Ablation of glutamate receptor GluRδ2 in adult Purkinje cells causes multiple innervation of climbing fibers by inducing aberrant invasion to parallel fiber innervation territory.
Ablation of Glutamate Receptor GluR2 in Adult Purkinje Cells Causes Multiple Innervation of Climbing Fibers by Inducing Aberrant Invasion to Parallel Fiber Innervation Territory

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Glutamate receptor GluR2 is exclusively expressed in Purkinje cells (PCs) from early development and plays key roles in parallel fiber (PF) synapse formation, elimination of surplus climbing fibers (CFs), long-term depression, motor coordination, and motor learning. To address its role in adulthood, we previously developed a mouse model of drug-induced GluR2 ablation in adult PCs (Takeuchi et al., 2005). In that study, we demonstrated an essential role to maintain the connectivity of PF–PC synapses, based on the observation that both mismatching of presynaptic and postsynaptic specializations and disconnection of PF–PC synapses are progressively increased after GluR2 ablation. Here, we pursued its role for CF wiring in adult cerebellum. In parallel with the disconnection of PF–PC synapses, ascending CF branches exhibited distal extension to innervate distal dendrites of the target and neighboring PCs. Furthermore, transverse CF branches, a short motile collateral rarely forming synapses in wild-type animals, displayed aberrant mediolateral extension to innervate distal dendrites of neighboring and remote PCs. Consequently, many PCs were wired by single main CF and other surplus CFs innervating a small part of distal dendrites. Electrophysiological recording further revealed that surplus CF-EPSCs characterized with slow rise time and small amplitude emerged after GluR2 ablation, and increased progressively both in number and amplitude. Therefore, GluR2 is essential for maintaining CF monoinnervation in adult cerebellum by suppressing aberrant invasion of CF branches to the territory of PF innervation. Thus, GluR2 fuels heterosynaptic competition and gives PFs the competitive advantages over CFs throughout the animal’s life.

Introduction

Cerebellar Purkinje cells (PCs) receive two distinct excitatory afferents (Palay and Chan-Palay, 1974). Each PC receives 105–106 inputs from parallel fibers (PFs) at distal dendrites, whereas each PF forms one or two synapses to individual PCs (Napper and Harvey, 1988). In contrast, each PC is innervated by a single climbing fiber (CF) that originates from the inferior olive and forms hundreds of synapses by twisting around proximal dendrites. This PC circuitry is established through heterosynaptic competition between PFs and CFs and homosynaptic competition among multiple CFs (Mariani et al., 1977; Crepel, 1982; Hashimoto et al., 2009). This competitive wiring is regulated by various signaling molecules expressed in cellular elements constituting PC synapses, including metabotropic glutamate receptor mGluR1, P/Q-type Ca2+ channels, glutamate receptor GluR2 (GluD2), and Cbln1 (or precerebellin) (Kano et al., 1995, 1997, 1998; Kashiwabuchi et al., 1995; Offermanns et al., 1997; Ichikawa et al., 2002; Miyazaki et al., 2004; Hirai et al., 2005).

Of these, GluR2 regulates heterosynaptic competition to the advantage of PF innervation (Watanabe, 2008; Yuzaki, 2009). GluR2 is dominantly expressed in PCs (Araki et al., 1993; Lomeli et al., 1993) and localized on spines contacting PFs (Takayama et al., 1995; Landsend et al., 1997). Targeted disruption of GluR2 gene in mice causes mismatching between presynaptic and postsynaptic specializations and disconnection at PF–PC synapses (Guastavino et al., 1990; Kashiwabuchi et al., 1995; Kurihara et al., 1997; Lalouette et al., 2001). The N-terminal domain of GluR2 has been demonstrated to mediate PF–PC synaptogenesis by interacting neurexin through Cbln1 (Uemura et al., 2007, 2010; Kakegawa et al., 2008, 2009; Uemura and
Mishina, 2008; Torashima et al., 2009; Matsuda et al., 2010). The loss of GluR2 further affects CF innervation; CFS extend distally to take over free spines of the target and neighboring PCs, causing multiple CF innervation (Hashimoto et al., 2001; Ichikawa et al., 2002). Intriguingly, mutant mice lacking the Ca,
2.1, a pore-forming subunit of P/Q-type Ca
2.2 channels, display almost reciprocal phenotypes that PF territory expands, and CF territory regresses, down to PC somata and basal dendrites (Miyazaki et al., 2004). Therefore, the construction of functional PC circuits stands on competitive equilibrium among afferents promoted by distinct molecular mechanisms.

