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Deficient Cerebellar Long-Term Depression and Impaired Motor Learning in mGluR1 Mutant Mice

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Summary

mGluR1 mutant mice are viable but show characteristic cerebellar symptoms such as ataxic gait and intention tremor. The anatomy of the cerebellum is not overtly disturbed. Excitatory synaptic transmission from parallel fibers (PFs) to Purkinje cells and that from climbing fibers (CFs) to Purkinje cells appear to be functional, and voltage-gated Ca^{2+} channels of Purkinje cells are normal. Both PF and CF synapses display normal short-term synaptic plasticity to paired stimuli. By marked contrast, long-term depression (LTD) is clearly deficient and conditioned eyeblink response is impaired. We conclude that mGluR1 is required for the induction of LTD and that the ataxic behavior and impaired eyeblink conditioning of the mGluR1 mutant mice are primarily due to deficient LTD.

Introduction

Persistent changes in synaptic strength, such as long-term potentiation (LTP) and long-term depression (LTD), are thought to be a cellular basis of learning and memory. In the cerebellum, LTD is found to occur in excitatory synapses on Purkinje cells, the sole output neurons of the cerebellar cortex. Each Purkinje cell has two distinct types of excitatory synapses: many weak synapses from parallel fibers (PFs) and a single strong synapse from a climbing fiber (CF; Ito, 1984). LTD occurs in PF–Purkinje cell synapses following repetitive activation of these synapses in conjunction with CF inputs (Ito et al., 1982; Ekerot and Kano, 1985; Sakurai, 1987).

Induction of LTD requires Ca^{2+} entry to Purkinje cells through voltage-gated Ca^{2+} channels that are activated by CF activity (Sakurai, 1990; Hirano, 1990; Crepel and Jaillard, 1991; Konnerth et al., 1992). In addition, LTD requires factors that result from stimulation of PFs that use glutamate as a transmitter (Ito, 1984). PF stimulation can be replaced by application of glutamate or quisqualate, but not aspartate or kainate, indicating that quisqualate-selective subtypes of glutamate receptors are involved (Kano and Kato, 1987, 1988). Later, it was revealed that activation of both ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors and metabotropic glutamate receptors (mGluRs) are necessary for LTD induction (Linden et al., 1991; Daniel et al., 1992). Involvement of mGluRs is further supported by two recent reports. Hartell (1994) showed that LTD in slice preparation is abolished by (RS)-α-methyl-4-carboxyphenylglycine (MCPG), a selective mGluR antagonist. Shigemoto et al. (1994) demonstrated that antibodies against mGluR1, a subtype of mGluRs that is abundant in Purkinje cells (Masu et al., 1991; Shigemoto et al., 1992; Fotuhi et al., 1993), block LTD induction in cell culture preparation.

Functional significance of cerebellar LTD in certain types of motor learning has been suggested by studies carried out using the lesion technique or the in vivo recording technique of neural activity in behaving animals (Ito, 1984, 1989; Thompson and Krupa, 1994). For example, the memory trace for classical conditioning of eyeblink responses seems to be stored in the cerebellum, either in the cortex or deep nuclei, or both (Thompson, 1986). The two excitatory inputs to the cerebellum, mossy fibers and CFs, are shown to carry conditioned stimulus (CS) and unconditioned stimulus (US), respectively. These two types of inputs converge in both the cerebellar cortex and the deep nuclei. In the cerebellar cortex, mossy fibers make excitatory synaptic contacts on granule cells whose bifurcated axons are PFs. Therefore, CS and US eventually converge on Purkinje cells. Since the induction of LTD requires conjunctive activation of PFs (CS pathway) and CFs (US pathway), it fulfills the condition for associative learning. Thus, LTD is a good candidate for a cellular mechanism of eyeblink conditioning. However, direct evidence for causal relationship between LTD at the cellular level and learning at the behavioral level is lacking. Attempts to link these two have been hindered by the difficulty to manipulate LTD selectively without affecting other brain functions. The recently developed gene-targeting technique to produce mutant mice defective in a particular gene product provides an opportunity for such a test. This approach has been used with some success for relating hippocampal LTP to spatial learning (Silva et al., 1992a, 1992b; Grant et al., 1992; Abeliovich et al., 1993a, 1993b).

In the present study, we have produced mice that lack mGluR1. The mice are viable but clearly ataxic. They show characteristic cerebellar symptoms such as ataxic gait and intention tremor. The anatomy of the cerebellum is not apparently disturbed. At the cellular level, excitatory synaptic transmission from PFs to Purkinje cells and that from CFs to Purkinje cells appear to be functional, and voltage-

