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Persistent Multiple Climbing Fiber Innervation of Cerebellar Purkinje Cells in Mice Lacking mGluR1

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Summary

Most of the cerebellar Purkinje cells (PCs) of an adult animal are innervated individually by a single climbing fiber (CF) that forms strong excitatory synapses with the PCs. This one-to-one relationship between a PC and a CF is a consequence of a developmentally regulated regression of the innervation of PCs by CFs. We found that, in mice deficient in the type 1 metabotropic glutamate receptor (mGluR1), the regression of supernumerary CFs ceases by the end of the second postnatal week, which is about one week earlier than in normal mice. Consequently, about one third of PCs in the mGluR1 mutant mice are innervated by multiple CFs in adulthood. We conclude that the regression of CFs normally occurs in two developmental phases and that mGluR1 plays a crucial role in the second phase.

Introduction

Establishment of specific neuronal connections in the mature nervous system includes the process by which redundant connections formed earlier during development are eliminated subsequently (Purves and Lichtman, 1980; Shatz, 1990). In the cerebellum of adult animals, each Purkinje cell (PC) is innervated by a single climbing fiber (CF) that originates from the inferior olive of the medulla and forms strong excitatory synapses onto proximal dendrites of the PC (Ito, 1984). This one-to-one relationship between a PC and a CF is preceded by a developmental stage in which each PC is innervated by multiple CFs (see Crepel, 1982, for review). Massive elimination of synapses formed by supernumerary CFs occurs postnatally, and the state of monoinnervation is established at around postnatal day 20 (P20). This developmental change at CF–PC synapses seems to depend on the presence of intact granule cells and of the excitatory synapses formed onto the dendritic spines of PCs by parallel fibers (PFs), bifurcated axons of granule cells (see Crepel, 1982, for review). Thus, multiple innervation of CFs has been shown to persist in the cerebella of adult animals that are devoid of granule cells (see Crepel, 1982, for review) or PF–PC synapses, such as X-irradiated rats (Woodward et al., 1974; Crepel and Delhaye-Bouchard, 1979), *weaver* mutant mice (Crepel and Mariani, 1976), *reeler* mutant mice (Mariani et al., 1977), and *staggerer* mutant mice (Crepel et al., 1980; Mariani and Changeux, 1980). The mechanisms by which the granule cells affect the regression of CF–PC synapses via PF–PC synapses is poorly known.

We have previously produced and analyzed null mutant mice deficient in the type 1 metabotropic glutamate receptor (mGluR1) (Aiba et al., 1994a, 1994b). The mGluR1 mutant mice are viable but show symptoms of cerebellar dysfunction such as ataxic gait, intention tremor, and dysmetria (Aiba et al., 1994b; Conquet et al., 1994) and are impaired in motor coordination and motor learning (Aiba et al., 1994b). These mutant mice are deficient in long-term depression (LTD) at PF–PC synapses (Aiba et al., 1994b; Conquet et al., 1994), a form of synaptic plasticity in the cerebellum that was proposed to be a cellular basis for motor learning (Ito, 1984, 1989). In this study, we show that innervation of multiple CFs onto a PC persists into adulthood in the mGluR1 mutant mice. Regression of synapses formed by supernumerary CFs appears to occur during the first and second postnatal weeks, but not in the third postnatal week. The PF–PC synapses appear to be intact morphologically and electrophysiologically. Thus, the effect of the mGluR1 mutation on the regression of CF–PC synapses does not seem to be through gross abnormalities at the PF–PC synaspes.

Results

Persistent Innervation of mGluR1 Mutant PCs by Multiple CFs

We measured excitatory postsynaptic currents (EPSCs) exhibited by PCs after stimulation of the CFs in the granule cell layer near the recorded PCs (50–100 μM away) (Konnerth et al., 1990; Llano et al., 1991; Kano et al., 1992, 1995; Aiba et al., 1994b). In most PCs (134 out of 147) from P22–P75 wild-type mice, large EPSCs were elicited in an all-or-none fashion as the stimulus intensity was gradually increased (pulse width, 0.1 ms; strength, 0–100 V) (Figure 1A). In contrast, in 32.6% (42 out of 129) of the mutant PCs, the EPSCs appeared in two

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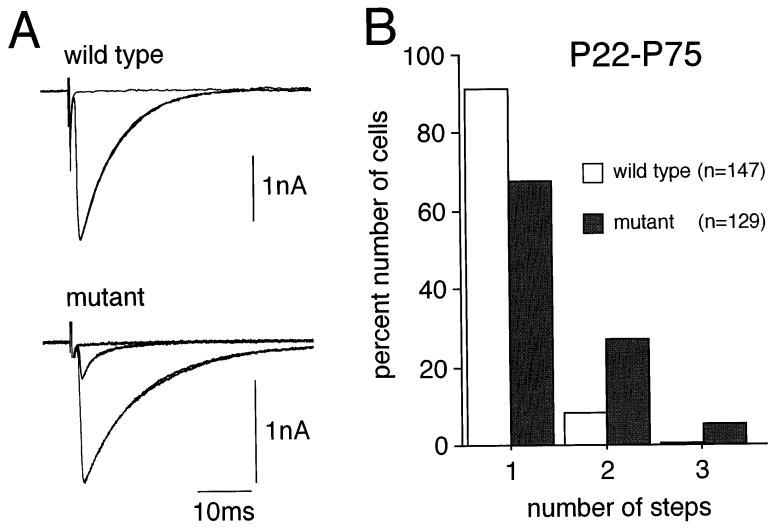

Figure 1. Multiple Innervation of PCs by CFs in mGluR1 Mutant Mice

(A) EPSCs elicited by stimulation of CFs in the granule cell layer in a wild-type (P24) and a mutant (P27) PC. With gradually increasing stimulus intensities, EPSCs of the wild type were obtained in an all-or-none fashion, while those of the mutant occurred at two discrete stimulus intensity steps, indicating that at least two CFs innervate this mutant PC. Two to three traces were superimposed at each threshold intensity. Stimuli were applied at 0.1 Hz. Holding potentials were −20 mV for both the wild-type and mutant PCs to inactivate voltage-dependent channels.