Heterosynaptic competition is still active in adult cerebellum, as surgical lesion to olivocerebellar projections or pharmacological blockade of cortical activities alters innervation territories of PFs and CFS reciprocally (Sotelo et al., 1975; Rossi et al., 1991a; Bravin et al., 1999; Cesa et al., 2005). To address the molecular mechanisms that maintain functional synaptic wiring in adult cerebellum, we previously developed a mouse model of CrePR/loxP-mediated GluR2 ablation in adult PCs (Takeuchi et al., 2005). In that study, we demonstrated that GluR2 plays an essential role to maintain the connectivity of PF–PC synapses. Here, we addressed that GluR2 is also indispensable to maintain CF monoinnervation. After induction of GluR2 ablation, CF innervation extended distally and mediolaterally in the cerebellar cortex, giving rise to aberrant surplus branches that caused multiple innervation.

**Materials and Methods**

**Animals.** We used littermates derived from crossing of GluR2
2flox/lox and GluR2
2flox/CrePR mice on the pure C57BL/6 genetic background, as reported previously (Takeuchi et al., 2005). GluR2
2flox/CrePR mice were produced by crossing a target mouse line carrying the GluR2 gene flanked by loxP sequences with a Cre mouse line carrying the Cre recombinase-progestosterone receptor (CrePR) fusion protein under the control of the GluR2 gene promoter. All animal experiments were performed according to the guidelines for the care and use of laboratory animals of the Hokkaido University School of Medicine. The GluR2
2CrePR allele was identified by PCR using primers 5'-AGCAACATCTAACCCT-CAAGAA-3' (FD2P3) and 5'-ATTACGTGGCCAGACGACAGACAA-3' (FD2P4). The GluR2
2CrePR allele was identified by PCR using the CreP1 and CreP2 primers (Tsujita et al., 1999). For induction of PC-specific gene recombination, 11B-[-(dimethylamino)phenyl]-17β-hydroxy-17-(1-propynyl)-estradiol (Sigma-Aldrich), suspended at a concentration of 50 mg/ml in water containing 0.25% (v/v) carboxymethyl cellulose and 0.5% (v/v) Tween 80, was injected intraperitoneally at a dose of 1 mg/g body weight RU-486 at postnatal day 42 (P42) to P45 for 2 consecutive days (Takeuchi et al., 2005). We also used global or null-type GluR2
2 knockout mice, which were produced as a Cre knock-in mouse line D2CreRN under the control of the GluR2 gene promoter (GluR2
2Cre/Cr) using the C57BL/6 ES cell line RENKA (Mishina and Sakimura, 2007). Details of the GluR2
2Cre/Cr mouse will be described elsewhere.

In each morphological analysis, we analyzed three GluR2
2flox/fox and three GluR2
2flox/CrePR as control and mutant mice, respectively. Under deep pentobarbital anesthesia, mice were perfused transcardially with 1% formaldehyde and 0.2% glutaraldehyde in PBS, pH 7.4. After perfusion, the brains were cut on a vibratome (VT1000S; Leica) into 30 μm thick coronal sections. The sections were either postfixed with 2% glutaraldehyde for electron microscopy or were immersed in 0.1 M cacodylate buffer containing 1% bovine serum albumin and 0.02% saponin for immunohistochemical detection. The sections were then incubated with primary antibodies for 2 h. Images of double or triple labeling were taken with a fluorescence microscope (AX-80; Olympus) equipped with a digital camera (DP70; Olympus), and analyzed with MetaMorph software (Molecular Devices). The reach of CFs was evaluated by the distance from the base of the molecular layer to the tips of VGLUT2-positive CF terminals relative to the total vertical height of the molecular layer.