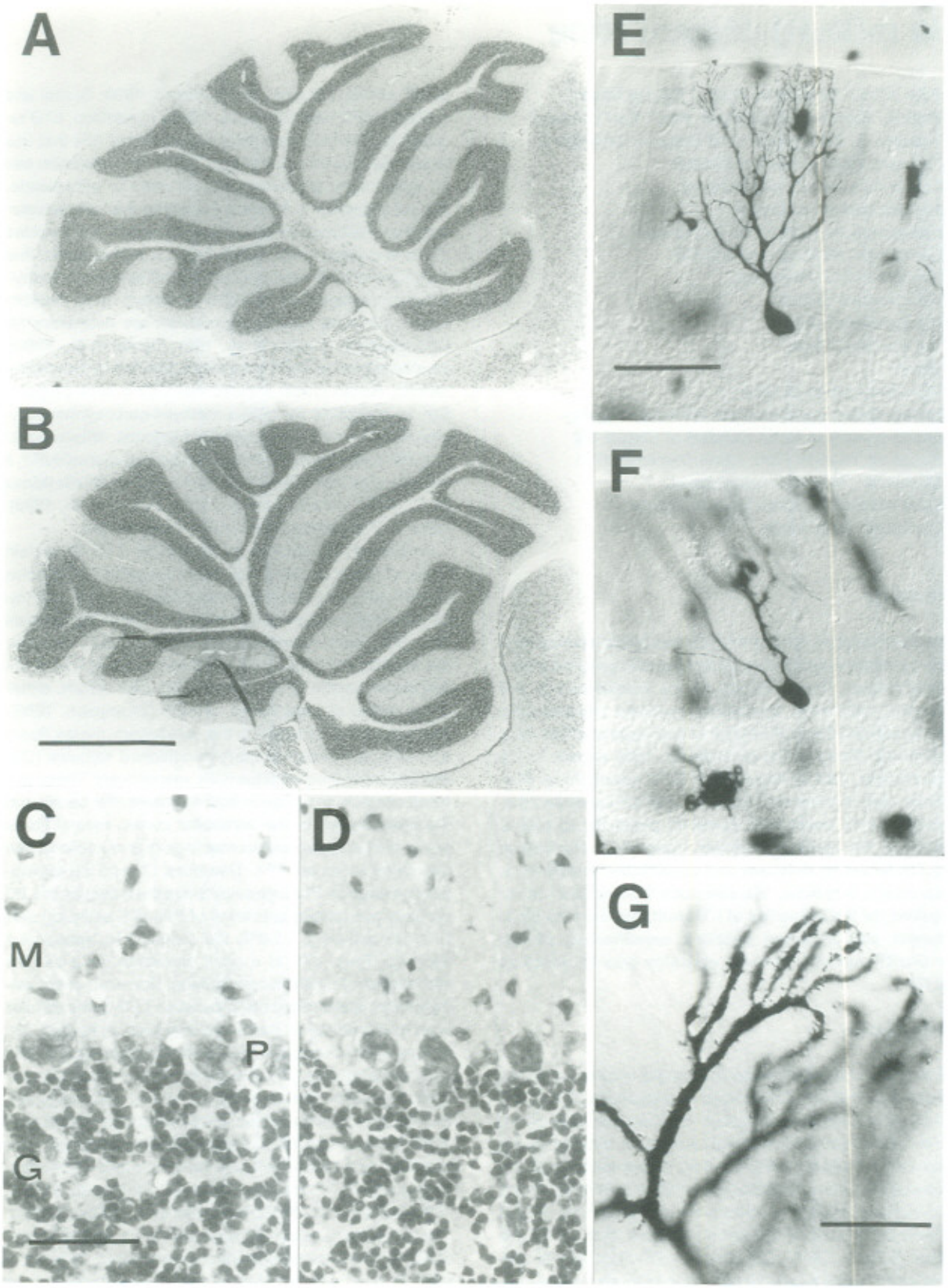

Figure 1. Anatomy of the mGluR1 Mutant Cerebellum

Comparison of a parasagittal section of the wild-type cerebellar cortex (A) and a slightly more lateral section from the mutant (B) illustrates that the size and cell density of this brain region are indistinguishable from the wild type, as is the pattern of cortical folding. This similarity is confirmed

gated Ca^{2+} channels of Purkinje cells are normal. Moreover, both PF and CF synapses display normal short-term synaptic plasticity to paired stimuli. By marked contrast, LTD is clearly deficient in the mutant mice. At the behavioral level, we found that the conditioned eyeblink response is impaired in the mutant mice. We thus conclude that mGluR1 is required for the induction of LTD and that the ataxic behavior and impaired eyeblink conditioning of the mGluR1 mutant mice is mainly due to deficient LTD.

Results

Anatomy of mGluR1 Mutant Cerebellum

In the cerebellum, mGluR1 is most abundant in the Purkinje cells, in which it is found at all levels of the dendritic tree (Martin et al., 1992). Nevertheless, the cellular composition of the cerebellum is unaffected by the mGluR1 mutation as viewed in cresyl violet–stained material (Figures 1A–1D). The size and cell structure of the cerebellum are normal. We further examined the Purkinje cell dendrites in the mutant animals. Two mutants and two wild-type controls were processed for Golgi impregnation with a modified Golgi–Cox stain (see Experimental Procedure). In general, neuronal dendrites throughout the brain of the mutants are well formed. Indeed, most qualitative features of the mutant Purkinje cell dendritic tree were unaltered (Figure 1E). The size of the dendrites appear normal, and the tertiary branchlets are studded with synaptic spines (Figure 1G). However, two minor abnormalities were noted in the mutants. There appears to be a moderate increase in the percentage of multipolar cells (cells with more than one dendrite emanating directly from the cell body) in the mutants (11.1%, n = 369), compared with the wild-type cerebellar cortex (5.9%, n = 152; Figure 1F). In addition, there might be a slight decrease in the overall complexity of Purkinje dendritic branches in the mutant brain (data not shown).

Excitatory Synaptic Transmission Is Functional but Slightly Modified

A characteristic feature of the excitatory synaptic transmission in cerebellar Purkinje cells is that it is exclusively mediated by a non-N-methyl-D-aspartate (non-NMDA) subtype of glutamate receptors (Kano and Kato, 1987; Kano et al., 1988; Llano et al., 1991b). We verified that this is also true for the mouse. Figure 2 presents sample records of PF-mediated (PF-) and CF-mediated excitatory postsynaptic currents (CF-EPSCs) of wild-type and mutant Purkinje cells from experiments in which effects of an NMDA blocker, DL-2-amino-5-phosphonopentanoate (AP5; 100 μM), and a non-NMDA antagonist, 6-cyano-7-nitroquinoxaline-2,3-dion (CNQX; 10 μM), were examined. We verified that both PF- and CF-EPSCs are mediated by non-NMDA receptors in wild-type and mutant mice as previously shown for other animal species (Figure 2). These results exclude the possibility of Ca^{2+} entry through NMDA receptor channels during excitatory synaptic transmission in mouse Purkinje cells. Thus, the main pathway of Ca^{2+} influx to Purkinje cells must be through voltage-gated Ca^{2+} channels, which were demonstrated to be normal in mutant neurons (see Figure 4).

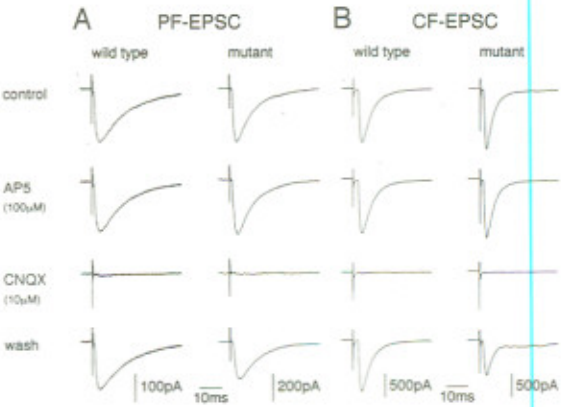

Figure 2. EPSCs in the Wild-Type and Mutant Purkinje Cells

(A) PF-EPSCs in a wild-type (25 days old) mouse and a mutant (17 days old) mouse. (B) CF-EPSCs in a wild-type (8 days old) mouse and a mutant (9 days old) mouse. Note that neither PF-EPSCs nor CF-EPSCs were blocked by a specific NMDA receptor antagonist, AP5 (100 μM), but both were totally suppressed by a non-NMDA receptor antagonist, CNQX (10 μM). In all records, the perfusate was Mg^{2+} free Ringer containing 10 μM glycine to maximize NMDA receptor mediated currents. AP5 and CNQX were bath applied. Stimuli were repeated at 0.5 Hz for PF-EPSCs and at 0.1 Hz for CF-EPSCs. Each trace is the average of five consecutive EPSCs. Holding potential was −60 mV except for the mutant in (B) (−70 mV).

PF-EPSCs in the mutant Purkinje cells appeared to be similar to those in wild-type cells, except for a slight difference in the decay time course (Figure 2A). The average decay time constant, which may be described by a single exponential function (Llano et al., 1991b), is 11.4 ± 0.5 ms in wild-type (n = 16 from nine mice, 17–28 days old) and 9.9 ± 0.4 ms in mutant (n = 16 from ten mice, 17–28 days old) Purkinje cells. This difference is significant ($p < 0.05$, t-test). The values of access resistance during recording of PF-EPSCs were 11.6 ± 0.3 MΩ (n = 16) for the wild-type and 11.8 ± 0.3 MΩ (n = 16) for the mutant, respectively, indicating that the recording conditions were identical. Thus, the synaptic transmission between PF and

at higher magnification where the microarchitecture of the cerebellar cortex of the wild-type mouse (C) can be seen to be comparable in every respect to that of the mGluR1 mutant (D). M, molecular layer; P, Purkinje cell layer; G, granule cell layer. The dendrites of the Purkinje cells were examined using a modified Golgi–Cox stain. Most cells in the mGluR1 mutant have a well-branched, iso-planar dendritic tree (E), although the branching density of the cells appeared reduced from the wild type. Despite this, the tertiary branchlets of the mutant Purkinje cell dendrites are studded with spines, the postsynaptic side of the PF–Purkinje cell synapse (G). An additional abnormality noted in the mutant animals was the presence of increased numbers of Purkinje cells with more than one dendrite issuing from the cell body (F). Scale bars indicate the following lengths: (A) and (B), 1 mm; (C) and (D), 100 μm; (E) and (F), 100 μm; (G), 20 μm.