(B) Summary histograms showing number of discrete steps of CF-EPSCs of the wild-type (open columns) and mutant (hatched columns) PCs. Data obtained from mice at P22–P75. Numbers of tested PCs are as follows: n = 147 (from 15 mice, 107 cells studied blind to the mouse genotype) for the wild type and n = 129 (from 13 mice, 97 cells studied blind) for the mutant.

to three discrete steps as the stimulus intensity was increased above the threshold (Figure 1A). In only 8.8% of the wild-type PCs, the EPSCs had more than one discrete step. The frequency distribution of PCs in terms of the number of discrete EPSC steps (Figure 1B) showed a significant difference between the wild-type and mutant mice ($p < 0.001$, χ^2 test). This indicates that most PCs in adult wild-type mice are innervated by a single CF, while about one third of the PCs are innervated by more than one CF in the adult mutant mice.

Other Electrophysiological Parameters of Mutant EPSCs Are Normal

Other electrophysiological parameters of EPSCs in the P22–P75 mGluR1 mutant mice appear normal (Table 1). First, we compared the 10%–90% rise time and the decay time constant of EPSCs of wild-type and mutant mice. The decay time constants were obtained by fitting the decay phases of EPSCs with single exponentials (Llano et al., 1991). The data from mutant mice were divided into two groups, those from monoinnervated PCs and those from polyinnervated PCs. In the latter group, measurements were made for the lowest of the multiple discrete EPSC steps. Although it was previously reported that EPSCs of the P7–P17 mGluR1 mutant mice have faster decay kinetics than those of wild type mice (Aiba et al., 1994b), the present measurements of P22–P75 mGluR1 mutant mice showed that there is no substantial difference in either the 10%–90% rise time or the decay time constant between the mutant and wild-type mice (Table 1).

Second, we examined the response to the second stimulus of a pair of pulses as reported previously in the rat (Konnerth et al., 1990) and in the mouse (Aiba et al., 1994b; Kano et al., 1995). EPSCs showed prominent paired-pulse depression in both monoinnervated and polyinnervated PCs derived from the mutant mice. The magnitude of paired-pulse depression was not significantly different from that of monoinnervated PCs from wild-type mice at varying interpulse intervals (Figure 2). Thus, short-term synaptic plasticity is unimpaired at CF synapses in the mutant mice. Paired-pulse synaptic plasticity is presumably caused by a change in transmitter release from presynaptic terminals (Zucker, 1989; K. H. and M.K., unpublished data). Therefore, the present results strongly suggest that presynaptic functions of CF terminals are unimpaired in the mutant mice.

Third, we verified that the current–voltage relations of

Table 1. Electrophysiological Parameters of Climbing and Parallel Fiber Synapses

Synapse and Data Set	Wild Type	mGluR1 Mutant	
		Mono	Multi
Climbing fiber synapse			
EPSC kinetics			
10%–90% rise time (ms)	0.5 ± 0.1 (n = 15)	0.5 ± 0.1 (n = 18)	0.6 ± 0.1 (n = 11)
Decay time constant (ms)	6.8 ± 2.6 (n = 15)	5.7 ± 1.7 (n = 18)	5.1 ± 2.5 (n = 11)
Parellel fiber synapse			
EPSC kinetics			
10%–90% rise time (ms)	1.4 ± 0.3 (n = 16)	1.2 ± 0.3 (n = 16)	
Decay time constant (ms)[a]	11.4 ± 2.1 (n = 16)[b]	9.9 ± 1.7 (n = 16)[b]	
Paired-pulse facilitation[a]	197.9 ± 29.0 (n = 8)	177.6 ± 30.1 (n = 7)	

Data are expressed as mean ± SD.

[a] These data are taken from Aiba et al. (1994b). Paired-pulse facilitation is defined as the percent of the first pulse, 20 ms interval.

[b] $p \leq .05$, t test.

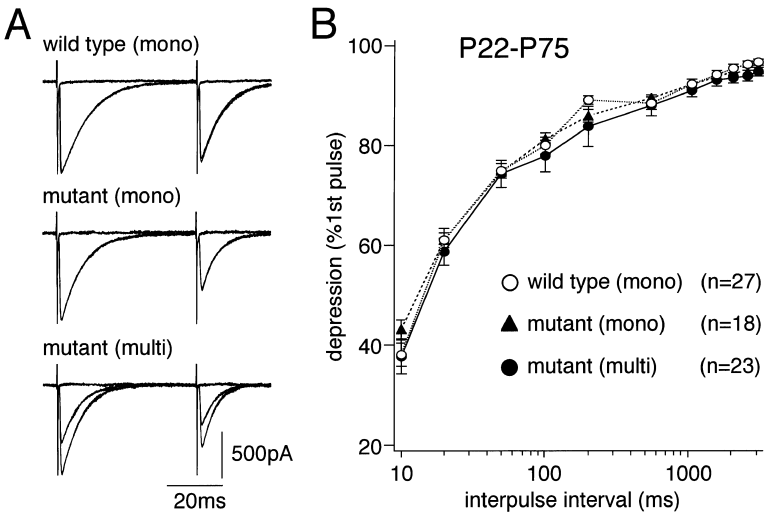

Figure 2. CF-EPSCs Display Normal Short-Term Synaptic Plasticity in Mutant PCs

(A) CF-EPSCs to pairs of stimuli separated by 20 ms in a wild-type monoinnervated (top, P55), a mutant monoinnervated (middle, P73), and a mutant double-innervated (lower, P73) PC. Three to five traces are superimposed. Note that the responses to each second stimulus were markedly depressed in all traces. Stimulus pairs were applied at 0.1 Hz. Holding potential was 0 mV for all PCs.