**Anterograde tracer labeling.** Under anesthesia with chloral hydrate (350 mg/kg body weight, i.p.), a glass pipette (G-1.2; Narishige) filled with 2–3 μl of 10% solution of biotinylated dextran amine (BDA) (3000 molecular weight; Invitrogen) or dextran Alexa 594 (DA-594) (Invitrogen) in PBS was inserted stereotaxically to the inferior olive by the dorsal approach. Tracers were injected by air pressure at 20 psi with 5 s intervals for 1 min (Pneumatic Pipetump; World Precision Instruments). After 4 d of survival, mice were anesthetized and fixed by transcardial perfusion. For bright-field light microscopy, BDA-labeled CFS were visualized by overnight incubation with peroxidase-labeled streptavidin (Nichirei) and colored in black using 3,3'-diaminobenzidine (DAB) and cobalt chloride. For combined labeling by tracer and immunofluorescence, DA-594-labeled microslicer sections were incubated with a mixture of calbindin and VGLUT2 antibodies followed by incubation with fluorescent secondary antibodies for 2 h. Images of double or triple labeling were taken with a confocal laser-scanning microscope. For quantitative analyses of transverse CF collaterals, six optical sections taken in the z-axis plane at an interval of 1 μm were stacked into a single image, and >15 images were obtained from each mouse. We measured the number of transverse collateral branches originating from ascending CF branches; the incidence was calculated as the number of per 100 μm of the parent ascending branches. We counted the number of VGLUT2-positive terminals per 100 μm of transverse branches. Quantitative analyses were performed using MetaMorph software, and all data were described as the mean ± SEM. Statistical significance was evaluated by Mann–Whitney U test. Statistical significance was assumed when p < 0.05.

For immunoelectron microscopy, BDA-labeled microslicer sections were incubated with VGLUT2 antibody diluted with Tris-buffered saline containing 1% bovine serum albumin and 0.04% saponin. Sections were first incubated with 1.4 nm gold particle conjugated anti-guinea pig antibody (1:200; Nanogold; Nanoprobes) for 3 h. Immunogold for VGLUT2 was silver-enhanced with an HQ-silver enhance kit (Nano probes), whereas BDA was detected by overnight incubation with peroxidase-labeled streptavidin (Nichirei) and visualized with DAB. Sections were postfixed with 1% osmium tetroxide for 15 min, dehydrated in graded alcohols, and embedded in Epox 812. Ultrathin sections (70 nm in thickness) were prepared with an ultramicrotome (Ultracut; Leica), and photographs were taken with an H-7100 electron microscope (Hitachi).

**Electrophysiology.** Whole-cell recordings were made from visually identified PCs using an upright microscope (BX51WI; Olympus) at 31°C. Patch pipettes had resistance of 2–4 MΩ when filled with an internal solution composed of the following (in mM): 60 CsCl, 10 Cs-glutamate, 20 TEA (tetraethylammonium)-Cl, 20 BAPTA, 4 MgCl2, 4 ATP, 0.4 GTP, and 30 HEPES, pH 7.3, adjusted with CsOH. The pipette access resistance was compensated by 80%. The composition of the standard bathing solution was as follows (in mM): 125 NaCl, 2.5 KCl, 2 CaCl2, 1 MgSO4, 1.25 NaH2PO4, 26 NaHCO3, and 20 glucose, bubbled with 95% O2 and 5% CO2. Bicuculline methochloride (10 μM; Tocris Bioscience) was always added to the bath solution for blocking inhibitory synaptic transmission. Ionic currents were recorded with a patch-clamp amplifier (EPC10; HEKA). The signals were filtered at 2 kHz and digitized at 20 kHz. On-line data acquisition and offline data analysis were been reported previously (Araki et al., 1993; Miyazaki et al., 2003; Miura et al., 2006). For GluR2 immunofluorescence, sections were digested with 1 mg/ml pepsin (Dako) in PBS, pH 7.4/0.2N HCl for 3 min at 37°C in a water bath. All immunohistochemical incubations were done at room temperature in a free-floating state. For immunofluorescence, cerebellar sections were incubated with 10% normal donkey serum for 20 min, a mixture of primary antibodies overnight, and a mixture of Alexa Fluor 488-, indocarbocyanine (Cy3)-, and indocarbocyanine (Cy5)-labeled species-specific secondary antibodies for 2 h at a dilution of 1:200 (Invitrogen; Jackson ImmunoResearch). Images were taken with a confocal laser-scanning microscope (FV1000; Olympus) or with a fluorescence microscope (AX-70; Olympus) equipped with a digital camera (DP70; Olympus), and analyzed with MetaMorph software (Molecular Devices). The reach of CFs was evaluated by the distance from the base of the molecular layer to the tips of VGLUT2-positive CF terminals relative to the total vertical height of the molecular layer.
performed using PULSE software (HEKA). Stimulation pipettes (5–10 μm tip diameter) were filled with the standard saline and used to apply square pulses for focal stimulation (duration, 0.1 ms; amplitude, 0–90 V). Decay time constant of CF-mediated EPSC (CF-EPSC) was measured by fitting the EPSC decay with single exponential (Llano et al., 1991). Passive membrane properties were calculated based on the equivalent circuit model proposed by Llano et al. (1991). The current in response to hyperpolarizing voltage steps from −70 to −80 mV was biphasic and fitted with two exponentials. Both time constants for the fast (τ₁) and slow (τ₂) components were not significantly different between control and mutant mice at any time points examined (supplemental Table S1, available at www.jneurosci.org as supplemental material). Statistical significance in frequency distribution was evaluated by χ² test or Mann–Whitney U test. Statistical significance was assumed when p < 0.05. For linear regression analyses, Pearson’s product moment correlation coefficient (r) was calculated.