Purkinje cell synapses is functional in mutant mice, but there is a slight modification.

Similar results were also observed in CF-EPSCs (Figure 2B). The average decay time constant, which may also be described by a single exponential function (Llano et al., 1991b), is significantly shorter in the mutant Purkinje cells (3.8 ± 0.3 ms, n = 13 from ten mice, 7–17 days old) in comparison with the wild-type cells (6.9 ± 0.8 ms, n = 7 from five mice, 7–17 days old; $p < 0.01$, t-test). Again, the values of access resistance were identical for the wild type (11.3 ± 1.0 MΩ, n = 7) and the mutant (11.9 ± 0.6 MΩ, n = 13). Since the decay time constant reflects electrotonic length between the recording electrode and the site of synapses, we suspect that this may be related to the minor morphological modifications observed in the mutant Purkinje dendritic trees (see Figure 1).

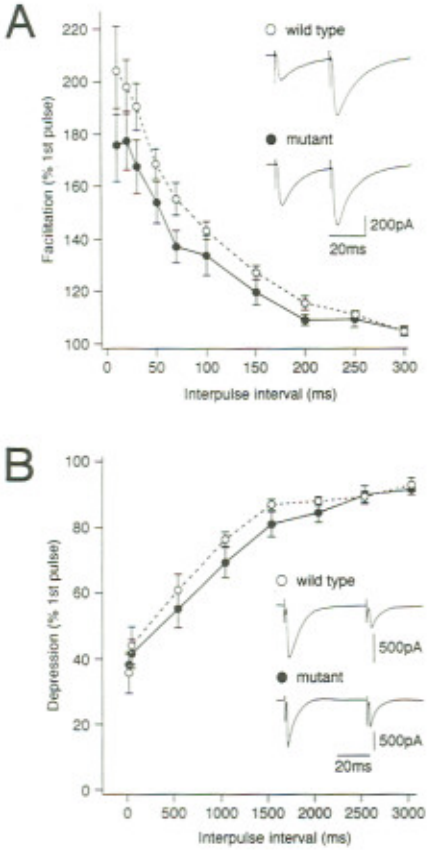

Figure 3. Short-Term Synaptic Plasticity Is Normal in the Mutant Purkinje Cells

(A) Paired-pulse facilitation of PF-EPSCs in the wild-type (open circles, n = 8 from eight mice) and mutant (closed circles, n = 7 from five mice) Purkinje cells. The second response (expressed as percentage of response to the first pulse, mean ± SEM) is plotted as a function of interpulse intervals. Stimulus pairs were applied at 0.5 Hz. Insets show example of PF-EPSCs to pairs of stimuli separated by 30 ms in transverse cerebellar slices from wild-type (19 days old) and mutant (17 days old) mice. Each trace is the average of five consecutive EPSCs. Holding potential was −60 mV.

(B) Paired-pulse depression of CF-EPSCs in the wild-type (open circles, n = 7 from three mice) and mutant (closed circles, n = 8 from seven mice) Purkinje cells, which is illustrated similarly to (A). Stimulus pairs were applied at 0.1 Hz. Insets show examples of CF-EPSCs to pairs of stimuli separated by 50 ms in sagittal cerebellar slices from wild-type (9 days old) and mutant (12 days old) mice. Each trace is the average of five consecutive EPSCs. Holding potential was −20 mV.

Short-Term Synaptic Plasticity Is Normal

Short-term historic effects, reflected as the response to the second stimulus of a pulse pair, are one important aspect of synaptic function. It has been reported that PF-EPSCs display a facilitation, while CF-EPSCs display a depression, to the second stimulus of a pulse pair (Konnerth et al., 1990). In the mutant mice, PF-EPSCs and CF-EPSCs show prominent paired-pulse facilitation and depression, respectively (Figures 3A and 3B). The amplitudes are not significantly different from those of the wild-type mice at all interpulse intervals tested (Figures 3A and 3B). Thus, short-term synaptic plasticity is unimpaired at both PF and CF synapses in the mutant mice. Paired-pulse synaptic plasticity is presumably caused by increase or decrease of transmitter release from presynaptic terminals (Zucker, 1989; M. K. and K. Hashimoto, unpublished data). Therefore, the present results strongly suggest that presynaptic functions of both CF and PF synapses are unimpaired in the mutant mice.

Voltage-Gated Ca^{2+} Currents Are Normal

The induction of LTD requires transient elevation of intracellular Ca^{2+} concentration mediated by the activation of voltage-gated Ca^{2+} channels (Sakurai, 1990; Hirano, 1990; Linden and Connor, 1991; Linden et al., 1991; Konnerth et al., 1992). Hence, we tested whether Ca^{2+} currents in

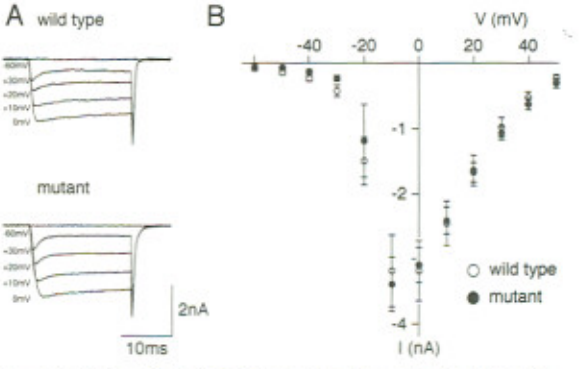

Figure 4. Voltage-Gated Ca^{2+} Currents Are Normal in the Mutant Purkinje Cells

(A) Whole-cell membrane currents elicited with 20 ms voltage steps from a holding potential of −80 mV to various test potentials of −60 mV, 0 mV, +10 mV, +20 mV, and +30 mV. Slices obtained from wild-type (6 days old) and mutant (5 days old) mice.

(B) Current–voltage relations for average peak Ca^{2+} currents in wild-type (open circles, n = 7 from three mice) and mutant (closed circles, n = 7 from three mice) Purkinje cells. Error bars display SEM. The external Ringer solution contained 0.5 μM tetrodotoxin (TTX) and 75 mM tetraethylammonium (TEA) chloride to block Na^+ and K^+ currents, respectively. The concentration of NaCl was reduced to 50 mM to keep the osmolarity constant. The pipette solution contained the following: 60 mM CsCl, 20 mM TEA–Cl, 20 mM BAPTA, 30 mM Cs D-gluconate, 30 mM HEPES, 4 mM $MgCl_2$, 4 mM ATP (pH 7.3, adjusted with CsOH).

Purkinje cells were normal in the mutant mice. Whole-cell membrane currents in response to voltage steps were recorded from wild-type and mutant Purkinje cells in cerebellar slices from young mice (5–6 days old). Young mice were used because Purkinje cells at this age are almost devoid of dendrites, and therefore, a good voltage control of the cell membrane can be achieved.

Voltage-gated Ca^{2+} currents are similar in their kinetics and amplitudes between wild-type and mutant Purkinje cells (Figure 4A). The current–voltage relations of Ca^{2+} currents are virtually identical between the two groups (Figure 4B). These results indicate that voltage-gated Ca^{2+} currents are normal in the mutant Purkinje cells.

