P28 and P35. There was no correlation between age and magnitude of LTP for either wild-type or mutant mice. A cut was made between the CA1 and the CA3 region, and the slices were incubated in a storage chamber for at least 1 hr before recording. Storage and recording solution contained 120 mM NaCl, 2.5 mM KCl, 1.3 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 10 mM glucose, 50 μM picrotoxin, and the solution was pregassed with 95% O_2 and 5% CO_2. The flow rate was 2 ml/min. All experiments were performed at room temperature (21°C–24°C).

Neonatal mouse hippocampal cultures were made as described in Rosenmund and Westbrook (1993). To isolate calcium currents, we added 10 mM tetraethyl-ammonium–HCl, 1 μM tetrodotoxin, and 5 mM $BaCl_2$ to the bath solution. For LTP and LTD measurements, field potential recordings in the CA1 region of the hippocampus were made with a glass electrode filled with recording solution or with 1 M NaCl. Whole-cell recordings were made with pipettes with a resistance of 2–5 MΩ utilizing the "blind" method (Blanton et al., 1990). The pipettes were filled with a solution containing 130 mM Cs–gluconate, 10 mM HEPES, 0.5 mM EGTA, 2 mM MgATP, 0.3 mM LiGTP, 1 mM $MgCl_2$, 5 mM NaCl adjusted to pH 7.2, and 300 mOsm. Field potentials were evoked by placing bipolar tungsten electrodes in the region of the Schaffer collateral commissural pathway. Minimal stimulation recorded in whole-cell mode were evoked by using a single glass electrode placed close to the proximal dendritic field near the recorded neuron. Responses were evoked in a frequency of 0.1 Hz. Tetanus to evoke LTP consisted of five trains of 100 Hz stimulations lasting for 200 ms at an intertrain interval of 10 s. LTD was evoked by 900 stimuli at a frequency of 1 Hz (Dudek and Bear, 1992). Currents or field potentials were recorded using an Axopatch 200A amplifier (Axon Instruments). Patch pipettes were fabricated from borosilicate glass (TWF 150, WPI Incorporated) pulled with a 2-step puller (Narishige). The series resistance was 60%–90% compensated. Data were acquired on an IBM 386 clone using Axobasic software (Axon Instruments). Acquisition rate was 2–5 kHz, and data were filtered at half the acquisition rate with an 8-pole Bessel filter (Frequency Devices). (1S,3R)-ACPD, AP5, and MCPG were purchased from Tocris Neuramin. Data is expressed as mean ± SEM. Significance is tested using Student's t-test or the Kolmogorov–Smirnov two-sample test.

Fear Conditioning

Naive male or female adult mice (10–14 weeks old) were housed individually for at least 1 week prior to behavioral testing. Mice were handled daily for 1 week prior to behavioral experiments to reduce stress. Fear conditioning and testing were conducted in a small rodent cham-

ber (Coulbourn) containing a stainless steel rod floor (5 mm diameter, spaced 1 cm apart) through which scrambled foot shocks could be administered. The chamber was placed inside a sound-attenuating chest (Coulbourn) with a ventilation fan providing background noise. The chamber was cleaned with 1% acetic acid and dried completely before each animal was placed inside. Freezing was assessed by a time-sampling procedure in which an observer blind to mouse genotype scored each mouse every 2 s. Percent freezing was calculated per minute.

In the contextual conditioning phase, animals were placed in the shocking chamber for 3 min and subsequently subjected to three foot shocks (0.5 mA intensity, 1 s duration, 1 min apart). Mice were removed from the chamber 1 min after the last foot shock. In the testing phase (the next day), animals were returned to the chamber. In the absence of foot shocks freezing was monitored for 6 min.

During the training phase of tone fear conditioning, animals were placed in the shocking chamber for 3 min and then presented with three loud tones of 20 s each (approximately 75 db, 1000 Hz, 2 min apart) through a speaker mounted on the chamber. Each tone was coterminated with a foot shock (0.5 mA intensity, 1 s duration). Mice were removed from the chamber 1 min after the last foot shock. In the testing phase (the next day), mice were placed in an empty plastic cage (similar to their home cage but different from the shocking chamber) to minimize context-specific freezing. Freezing was scored for 3 min prior to the tone and subsequently for 6 min in the presence of the tone.

Behavioral data were analyzed by a two-way analysis of variance.

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