(B) Summary graphs showing paired-pulse depression of CF-EPSCs in the wild-type monoinnervated (open circles), mutant monoinnervated (closed circles), and mutant multiple-innervated (closed triangles) PCs from mice at P22–P75. The second response (expressed as a percentage of response to the first pulse, mean ± SEM) is plotted as a function of interpulse interval. Stimulus pairs were applied at 0.1 Hz. Numbers of tested PCs are as follows: n = 27 (from 9 mice, 14 cells studied blind) for the wild type (mono), n = 18 (from 9 mice, 15 cells studied blind) for the mutant (mono), and n = 23 (from 8 mice, 21 cells studied blind) for the mutant (multi).

CF-EPSCs were linear in the wild-type monoinnervated, the mutant monoinnervated, and the mutant double-innervated PCs (data not shown). Futhermore, EPSCs in both P22–P75 wild-type and P22–P75 mutant mice were not affected by an NMDA receptor blocker, DL-2-amino-5-phosphonopentanoate (AP5, 100 μM), but were totally suppressed by an AMPA receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dion (CNQX, 10 μM) (data not shown). These results indicate that the EPSCs of mature PCs from wild-type and mutant mice are exclusively mediated by the non-NMDA subtype of glutamate receptors.

CF Innervation during Early Postnatal Days

The persistent innervation of PCs by multiple CFs in mature mGluR1 mutant mice may result from either augmented CF innervation during the early postnatal period or from an impaired CF elimination at a subsequent stage, or both. To examine these possibilities, we followed the development of CF innervation during the first three postnatal weeks. During P1–P7, the majority of PCs were polyinnervated by CFs in both wild-type and mutant mice (Figure 3A). In particular, more than one third of PCs were innervated by four or more CFs (Figure 3A). The frequency distributions of EPSC steps among PCs were not significantly different ($p > 0.05$, χ^2 test) between the wild-type (Figure 3A, right, open columns) and the mutant mice (Figure 3A, right, hatched columns).

During P8–P14, the percentage of PCs with multiple EPSC steps markedly decreased in both wild-type and mutant mice (Figure 3B). The difference between the frequency distributions of PCs of P8–P14 mice (Figure 3B, right) and of P1–P7 mice (Figure 3A, right) was highly significant in both the wild-type and the mutant mice ($p < 0.001$ for both, χ^2 test). During P8–P14, about half of the PCs remained polyinnervated, and the frequency distributions of EPSC steps showed no significant difference between the wild-type and the mutant PCs ($p > 0.05$, χ^2 test) (Figure 3B, right). During P15–P21, the percentage of PCs with multiple EPSC steps further decreased in the wild-type mice (Figure 3C). On the other hand, the percentage of PCs with multiple EPSC steps did not significantly change in the mutant mice during P15–P21 compared with during P8–P15 (Figure 3C).

In the wild-type mice, regression of CF innervation appears to continue during the third postnatal week, and the CF innervation pattern observed in adult mice is established by P21. Thus, the distribution of PCs in P15–P21 mice (Figure 3C, right) is significantly different from that in P22–P75 mice (see Figure 1B) ($p < 0.01$, χ^2 test). By contrast, PCs in P15–P21 mutant mice (Figure 3C, right) had similar distributions ($p > 0.05$, χ^2 test) as those in P22–P75 mutant mice (see Figure 1B), indicating that no significant change occurred in CF innervation during the third postnatal week.

These results suggest that during the early postnatal period (P1–P14), regression of CF innervation appears to occur normally in mGluR1 mutant mice. In wild-type mice, the regression continues during P15–P21, while in mGluR1 mutant mice, it does not. Thus, in terms of CF innervation, mutant cerebella remain immature throughout life, being arrested at the developmental stage corresponding to the second postnatal week.

Morphology and Electrophysiology of Parallel Fiber–PC Synapses

Previous studies suggested that the elimination of supernumerary CFs depends on the formation of PF–PC synapses (see Crepel, 1982, for review). It is, therefore, of interest to examine whether these synapses are normal in mGluR1 mutant mice. In Nissl-stained sections, the cerebellum of the mGluR1 mutant mouse was indistinguishable from that of the wild type, in terms of foliation and laminar organization of the cerebellar cortex (Figures 4A and 4B). Immunohistochemistry with antibodies against spot 35/calbindin, a cytosolic Ca^{2+}-binding protein specific for cerebellar PCs (Garcia-Segura et al., 1984; Yamakuni et al., 1984), revealed, in both wild-type and mutant mice, well-differentiated dendritic arbors extending from the apical pole of the cell body toward the pial surface; they were studded with numerous spines (Figures 4C and 4D).