LTD Is Deficient

To assess fully the effect of mGluR1 gene deletion on the induction of cerebellar LTD, we used two different protocols to induce LTD. The first protocol is conventional and similar to the ones used previously to induce LTD in both whole-animal and slice experiments (Ito et al., 1982; Ekerot and Kano, 1985; Sakurai, 1987). This protocol is composed of 480 single PF stimuli in conjunction with a depolarizing pulse (for 50 ms from a holding potential of −60 mV to 0 mV) repeated at 4 Hz (CJ-4Hz). The second protocol is analogous to the one used by Hartell (1994) to demonstrate that the mGluR antagonist MCPG blocks LTD induction. This consists of 96 trains of PF stimuli (five pulses at 100 Hz) in conjunction with a depolarizing pulse (for 50 ms from a holding potential of −60 mV to 0 mV) delivered every 2 s (CJ-train).

We substituted CF stimulation with postsynaptic depolarization, which has been shown to be effective for the induction of LTD (Hirano, 1990; Crepel and Jaillard, 1991; Linden and Connor, 1991; Linden et al., 1991; Konnerth et al., 1992). This procedure ensures that mGluR1 in the Purkinje cells would be activated by glutamate released from PFs but not from CFs. In addition, this method eliminates complications arising from white matter stimulation, which may also activate the mossy fiber–granule cell–PF pathway.

Whole-cell recording was made from Purkinje cells in transverse cerebellar slices from 18- to 25-day-old mice. PFs were stimulated in the middle molecular layer (pulse width 0.1 ms, strength 1–10 V) about 200 μm away from the recorded Purkinje cells. Conjunctive stimulation was applied after stable recording of PF-EPSCs for 10 min. Passive membrane properties of Purkinje cells were monitored by applying 10 mV hyperpolarizing pulses. Series resistance was monitored intermittently every 3 min and was kept constant around 10–15 MΩ.

In the wild-type Purkinje cells, both protocols induced significant LTD of PF-EPSCs (Figures 5A and 5C). The amplitudes of depression measured during 25–35 min after conditioning ranged from 46.0%–97.5% (76.5 ± 5.6%, n = 9 from seven mice) for the CJ-4Hz, and from 60.6%–87.5% (77.2 ± 3.7%, n = 7 from four mice) for the CJ-train (Figure 6). In contrast, neither the CJ-4Hz nor the CJ-train protocol induced LTD in mutant slices (see Figures 5B and 5D). The amplitudes of depression measured during 25–35 min after conditioning ranged from 83.1%–116.7% (104.1 ± 4.7%, n = 7 from five mice) for the CJ-4Hz, and from 98.0%–127.4% (107.4 ± 3.8%, n = 8 from six mice) for the CJ-train (Figure 6). In mutant slices, the average amplitudes tend to be potentiated rather than depressed for both the CJ-4Hz and CJ-train protocols. Thus, cerebellar LTD is clearly deficient in the mGluR1 mutant mice.

Motor Coordination Is Impaired

The mutant mice are viable but clearly ataxic. They show characteristic cerebellar symptoms such as ataxic gait and intention tremor. At rest, the mice sit quietly, but on initia-

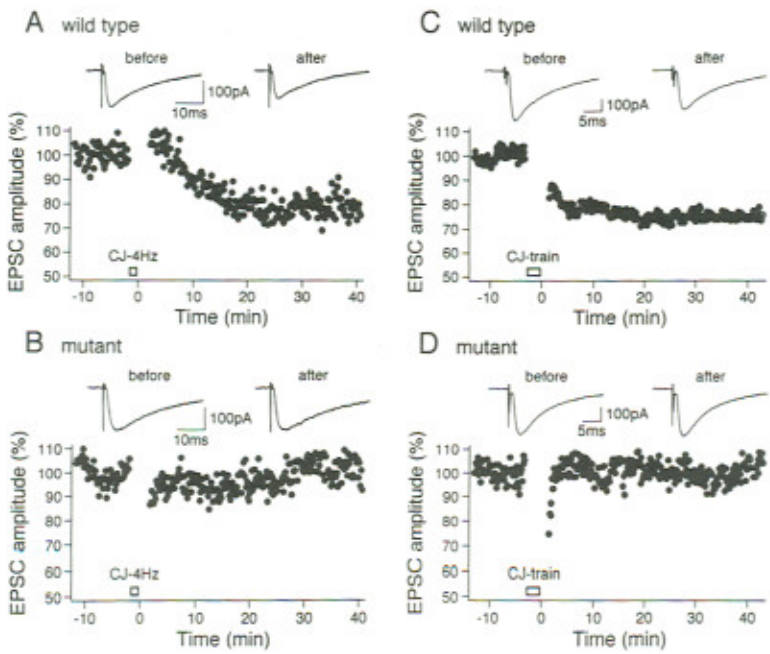

Figure 5. LTD Is Deficient in Mutant Purkinje Cells

(A) LTD was induced by a conventional conjunction protocol (CJ-4Hz) in a wild-type mouse (19 days old). This protocol comprises 480 single PF stimuli in conjunction with a depolarizing pulse (for 50 ms from a holding potential of −60 mV to 0 mV) repeated at 4 Hz.

(B) The CJ-4Hz protocol failed to induce LTD in a mutant mouse (20 days old). Test PF stimulation was delivered at 0.5 Hz. Each point represents the amplitude of the averaged PF-EPSC of five consecutive stimuli expressed as percentage of the baseline before conditioning. Sample PF-EPSC traces are taken before and 30 min after LTD induction.

(C) LTD was induced by another conjunction protocol (CJ-train) in a wild-type mouse (20 days old). The CJ-train protocol consists of 96 trains of PF stimuli (five pulses at 100 Hz) in conjunction with a depolarizing pulse (for 50 ms from a holding potential of −60 mV to 0 mV) delivered at an intertrain interval of 2 s.

(D) The CJ-train protocol failed to induce LTD in a mutant mouse (19 days old). Data were sampled and illustrated similarly to (A) and (B).

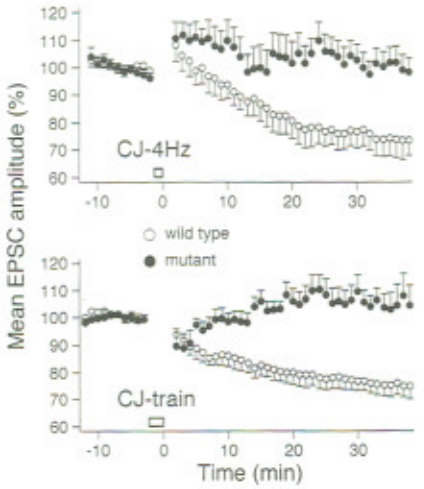

Figure 6. Average Changes in PF-EPSC Amplitudes Following Two Conjunction Protocols in Wild-Type and Mutant Purkinje Cells

Summary of experiments in which the CJ-4Hz (upper panel) and CJ-train (lower panel) protocols were applied to the wild-type (open circles) and mutant (closed circles) Purkinje cells. The amplitude of PF-EPSC is normalized to the baseline value before conditioning. Error bars display SEM. Each point represents average of thirty consecutive PF-EPSCs. Number of tested Purkinje cells are the following: n = 9 (seven slices from seven mice) for wild-type CJ-4Hz, n = 7 (five slices from five mice) for mutant CJ-4Hz, n = 7 (five slices from four mice) for wild-type CJ-train, and n = 8 (seven slices from six mice) for mutant CJ-train.