Rotarod test. Control (n = 15) and mutant (n = 11) mice were used for motor behavioral test at 8, 12, 16, 20, 24, and 28 weeks after RU-486 administration. Mice were housed individually and were handled for ~1 min a day for 7–10 d before behavioral tests. An animal was placed on the rod (3.2 cm diameter) rotating at 15 rpm (RRAC-3002; O’Hara), and given three trials with 45–60 min intertrial intervals. The latency to fall (retention time) was measured with cutoff time of 5 min. Rotarod test was performed in a blind fashion. Statistical significance was evaluated by ANOVA with repeated measures followed by Student’s t test. Statistical significance was assumed when p < 0.05.

Results

The global GluR2-knock-out mice display two major anatomical phenotypes in terms of CF–PC wiring: distal extension of CF territory and multiple CF innervation by surplus innervation to distal dendrites (Kashiwabuchi et al., 1995; Ichikawa et al., 2002). We first examined whether and how these phenotypes were reproduced after GluR2 ablation in the adulthood by anatomically investigating GluR2 flox/flox (control) and GluR2 flox/CrePR (mutant) mice at 2, 8, 16, and 24 weeks after RU-486 administration on 2 consecutive days during P42–P45 of age.

Distal extension of CF innervation is induced after GluR2 ablation

Using parasagittal cerebellar sections, we applied double immunofluorescence for calbindin and vesicular glutamate transporter VGlut2 (red) in GluR2 flox/flox at 2 weeks after RU-486 administration (A) and GluR2 flox/CrePR mice at 2 weeks (B), 8 weeks (C), and 24 weeks (D) after RU-486 administration. A–D are separated images of A–D, respectively. Note atrophied distal dendrites of PCs and aberrant distal extension of CF innervation in GluR2 flox/CrePR mice at 24 weeks after RU-486 administration (F), compared with normal territorized innervation in GluR2 flox/flox mice (E). G, Changes in the thickness of the molecular layer after RU-486 administration. The mean thickness (in micrometers) is 191.0 ± 2.0 (n = 31 sites), 180.1 ± 2.6 (n = 43), 184.7 ± 3.6 (n = 21), and 181.9 ± 2.7 (n = 27) in GluR2 flox/flox mice, and 189.9 ± 2.3 (n = 47), 166.6 ± 2.3 (n = 49), 158.5 ± 2.2 (n = 44), 153.4 ± 1.8 (n = 31) in GluR2 flox/CrePR mice at 2, 8, 16, and 24 weeks after RU-486 administration, respectively (mean ± SEM; p = 0.9146 at 2 weeks; p < 0.0001 at 8, 16, and 24 weeks, U test). H, Changes in the vertical height to the tips of VGlut2-positive CF terminals relative to the thickness of the molecular layer. Scores (in percentage) are 78.7 ± 0.4 (n = 49 sites), 78.2 ± 0.4 (n = 49), 80.6 ± 0.6 (n = 29), and 79.4 ± 0.5 (n = 27) in GluR2 flox/flox mice, and 77.5 ± 0.5 (n = 43), 81.7 ± 0.4 (n = 52), 88.1 ± 0.5 (n = 44), and 93.1 ± 0.3 (n = 33) in GluR2 flox/CrePR mice at 2, 8, 16, and 24 weeks after RU-486 administration, respectively (mean ± SEM; p = 0.0979 at 2 weeks; p < 0.0001 at 8, 16, and 24 weeks, U test). ***p < 0.001. Scale bars: A–E, 20 μm.