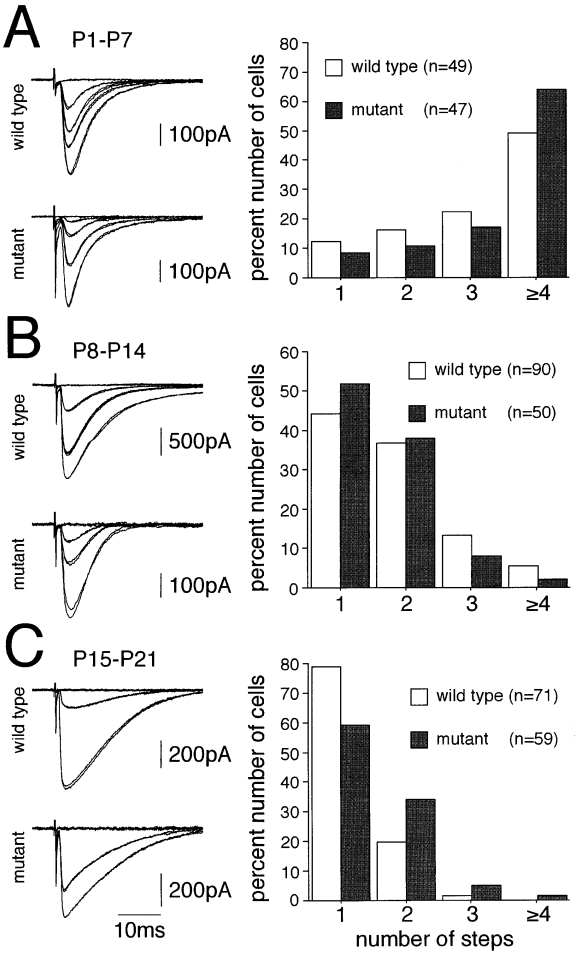

Figure 3. Postnatal Development of CF Innervation

(A, left) CF-EPSCs of the wild-type (P5, holding potential −60 mV) and mutant (P3, holding potential −60 mV) PCs. One to three traces each were superimposed at threshold intensities. Stimuli were applied at 0.1 Hz. (Right) Summary graph showing numbers of discrete steps of CF-EPSCs of the wild-type (open columns) and mutant (hatched columns) PCs from mice at P1–P7. Numbers of tested PCs are as follows: n = 49 (from nine mice, 14 cells studied blind) for the wild type and n = 47 (from six mice, all cells studied blind) for the mutant.

(B) Similar to (A), but from mice at P8–P14 of CF-EPSCs of the wild-type (P9, holding potential −60 mV) and mutant (P8, holding potential −20 mV) PCs and summary graph of the data. Numbers of tested PCs are as follows: n = 90 (from 13 mice, 30 cells studied blind) for the wild type and n = 50 (from five mice, 46 cells studied blind) for the mutant.

(C) Similar to (A) and (B), but from mice at P15–P21 of CF-EPSCs of the wild-type (P17, holding potential −10 mV) and mutant (P18, holding potential −10 mV) PCs and summary graph of the data. Numbers of tested PCs are as follows: n = 71 (from five mice, 12 cells studied blind) for the wild type and n = 59 (from six mice, 30 cells studied blind) for the mutant.

Electron microscopy revealed numerous synaptic structures in the molecular layers of both mutant and wild-type mice. The majority of these synaptic structures were asymmetric, being formed by presynaptic terminals containing clear round vesicles and dendritic spines with marked postsynaptic densities (Figure 5). The morphological features indicate that these structures are PF–PC synapses. The number of synapses with these characteristics was 20.7 ± 0.5 per 100 μm^2 (mean ± SEM, n = 10), 21.8 ± 0.8, and 22.1 ± 0.7 for three mutant mice, and 19.7 ± 0.8, 21.3 ± 0.7, and 22.4 ± 0.6 for three wild-type mice, with no significant difference between the two groups (t test, p = 0.36). Moreover, no significant difference was found in the mean profile diameter of presynaptic terminals or postsynaptic spines (data not shown) between the wild-type and mutant mice. Thus, as far as these analyses are concerned, no morphological abnormalities were found in the mutant mice.

It was previously reported that electrophysiology of PF-mediated EPSCs (PF-EPSCs) was normal in mature mGluR1 mutant PCs (Aiba et al., 1994a; Conquet et al., 1994). The only abnormality observed was the slightly reduced decay time constants of PF-EPSCs (Table 1; Aiba et al., 1994a). The 10%–90% rise time of the mutant mice did not differ significantly from that of the wild-type mice (Table 1). PF-EPSCs of both wild-type and mutant PCs displayed prominent facilitation to the second pulse of a stimulus pair at varying interpulse intervals between 10 ms and 300 ms. The magnitude of paired-pulse facilitation in the mutant mice was not significantly different from that of the wild-type mice (Aiba et al., 1994b; Table 1). Thus, presynaptic functions of PF terminals were unimpaired in the mutant mice. Furthermore, PF-EPSCs were not affected by AP5 (100 μM) in either wild-type or mutant mice and were totally suppressed by CNQX (10 μM) (Aiba et al., 1994b), indicating that PF-EPSCs of mature PCs from the wild-type and mutant mice are exclusively mediated by the non-NMDA subtype of glutamate receptors.

CFs are provided by the cells in the inferior olivary nucleus (Desclin, 1974). Since it is possible that the developmental abnormality observed regarding the CF innervation results from abnormal inferior olive inputs, we applied a histological examination to the inferior olivary nucleus. With Nissl staining, the inferior olivary nucleus in the mutant and wild-type mice was found as a folded gray mass of cells within the ventromedial medulla oblongata, and no differences were detected in the size and shape of the nucleus (Figure 6). In both mice, neurons of the inferior olivary nucleus were oval in shape and showed no signs of degeneration and cell loss (insets in Figure 6).

Taken together, these results strongly suggest that the persistent innervation of multiple CFs observed in the mGluR1 mutant mice is not due either to gross morphological or electrophysiological abnormalities at the PF–PC synapses or to gross morphological changes in the interior olive.