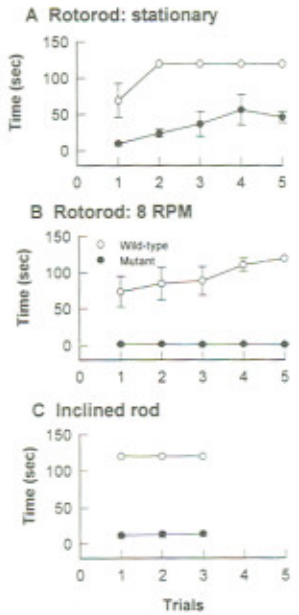

Figure 7. Mutant Mice Are Impaired in Motor Coordination

(A) Stationary rotorod test. Wild-type mice (n = 6) were able to stay and move around on the roller for an extended period of time; mutant mice (n = 6), although initially able to cling to the rod, fell off immediately once they initiated any movement ($F[1, 59] = 102.5$, $p < 0.0001$). Maximal allowed time was 120 s.

(B) Running rotorod. Wild-type mice (n = 6) managed to stay on the roller for the entire 2 min by the fifth trial; mutant mice (n = 6) fell immediately after the rotorod started rotating ($F[1, 59] = 156.8$, $p < 0.0001$). The speed was 8 rpm.

(C) Inclined rod. Wild-type mice (n = 6) were able to remain and move around on the rod; mutant mice (n = 6) had difficulty staying on the rod ($F[1, 35] = 3523$, $p < 0.0001$).

tion of movement, they begin to shake. In testing their motor coordination, we subjected wild-type and mutant mice to two motor tests, the rotorod and the inclined rod. Mutant mice had difficulty staying on the rotorod, even if it was stationary (Figure 7A). Once the rotorod began to turn, mutant mice fell off immediately, while wild-type mice managed to stay on the rod (Figure 7B). Similarly, on being placed in the center of the inclined rod, mutant mice had difficulty staying on it (Figure 7C).

The mutant mice walk with a wide base rolling motion from side to side. Their motion typically ends with a large amplitude tremor that involves the entire body. Analysis of hind footprints showed that mutant mice cannot walk along a straight line and that their feet tend to sweep along the floor (Figure 8). The length of steps appears to be shorter in mutant animals (Figure 8). These results further suggest neuronal impairments in the cerebellum (Brunner and Altman, 1973; Pellegrino and Altman, 1979).

Associative Learning of Eyeblink Response Is Impaired

Introduced by Gormezano et al. (1962), rabbit eyeblink conditioning has become a prevalent and extensively used technique in the study of classical conditioning. Others have shown that reliable eyeblink conditioning could be obtained with rats with paired but not with unpaired trials (Hall, 1973; Disterhoft et al., 1977; Skelton, 1988; Schmajiuk and Christiansen, 1990). As in the case of rabbits (Thompson, 1986), the cerebellum is also critically involved with eyeblink conditioning in rats (Skelton, 1988; Stanton and Freeman, 1994). The mouse eyeblink conditioning method utilized in this study is similar to the one used in conditioning freely moving adult rats (Skelton, 1988; Stanton and Freeman, 1994). In this procedure, mice were surgically implanted with two electrodes: one for measuring eyelid electromyographic (EMG) activity and the other for delivering a brief electrical stimulation in the vicinity of the eye.

We found that wild-type mice showed eyeblink conditioning following pairings of an initially neutral tone with a 5 ms perioccular shock (Figure 9). The general features of the eyeblink response resembled that previously reported for rats (Skelton, 1988) and rabbits (Schneiderman et al., 1962). On some trials, mice showed responses with relatively short onset latencies (20–100 ms) like those described as "startle responses" in adult rats (Skelton, 1988). However, these responses were lower in amplitude and longer in duration, and, unlike rats, they appeared to have an associative component, in that they were less prevalent in unpaired animals. In the paired group, conditioned response (CR) amplitudes (not normalized) in the first training block of the first session did not seem to differ between the wild-type (1.248 ± 0.699 [mean ± SEM], n = 10) and mutant (1.52 ± 0.5288 [mean ± SEM], n = 10; p =

Figure 8. Hind Footprint Pattern of Wild-Type and Mutant Mice

The mutant animal walks with a wide base rolling motion from side to side. Motion typically ends with a large amplitude tremor that involves the entire body. The mutant mouse is unable to walk along a straight line. Walking steps appear to be shorter in the mutant mouse, and their feet tend to sweep along the floor.

0.699, t-test) mice, nor did the amplitudes of unconditioned responses (UR; wild type: 7.996 ± 0.5483 [mean ± SEM]; mutant: 9.22 ± 1.1574 [mean ± SEM]; p = 0.3514). Similar results were observed in the unpaired groups (wild-type CR: 0.26 ± 0.1448 [mean ± SEM]; mutant CR: 0.4533 ± 0.2915 [mean ± SEM]; p = 0.5657, n = 6 each). Thus, CR baseline responses were not significantly different between the wild-type and mutant mice and in both types of mice, the responses were lower in the unpaired groups compared with the corresponding paired groups. For each mouse, we calculated the average CR and UR amplitudes of the first session and took these as the baseline CR and UR responses, respectively, of that mouse. We then normalized amplitudes of the blocks in the subsequent sessions of each mouse against its corresponding baseline.

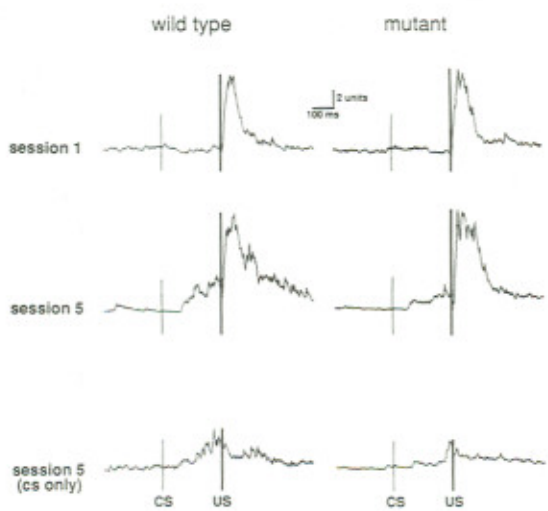

Figure 9. EMG Records of Associative Eyeblink Conditioning of Wild-Type and Mutant Mice

Examples of eyeblink response to tone and shock in the initial (session 1) and well-trained (session 5) stages in wild-type (left panel) and mutant (right panel) mice. Onsets of tone (CS) and shock (US) are indicated in the bottom of the figure. The interstimulus interval between the CS and US was 280 ms. The last trace is a tone only trial. Conditioned eyeblink response (CR) was elicited by tone.