Discussion

Intact mGluR1 Is Required in the Late Phase of the Regression of Multiple CFs

The present electrophysiological results suggest that regression of multiple CF innervation occurs in at least two distinct steps (Figure 3). In the rat and probably also in the mouse, dendrites of PCs have not yet developed at the end of the first postnatal week. At this time point,

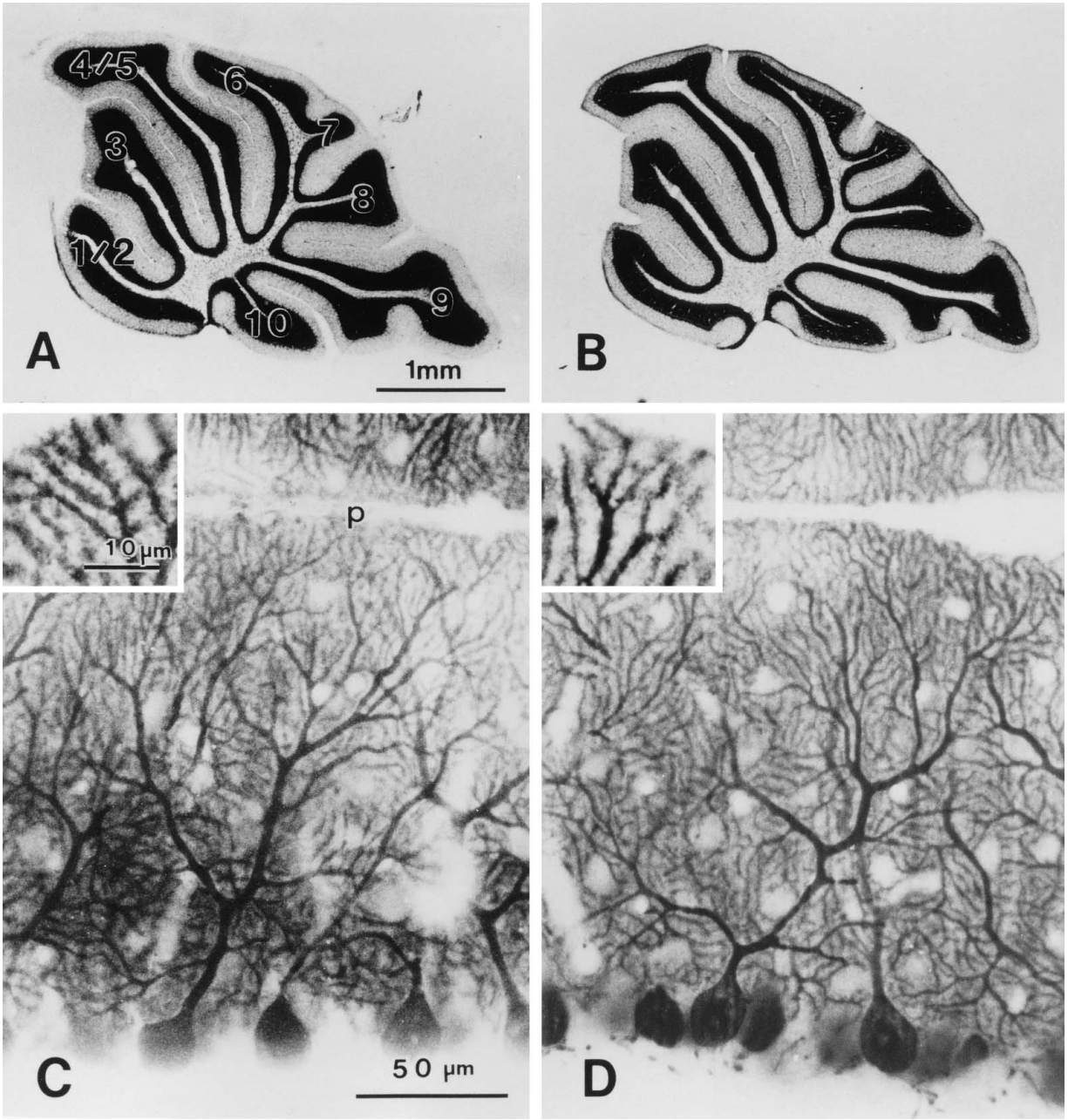

Figure 4. Histological and Immunohistochemical Analyses of the Mutant and Wild-Type Cerebella

(A) and (C) are the mutants, (B) and (D) the wild type.

(A and B) Toluidine blue–stained parasagittal sections through the cerebellar vermis.

(C and D) Cerebellar cortex immunostained for spot 35/calbindin. The lobule number of the mouse cerebellum is indicated as 1 through 10, according to Marani and Voogd (1979). Rostral is to the left, and dorsal is to the top in (A) and (B). P, pia matter.

CFs form synaptic contacts on lateral processes around the soma (Altman, 1972). Thereafter, as the dendrites grow, the somatic processes and the CF synapses on them disappear, and the CFs form synaptic contacts on the dendrites (O'Leary et al., 1971; Palay and Chan-Palay, 1974). The massive reduction of multiple CF innervation that occurs during the first 10 postnatal days (Figures 3A and 3B) corresponds well with these morphological changes. During the second 10 postnatal days, the dendritic trees of PCs develop dramatically, and PFs form synaptic contacts on dendritic spines. We have demonstrated electrophysiologically the continuous reduction of the number of polyinnervated PCs during this period (Figures 3B and 3C), which reflects a change of CF–PC connections from an immature to a mature state. Our results indicate that regression of multiple CF input occurs normally in the mGluR1 mutant mice at least up to P14 (Figures 3A and 3B). However, the regression does not appear to progress further during P15–P21. These results strongly suggest that mGluR1

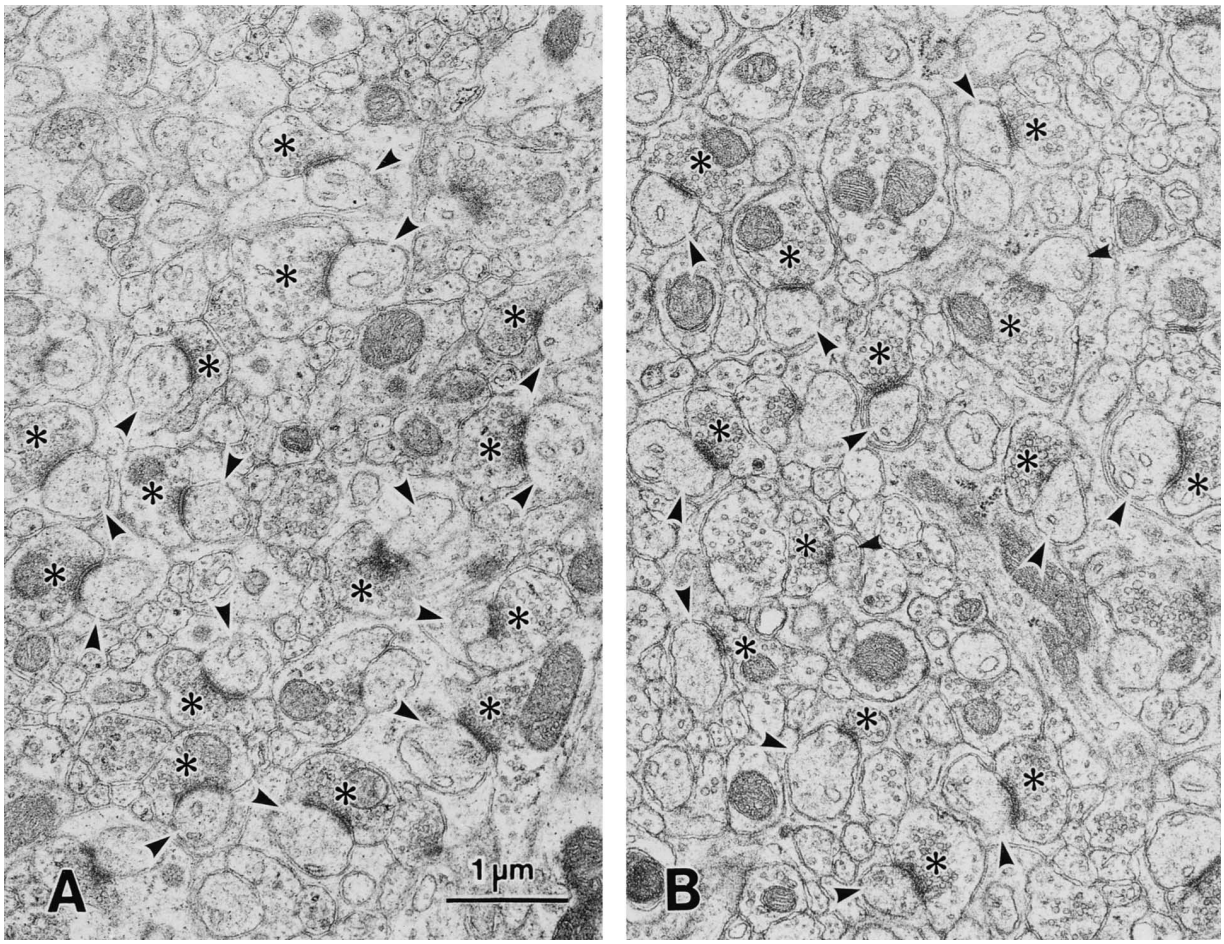

Figure 5. Electron Micrographs of the Molecular Layer in the Mutant and Wild-Type Cerebella

(A) mutant; (B) wild type. Asterisks and arrowheads indicate presynaptic terminals and postsynaptic dendritic spines, respectively. The electron micrographs were taken at the deeper one third of the molecular layer from the pial surface in the lobule 4+5 (culmen).

has a stage-specific role in the CF synapse elimination process during the third postnatal week. A similar observation was made in mice in which the gene encoding the γ isoform of PKC (PKCγ) was knocked out (Kano et al., 1995).

Abnormal Regression of CF Innervation without a Gross Defect of PF–PC Synapses

Previous reports showed that the innervation of multiple CFs persists in adult animals that lack granule cells or functional PFs during early postnatal development (see Crepel, 1982, for review). The animal models studied include rats in which granule cells have been destroyed by X-irradiation (Woodward et al., 1974; Crepel and Delhaye-Bouchard, 1979), *weaver* mutant mice in which granule cells die during their migration (Crepel and Mariani, 1976), *reeler* mice in which some PCs are devoid of synaptic contacts with granule cells (Mariani et al., 1977), and *staggerer* mutant mice in which granule cells develop normally, but subsequently degenerate without making synaptic contacts with PCs (Crepel et al., 1980; Mariani and Changeaux, 1980). In addition, the multiple CF innervation was recently reported in the mutant adult mice deficient in the δ2 subunit of glutamate receptors (GluRδ2) in which the number of synaptic contacts from PFs onto dendritic spines of PCs is less than half of that of the wild type (Kashiwabuchi et al., 1995). These results are consistent with the notion that the presence of granule cells or of PF–PC synapses is essential for the regression of multiple CF innervation.