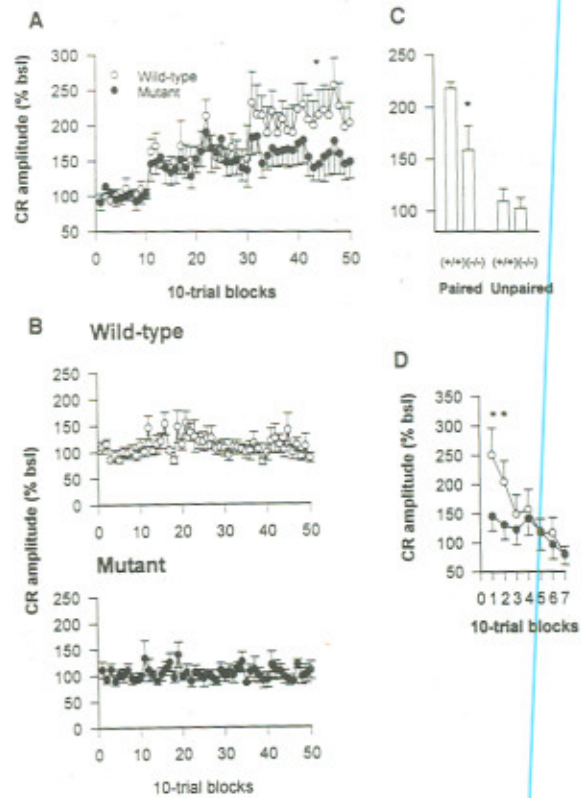

Figure 10. Mutant Mice Show Less Eyeblink Conditioning Than Wild-Type Mice

(A) CR amplitudes expressed as percent of the first averaged session during CS–US paired trials in wild-type (n = 10) and mutant (n = 10) mice. Learning is evident in increasing amplitudes over training blocks in both groups (session 1 versus session 5: $F[1, 395] = 130.6$, $p < 0.0001$) with a paired paradigm. Mutant CR amplitudes are significantly less in the fifth session compared with the wild type ($F[1, 395] = 15.8$, $p < 0.0001$). Each session contains ten blocks; each block contains nine paired trials and one tone-only trial. The peak amplitudes were measured within a 100 ms window before the US onset in paired trials.

(B) Conditioned responses were observed in neither wild-type (n = 6) nor mutant (n = 6) mice when they were trained with an unpaired paradigm (see the Experimental Procedures). CR amplitudes were measured in tone-only trials.

(C) Average CR amplitudes in the fifth session are compared between the paired and the unpaired paradigms. The training-related CR impairment in mutant animals is evident in paired groups ($t_{18} = 2.48$, $p = 0.023$) but not in unpaired groups (see B).

(D) The CR amplitudes in both the wild-type and mutant mice after five sessions of paired training were extinguished to preconditioning baselines when CS-only trials were presented. Each block contains ten CS-only trials. The mutant CR amplitudes are initially less than the wild-type group (block 1: $t_{18} = 2.55$, $p = 0.022$; block 2: $t_{18} = 2.09$, $p = 0.053$).

The CR appeared only in the paired groups (Figure 10A) but not in the unpaired groups (Figure 10B), and it disappeared during extinction trials in which the CS alone was repeatedly presented to trained animals (Figure 10D). Thus, the CR is associative and pairing specific. Significant conditioning occurred in the second session (blocks 11–20, Figure 10A) in wild-type and mutant animals. However, the two groups differed in their CR amplitudes in the fifth session (blocks 41–50), in which the wild-type mice surpassed the mutant group ($p < 0.0001$, also Figure 10C). Normalized CR amplitudes of wild-type animals seemed to increase over the entire period of training sessions, while those of mutant mice reached a plateau after the second session.

Neither wild-type nor mutant animals in unpaired groups showed any noticeable conditioning (Figure 10B). Furthermore, the UR, i.e., eyeblink elicited by just a shock, did not differ between the wild-type and mutant animals in the two paired groups or in the unpaired groups (data not shown). Thus, the eyeblink reflex did not seem to be modified by the gene knockout as UR amplitudes did not differ between wild-type and mutant mice.

Discussion

Anatomical Alterations in the Mutant Cerebellum Are Minor

At the light microscope level, few changes in cytoarchitecture, cell density, or cell size have been observed in the cerebellum of the mGluR1 mutant mice (Figures 1A–1D). Minor abnormalities observed are a moderate increase in the overall percentage of multipolar Purkinje cells and a small decrease in the overall extent of branching complexity of their dendrites (Figures 1E–1G). Thus, the well-studied developmental influence of the granule cell axon on the Purkinje cell dendrite cannot be entirely mediated by the mGluR1 protein. The quality and quantity of the afferent input of a neuron is perhaps the most important influence on the final shape of its dendritic arbor. Studies of early postnatal interference with cerebellar granule cell input using either X-rays (Altman, 1973; Crepel et al., 1980; Woodward et al., 1975), drugs (Hartkop and Jones, 1977), or genetic lesions such as weaver or reeler (Caviness and Rakic, 1978; Rakic, 1975; Rakic and Sidman, 1973) have shown that many features of the size, shape, and orientation of the Purkinje cell dendrite depend on the presence of healthy granule cells. The changes in the Golgi impregnation material from the mGluR1 mutant mice described in this paper are far less dramatic than those observed in these earlier studies. Although they are subtle, we cannot exclude the possibility that the morphological changes are associated with the observed failure of LTD, behavioral abnormalities, or both.

LTD Deficiency Is the Major Electrophysiological Abnormality in the Mutant Cerebellum

Electrophysiological studies confirmed that basic neural connections in the mGluR1 mutant cerebellum are largely normal. Thus, excitatory synaptic transmission from both PFs and CFs is functional, and their pharmacological nature is identical to that of wild-type animals (Figure 2). Short-term synaptic plasticity characterized by responses to paired stimuli is essentially normal in both PF and CF synapses (Figure 3), suggesting that presynaptic functions are normal in the mutant mice. Moreover, voltage-gated Ca^{2+} channels are normal (Figure 4). The only difference we could detect is that the time courses of EPSCs are faster in the mutant, which might be attributable to the slight modification of the Purkinje cell dendritic tree (Figures 1E–1G). Thus, LTD deficiency is the most prominent feature in the mutant cerebellum (Figures 5 and 6). These results suggest that mGluR1 is not essential for basic excitatory synaptic transmission, but it is for the induction of LTD. Our results obtained with slices of mGluR1 mutant cerebellum confirm the conclusion of the recent study carried out using anti-mGluR1 antibody in cultured neurons (Shigemoto et al., 1994).

mGluR1 at PF Synapses Is Required for LTD Induction

In wild-type mice, both CJ-4Hz and CJ-train protocols induced a stable LTD of PF-EPSCs (Figures 5 and 6). In contrast, neither protocol induced LTD in the mutant animals (Figures 5 and 6), and the average amplitudes tended to be potentiated rather than depressed (Figure 6). These results suggest that activation of both AMPA receptors and mGluR1 at PF synapses leads to LTD when paired with depolarization of Purkinje cells. On the other hand, activation of AMPA receptors alone in conjunction with depolarization does not depress, but slightly potentiates PF–Purkinje cell synapses. This is consistent with the previous findings in cell culture preparations: iontophoretic application of both AMPA and trans-1-aminocyclopentane-1,3-dicarboxylic acid (trans-ACPD) induced LTD, while AMPA alone caused slight potentiation when paired with depolarization of Purkinje cells (Linden et al., 1991).

A recent study showed that slight potentiation rather than depression is induced when the culture is given glutamate depolarization conjunction in the presence of antibodies against mGluR1 (Shigemoto et al., 1994). This study, however, did not address the issue of which of the two types of Purkinje cell synapses requires mGluR1 for LTD induction. mGluR1 is abundant not only in dendritic spines where PF synapses are present, but also in cell bodies and dendritic shafts where CFs make synaptic contacts (Martin et al., 1992; Shigemoto et al., 1994). Since we demonstrated LTD deficiency of the mutant mice by adopting depolarizing pulses rather than CF stimulation, we can conclude that mGluR1 at PF–Purkinje cell synapses are essential for LTD induction. Whether or not mGluR1 at the CF–Purkinje cell synapse is involved in LTD induction remains to be seen.

What cellular processes mediate LTD induction following activation of mGluR1? It has been shown that mGluR1 activation results in formation of 1,2-diacylglycerol (DG), inositol trisphosphate, arachidonic acid, and cAMP (Masu et al., 1991; Aramori and Nakanishi, 1992). Furthermore, activation of protein kinase C by DG and Ca^{2+} has been shown to mediate LTD (Crepel and Krupa, 1988; Linden and Connor, 1991). Whether some intracellular responses

other than protein kinase C mediate LTD remains to be investigated (for review see Linden, 1994).

LTD Deficiency and Cerebellar Symptoms in the Mutant Mice

The mGluR1 mutant mice have symptoms that are typical for cerebellar dysfunction. The anatomy of the cerebellum is essentially normal, and basic excitatory synaptic transmission is almost unimpaired, nevertheless LTD is deficient. This suggests that LTD deficiency is a main cause of cerebellar symptoms in the mutant mice. In other animal models with cerebellar symptoms such as reeler, staggerer, and nervous mice, the animals have clear morphological deficits (Sotelo, 1990). Therefore, the mGluR1 mutant mouse is an animal model in which cerebellar symptoms are linked to LTD deficiency rather than just abnormal morphology in the cerebellum.

It has been proposed that the role of the cerebellar cortex is to combine simpler elements of movement into complex coordinated acts (Thach et al., 1992). The PF, by virtue of its connection through Purkinje cells to the deep nuclei, appears optimally designed for conveying sensory signals needed for such coordination at several joints. CF signals may represent error control signals in the performance of the motor systems. When the animal makes "errors" during movements, CFs would fire and depress the transmission of PF signals responsible for error generation, through induction of LTD. Repetition of such trials would lead to elimination of wiring of the cerebellum that generates "errors" and, in parallel, improvements of motor skills (Marr, 1969; Albus, 1971; Ito, 1984, 1989). According to this hypothesis, mGluR1 mutant mice lacking cerebellar LTD would not improve motor skills during development, and the cerebellar circuitry would remain naive and unlearned.

LTD Deficiency and Impaired Eyeblink Conditioning in the Mutant Mice

The essential memory trace circuit for long-term associative memory such as classical conditioning of sensorymotor responses is thought to include the cerebellum and its associated brain stem circuits (Thompson, 1986). Although other sites such as the brain stem have also been suggested (Welsh and Harvey, 1989, 1991; Bloedel, 1992), the cerebellum is thought to be the site of the formation and storage of this type of memory (see Thompson and Krupa, 1994). For instance, lesions of one of the deep cerebellar nuclei (interpositus nucleus) prevent acquisition and abolish retention of the conditioned eyeblink response (McCormick et al., 1982; Lincoln et al., 1982; Yeo et al., 1985a; Steinmetz et al., 1992; Clark et al., 1992; Krupa et al., 1993). Although the question of whether or not the cerebellar cortex is essential is unresolved, it is clear that it is critically important for normal learning of the conditioned eyeblink response. Thus, if lesions were made before training, lesions limited to lobule HVI slowed acquisition somewhat whereas larger lesions markedly impaired acquisition (Yeo et al., 1985b; Lavond et al., 1987; Yeo and Hardiman, 1992; Perrett et al., 1993). Although these lesion studies are effective in mapping neural circuitry involved in learning and identifying the site of memory traces, they do not illuminate synaptic mechanisms responsible for learning and memory.

Although the associative nature of cerebellar LTD makes it an attractive candidate for a synaptic mechanism for associative learning of eyeblink response, direct evidence is lacking. mGluR1 mutant mice that exhibit cerebellar LTD deficit provided an excellent opportunity to test this hypothesis. We thus adapted the eyeblink conditioning paradigm previously developed for rabbits (Thompson, 1986) and rats (Skelton, 1988; Stanton and Freeman, 1994) to mice. We found that, like wild-type mice, mutant mice are responsive to CS (tone) and US (shock). This finding is in agreement with electrophysiological data (Figure 2) that showed that the excitatory synaptic transmission is essentially normal at both PF–Purkinje and CF–Purkinje synapses. Thus, it is unlikely that, during eyeblink conditioning, sensory inputs could not reach Purkinje cells. Furthermore, execution of the unconditioned eyeblink response (UR) per se is normal in both its amplitude and shape (Figure 9). Thus, the mutant mice do not exhibit obvious performance deficits; they seem to have an unaffected motor reflex pathway.

We demonstrated that wild-type mice show eyeblink conditioning that resembles the conditioning previously found in rabbits (Thompson, 1986) or rats (Skelton, 1988). Conditioned responses in these mice were associative, in that they appeared only when the CS and US were temporally paired and that they disappeared during extinction training involving repeated presentations of the CS alone. Compared with wild-type mice, the training-related growth in the CR amplitude was impaired in the mGluR1 mutant mice, although some degree of eyeblink conditioning was present. This impairment was most evident toward the end of training in our experiment (Figure 10A). There are two possible explanations for the observed impairment: either the maximal level of learning (the asymptote) is reduced by the mutation, or the rate of learning is retarded. Further studies involving many more training sessions are required to find out which of these two possibilities is correct.

Although the conditioned eyeblink response seemed to be reduced in mutant mice compared with wild-type mice, they clearly still can learn the task. This partial impairment appears to be similar to that exhibited by animals with restricted cortical lesions (Lavond and Steinmetz, 1989). Thus, we conclude that mGluR1-mediated LTD in the PF–Purkinje synapses is a cortical mechanism important for the acquisition of the conditioned eyeblink response, but that it is not essential. Thus, the role of cortical LTD may be to optimize the learning that is mediated by other brain mechanisms. Perhaps learning-induced synaptic plasticity in the interpositus nucleus plays an essential role, although little is known about it (Racine et al., 1986). If such a mechanism exists, our data suggest that mGluR1 is not its essential component.

It is significant that we observed impairment of the conditioned eyeblink response in the mGluR1 mutant mice despite the fact that these mice exhibited virtually normal morphology and synaptic function in the cerebellum. The

LTD deficiency appears to be a major cerebellar feature that correlates with the learning impairment. Our results, therefore, demonstrate that deficiency of cerebellar LTD is correlated with impairment of associative learning of eyeblink response.

In the present study, we did not examine the inhibitory synapses in the mutant cerebellum. It has recently been revealed that inhibitory synapses also undergo long-term change. For example, long-lasting rebound potentiation is induced in GABAergic inhibitory synapses on Purkinje cells following activation of CFs (Kano et al., 1992). It remains to be seen whether inhibitory synapses are normal and display long-term change in the mutant Purkinje cells and whether they contribute to synaptic mechanisms for associative learning.

Experimental Procedures

Production of mGluR1 Mutant Mice

Mutant mice were produced as described in the accompanying paper (Aiba et al., 1994 [this issue of *Cell*]). Both wild-type and mutant mice utilized were of 129/Sv × C57BL/6 genetic background and were kept in the same room at the animal facility of the Massachusetts Institute of Technology with a 12 hr light–dark cycle.

Golgi Impregnation

Animals were sacrificed by CO_2 inhalation, and their brains were rapidly dissected. The tissue, freed of membranes, was immersed in a vial containing 20 ml of a solution of 1% potassium dichromate, 1% mercuric chloride, 0.45% potassium chromate in water. The vials were wrapped in aluminum foil and stored at room temperature for 8 weeks. After rinsing, the impregnated tissue was bisected on the midline, dehydrated, and embedded in 12% parlodian (Fisher). Blocks were hardened in chloroform vapors and sectioned on a sledge microtome at 100 μm. The sections were collected in 70% ethanol, then rehydrated in tap water, developed in 5% Na_2SO_3, rinsed, dehydrated, cleared in terpineol, and mounted on glass slides using Permount mounting medium. All photographs were done on a Leica DM RB microscope under either bright field or DIC optics.