In contrast with these previously reported animal models of abnormal CF regression, no obvious morphological abnormalities were observed in the PF–PC synapses of mGluR1 mutant mice. Although the percentage of multipolar PCs (PCs with more than one main dendrite emanating directly from the cell body) is higher in the mGluR1 mutants (11.1%, n = 369) than in the wild-type mice (5.9%, n = 152) (Aiba et al., 1994b), this difference cannot account for the defective regression of CFs, because the difference in the proportion of multiply-innervated PCs between the mutant mice (32.6%, n = 129 at P22–P75) and the wild-type mice (8.8%, n = 147 at P22–P75) is substantially greater. Moreover, our electrophysiological analysis showed that PF-mediated synaptic transmission is normal. Thus, mGluR1 mutant mice are similar to the recently described PKCγ mutant mice

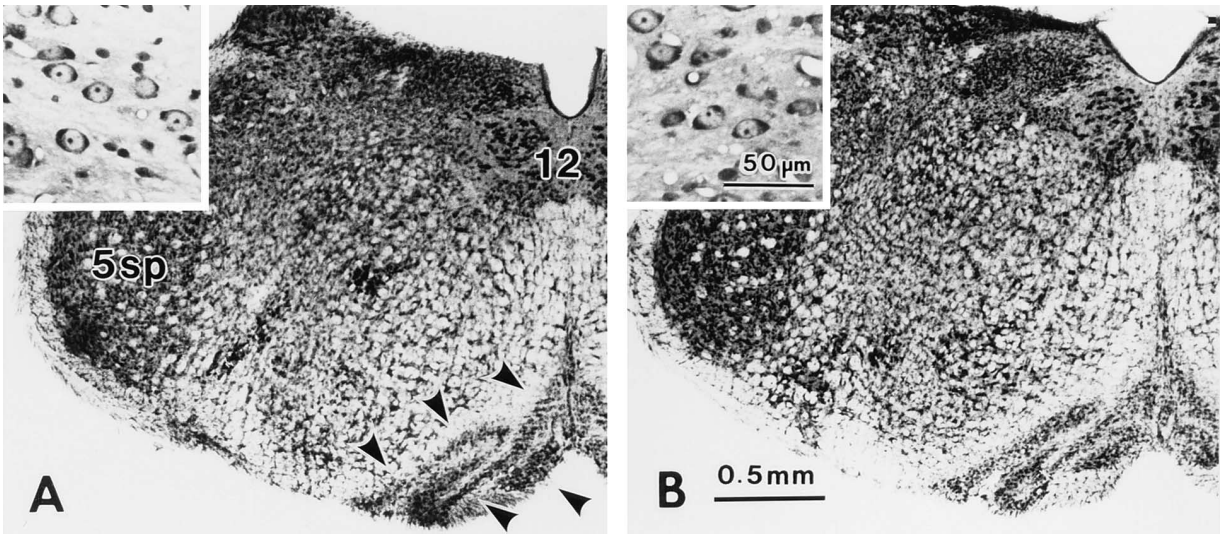

Figure 6. Nissl Staining of the Medulla Oblongata in the Mutant and Wild-Type Mice

(A) mutant; (B) wild type. Arrowheads indicate the inferior olivary nucleus. Insets are high power images of cell bodies in the inferior olivary nucleus, taken from paraffin sections. Dorsal is to the top. 5sp, trigeminal spinal tract nucleus; 12, hypotlassal nucleus.

in that they exhibit abnormality in the regression of multiple CF innervation with no gross defects in the PF–PC synapses (Kano et al., 1995).

Role of mGluR1 in the Elimination of Surplus CFs

What role do PF–PC synapses play in the developmental regression of polyinnervating CFs? One previous study suggests that the activity at PF–PC synapses is important. Rabacchi et al. (1992) reported that multiple innervation persists to a significant extent in rats chronically treated with the NMDA receptor antagonist AP5. The authors suggest that NMDA receptors, present on PCs, granule cells, or both, are involved in the elimination of multiple CF innervation. However, electrophysiological data in the rat (Llano et al., 1991) and the mouse (Aiba et al., 1994b; Kano et al., 1995) did not reveal an NMDA-receptor component in either PF-EPSCs or CF-EPSCs in immature cerebellum, as might have been expected if NMDA receptors in PCs played a role in the synapse elimination. In contrast, expression of NMDA receptors is well established in granule cells (Watanabe et al., 1994; Akazawa et al., 1994). Therefore, if NMDA receptors play a role in the elimination of multiple CF innervation, it would be through an indirect mechanism involving granule cells.

Previous histochemical examinations using specific antibodies to mGluR1α, the major splice variant of mGluR1, have revealed a strong immunoreactivity at dendritic spines of PCs on which PF terminals form synaptic contacts (Martin et al., 1992; Baude et al., 1993; Gorcs et al., 1993; Grandes et al., 1994; Nusser et al., 1994). In contrast, presynaptic terminals of PFs are apparently devoid of mGluR1 immunoreactivity (Martin et al., 1992; Baude et al., 1993; Gorcs et al., 1993; Grandes et al., 1994; Nusser et al., 1994). These results are consistent with the notion that the neural activity mediated by mGluR1 present at the dendritic spines of PF–PC synapses generates a signal that leads to the elimination of multiple CFs in a heterosynaptic fashion. PKC is a known signal transducer of mGluR1 and PKCγ is the major PKC isoform present in PCs. Thus, it is likely that PKCγ plays a role in the biochemical cascade downstream of the mGluR1 expressed at the dendritic spines of PF–PC synapses. Consistent with this hypothesis is the observation that regression of CF innervation is also impaired in PKCγ mutant mice (Kano et al., 1995).

Besides dendritic spines at PF synapses, mGluR1α immunoreactivity is also detected on PC dendrites at putative CF synapses (Nusser et al., 1994) or on the plasma membrane of PCs where no synaptic contacts are formed (Baude et al., 1993; Nusser et al., 1994). Thus, it is possible that mGluR1α in these regions is activated by either glutamate released from CF terminals or ambient glutamate in the extracellular space and thereby transduces signals crucial for the elimination of CFs.

It has been reported that granule cells are moderately immunoreactive to mGluR1γ antibody (Grandes et al., 1994). It has also been reported that subsets of granule cells, but not all, are immunoreactive to mGluR1α antibodies (Baude et al., 1993). Moreover, neurons in the inferior olive are reported to express mGluR1 (Shigemoto et al., 1992; Baude et al., 1993). Therefore, we cannot exclude the possibility that mGluR1 expressed in granule cells, inferior olivary neurons, or both are crucially involved in the regression of multiple CF innervation. To address this point, we intend to generate mutant mice in which mGluR1 is uniquely deficient in PCs.

Behavioral Correlate of Multiple CF Innervation

We previously reported that PKCγ mutant mice are impaired in motor coordination despite that they are capable of learning a discrete motor act such as a conditioned eyeblink response (Chen et al., 1995). We also

reported that these mutant mice, as mGluR1 mutant mice, retain polyinnervation of CFs into adulthood in a substantial (~30%) proportion of PCs (Kano et al., 1995). We proposed that this innervation of PCs by multiple CFs disrupts the functional organization of olivary neurons and PCs such that a transfer of the signals necessary for the refinement of a motor program for a compound movement is compromised (Chen et al., 1995). As we reported previously (Aiba et al., 1994), mGluR1 mutant mice exhibit motor discoordination as PKCγ mutant mice. The present finding that mGluR1 mutant mice are defective in the regression of CF polyinnervation is consistent with the hypothesis that CF monoinnervation is crucial for normal motor coordination.

Experimental Procedures

Production of mGluR1 Mutant Mice

Mutant mice deficient in mGluR1 were produced as described previously (Aiba et al., 1994a). Both wild-type and mutant mice utilized were of the 129/Sv × C57BL/6 genetic background and were kept in the same room at the animal facility at the Massachusetts Institute of Technology with a 12 hr light–dark cycle.

Electrophysiology

Either sagittal or transverse cerebellar slices of 200–300 μm thickness were prepared from the wild-type and mutant mice as described previously (Edwards et al., 1989; Llano et al., 1991; Kano and Konnerth, 1992; Aiba et al., 1994b). Whole-cell recording was made from visually identified PCs by using a 40× water immersion objective attached to either Olympus (BH-2) or Zeiss (Axioskop) upright microscope (Edwards et al., 1989; Llano et al., 1991). In both the wild-type and the mGluR1 mutant mice, PCs were sampled from all the lobules in the parasagittal plane with 500 μm from the midline. Resistance of patch pipettes was 3–6 MΩ when filled with an intracellular solution composed of 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). The composition of standard bathing solution was 125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 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 μM) was always present in the saline to block spontaneous inhibitory postsynaptic currents (Konnerth et al., 1990; Kano et al., 1992). Ionic currents were recorded with an EPC-9 patch–clamp amplifier (HEKA, Germany) and stored on a DAT data recorder (Sony, PC204, Japan) for later analysis. The pipette access resistance was compensated as described by Llano et al. (1991). Stimulation and on-line data acquisition were performed with the PULSE program on a Macintosh computer (version 7.5, HEKA, Germany). The signals were filtered at 3 kHz and digitized at 20 kHz. Fitting of the decay phases of EPSCs was done with the PULSEFIT program (version 7.5, HEKA, Germany). 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, 0–100 V for CF stimulation, 1–10 V for PF stimulation) were applied for focal stimulation.

Histology

Under deep anesthesia with chloral hydrate (350 mg/kg body weight, intraperitoneally), mice at the fifth postnatal week were perfused transcardially with freshly prepared 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2). The brain was removed from the skull and immersed in the same fixative overnight. Cryostat (50 μm in thickness) and paraffin (5 μm) sections mounted on gelatin-coated slides were Nissl-stained with toluidine blue. Microslicer sections (100 μm) were immunostained with rabbit anti-rat spot 35/calbindin antibody (Yamakuni et al., 1984). Following visualization with the ABC kit (Vector, Burlingame), the sections were treated with 0.5% OsO_4 for 15 min to enhance the immunostaining.

Electron Microscopy and Morphometry

For electron microscopy, mice at the fifth postnatal week were anesthetized and perfused through the heart with 0.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). After sectioning at a thickness of 500 μm by microslicer in the parasagittal plane, sections through the cerebellar midline were chosen and immersed in the same fixative for an additional 12 hr. The sections were postfixed for 2 hr with 1% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.2), dehydrated with graded alcohols, and embedded in Epon 812. Silver–gold ultrathin sections through the culmen (lobule 4+5) were collected on copper grids and stained with 1% uranyl acetate for 5 min and mixed lead solution for 2 min.

From each mouse, 10 sets of electron micrographs were prepared, each set being taken serially from the deepest region of the molecular layer up to the pial surface, at an original magnification of 3000× with a Hitachi H7100 electron microscope. For quantitative measurements, the number of synaptic profiles was counted for each set of electron micrographs printed at a final magnification of 11,400×. The counted neuropil area, which excluded blood vessels, cell bodies, and dendritic shafts thicker than 2 μm in caliber, was measured by the point-counting method of Weibel (1979), using a double lattice system. The mean synaptic number per 100 μm^2 was calculated for each mouse, from the synaptic number and the counted neuropil area.

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