Electrophysiology

Cerebellar slices were prepared from the wild-type and mutant mice as described previously (Edwards et al., 1989; Llano et al., 1991a, 1991b; Kano and Konnerth 1992). In brief, animals were decapitated by cervical dislocation, and the cerebellum was rapidly isolated and placed in ice-cold bicarbonate-buffered standard saline (for composition, see below). Either sagittal or transverse cerebellar slices of 200 μm thickness were cut with a vibratome and kept at 34°C for at least 1 hr in a chamber containing standard saline that was bubbled with 95% O_2 and 5% CO_2. One slice was then transferred to a recording chamber in which it was continuously perfused with the oxygenated standard saline. Recognition of layers within the cerebellar cortex and identification of Purkinje cells were easily achieved on slices when viewed using a 40× water immersion objective attached to a Zeiss upright microscope (Axioscope; Edwards et al., 1989; Llano et al., 1991a, 1991b). Cleaning of the surface of Purkinje cells and patch clamping of individual cells were the same as previously described (Edwards et al., 1989; Llano et al., 1991a, 1991b). All experiments were performed using whole-cell configuration of the patch-clamp technique with borosilicate pipettes (resistance of 2–4 MΩ when filled with an intracellular solution; see below). Ionic currents were recorded with an EPC-9 patch-clamp amplifier (HEKA) and stored on a DAT data recorder (Sony) for later analysis. The pipette access resistance was compensated as explained by Llano et al. (1991b). Stimulation and on-line data acquisition were performed using the PULSE program on a Macintosh computer (HEKA). The signals were filtered at 3 kHz and digitized at 20 kHz. Fitting of the decay phases of EPSCs was done with the PULSE-FIT program (HEKA). For stimulation of CFs and PFs, a glass pipette with 5–10 μm tip diameter filled with standard saline was used. Square pulses (duration, 0.1 ms; amplitude, 1–10 V) were applied for focal stimulation.

The composition of standard saline was the following: 125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 20 mM glucose, which was bubbled continuously with a mixture of 95% O_2 and 5% CO_2. Bicuculline (10 mM) was always present in the saline to block spontaneous inhibitory postsynaptic currents (Konnerth et al., 1990; Kano et al., 1992). For measuring voltage-gated Ca^{2+} currents, tetrodotoxin (TTX; 0.5 μM) and tetraethylammonium (TEA; 75 mM) were included to block Na^+ and K^+ currents, and the concentration of NaCl was reduced to 50 mM to keep the osmolarity constant. The standard pipette solution contained the following: 70 mM KCl, 60 mM K D-gluconate, 0.5 mM EGTA, 4 mM $MgCl_2$, 4 mM Na–ATP, 0.4 mM Na–GTP, and 30 mM HEPES (pH 7.3, adjusted with KOH). In experiments for measuring voltage-gated Ca^{2+} currents and CF-EPSCs, a Cs-based pipette solution with the following composition was used: 60 mM CsCl, 30 mM Cs D-gluconate, 20 mM TEA–Cl, 20 mM BAPTA, 4 mM $MgCl_2$, 4 mM ATP, and 30 mM HEPES, (pH 7.3, adjusted with CsOH). All experiments were carried out at a bath temperature of 32°C, except those for voltage-gated Ca^{2+} currents that were performed at room temperature.

Eyeblink Conditioning

Mouse eyeblink conditioning was adapted from a method for conditioning freely moving adult and infant rats (Skelton, 1988; Stanton et al., 1992; Stanton and Freeman, 1994). Mice were surgically implanted with two electrodes: one for measuring eyelid electomyographic (EMG) activity and the other for delivering brief electrical stimulation in the vicinity of the eye. Five to seven days were allowed for recovery from surgery, and operated mice were handled daily for three days prior to training. Subjects were placed in a sound-attenuated enclosure containing a speaker for delivering the auditory CS, and their electrodes were connected via a headstage and commutator to peripheral devices and a personal computer that collects data and controls experimental events (Stanton and Freeman, 1994).

Animals were trained with delay conditioning or unpaired control procedures. Delay conditioning trials involved pairings of a 285 ms tone CS (intensity 75 db, frequency 2.8 KHz) and a 5 ms perioccular shock as a US (intensity 4 mA). UR amplitudes ranged from 5–20 U. In the unpaired control condition, the CS and US were presented in a "pseudorandom" order such that no more than three presentations of either stimulus occurred consecutively. The paired and unpaired groups received the same number of stimulus presentations at the same average rate across each session. This unpaired group is an important control because it indicates levels of sensitization, pseudoconditioning, and/or spontaneous EMG activity that could lead one to overestimate the amount of associative learning in the paired condition.

Animals were trained over five daily sessions. Each session consisted of ten blocks of ten trials. In the paired condition, the first nine trials within each block involved pairings of the CS and US, whereas on the tenth trial, only the CS was presented (CS-alone trial). Trials were separated by variable intertrial intervals (ITI) with a mean of 60 s and a range of 36–84 s. In the unpaired groups, 100 CS-alone trials were mixed randomly with 100 US-alone trials in a pseudorandom order with an average of 30 s between trials.

The amplitude of integrated EMG activity was recorded for 1 s (Figure 9). Recording was interrupted during the 5 ms presentation of the US. Trials in which significant activity (>0.5 U) occurred during the pre-CS period were excluded from analysis. To minimize the contribution of short-latency responses to the CS (perhaps startle responses; see Skelton, 1988) to the CR-amplitude measure, only the final 100 ms of the CS period (prior to the US presentation) was used in determining CR amplitudes. In comparison with rats, both the wild-type and mutant mice exhibited more animal to animal variability in EMG activity and responsiveness to stimuli (CS and US). For each mouse, we calculated the average CR and UR amplitudes of the first session and took these as the baseline CR and UR responses, respectively, of that mouse. We then normalized amplitudes of the blocks in the subsequent sessions of each mouse against its corresponding baseline. Group-averaged CR amplitudes in the first session were 1.37 ± 0.42 (mean ± SEM, wild type) and 1.85 ± 0.67 (mean ± SEM, mutant) with paired training, 0.17 ± 0.06 (mean ± SEM, wild type) and

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0.28 ± 0.13 (mean ± SEM, mutant) with unpaired training; group-averaged UR amplitudes were 7.40 ± 0.54 (mean ± SEM, wild type) and 8.31 ± 1.11 (mean ± SEM, mutant). A factor of 0.5 U (noise level) was added to all amplitude measurements (block average) before normalization. The peak UR amplitudes were measured after the US onset.

Motor Skill Test

The rotorod (Ugo Basile Biological Research Apparatus, Milan, Italy) consists of a gritted plastic roller (4 cm diameter, 10 cm long) flanked by two large round plates (50 cm diameter) to prevent animals from escaping. The roller is driven by a motor. A mouse was placed on the roller, and the time it remained on the roller was measured. The inclined rod consists of a smooth plastic stick (1.5 cm diameter, 50 cm long, 30°). A mouse was placed in the midpoint of the rod, and the time remaining on the rod was measured. Footprints were made with ink and water color paper. Ink was applied to the hind paws of individual mice, and they were induced to walk forward, leaving a record on the paper.

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