In mutant mice at 2 weeks after RU-486 administration, the distribution of CF terminals was comparable with that in control mice (Fig. 1B). At later time points, however, CF terminals were distributed closer to the pial surface (Fig. 1C,D). At high magnifications, distal dendrites of mutant PCs were stunted and studded with shrunked spines (Fig. 1F). Furthermore, CF terminals in mutant mice were not confined to proximal shaft dendrites, but also associated with the atrophied distal dendrites (Fig. 1F). These phenotypic alterations were confirmed by measuring the thickness of the molecular layer (Fig. 1G) and the height of VGlut2-positive CF terminals (Fig. 1H). The mean thickness of
the molecular layer decreased progressively (Fig. 1G), whereas the mean relative height of CF terminals in molecular layer increased progressively (Fig. 1H), both showing significant differences at 8, 16, and 24 weeks \((p < 0.00001\) for each comparison, \(U\) test). These results suggest that, after induction of GluR2 ablation, CF territory extends distally along atrophied distal dendrites.

After RU-486 administration, the ablation of GluR2 protein in mutant mice proceeds unevenly among PC populations (Takeuchi et al., 2005). We examined the relationship between distal extension of CF territory and GluR2 ablation by triple immunofluorescence for calbindin (blue), VGluT2 (red), and GluR2 (green) at 12 weeks after RU-486 administration (Fig. 2A–E). In this analysis, we used horizontal cerebellar sections, in which PC dendrites are seen as single straight bars and GluR2 is detected, if expressed, on the surface of dendritic bars. In control mice, the reach of VGluT2-positive CF terminals up to basal four-fifths of the molecular layer was confirmed in horizontal sections (mean ± SEM, 77.2 ± 0.5% of the molecular layer; \(n = 54\)) (Fig. 2A, B, E). In mutant mice, the relative reach was significantly extended along dendritic bars lacking GluR2 (88.2 ± 0.4%; \(n = 38\)), compared with that along dendritic bars retaining GluR2 (75.2 ± 0.8%; \(n = 38\); \(p < 0.00001\), \(U\) test) (Fig. 2C–E). Thus, distal extension of CF innervation selectively occurs along GluR2-ablated PC dendrites.

**Figure 2.** Aberrant extension and innervation of CFs along and against GluR2-negative PC dendrites. All data were obtained using horizontal cerebellar sections of in GluR2flox/flox (A, B) and GluR2flox/CrePR (C, D, F, G) mice at 12 weeks after RU-486 administration. The boxed regions in A, C, and F are enlarged in B, D, and G, respectively. A–D, Triple immunofluorescence for calbindin (blue), GluR2 (green), and VGluT2 (red). Note that the reach of VGluT2-positive CF terminals selectively extends along GluR2-lacking dendrites, compared with that along GluR2-positive dendrites (encircled by dotted lines in G). The broken lines in A and C indicate the pial surface. E, A histogram showing the mean height to the tips of VGluT2-positive CF terminals relative to the height of the molecular layer. White bar, GluR2flox/flox mice; gray bar, GluR2flox/CrePR mice (along GluR2-positive dendrites); black bar, GluR2flox/CrePR mice (along GluR2-negative dendrites). Error bars indicate SEM. F, G, Triple fluorescent labeling for GluR2 (blue), anterograde tracer DA-594 (red), and VGluT2 (green). Note that VGluT2-positive terminals are preferentially differentiated where transverse CF branches cross with GluR2-negative dendrites, whereas such terminal differentiation is rarely found around GluR2-positive dendrites (encircled by dotted lines in G). *** \(p < 0.001\). Scale bars: A1, C1, F, 20 μm; B1, D1, G1, 10 μm.
GluR62 ablation induces multiple innervation by ascending CF branches

Then we tested whether the distally extended ascending branches caused multiple innervation. To this end, we applied triple fluorescent labeling for calbindin (blue), VGluT2 (green), and anterograde tracer DA-594 (red) to distinguish anatomical forms of CF innervation (Fig. 3). In this analysis, PC dendrites that were associated with CF terminals either double-labeled for DA594 and VGluT2 or single-labeled for VGluT2 only were judged to be monoinnervated (supplemental Fig. S1A, available at www.jneurosci.org as supplemental material), whereas those associated with double- and single-labeled CF terminals simultaneously were to be multiply innervated (supplemental Fig. S1B, available at www.jneurosci.org as supplemental material).

In parasagittal cerebellar sections, monoinnervation pattern was overwhelming in control mice at each time point examined (Fig. 3A) and in mutant mice at 2 weeks after RU-486 administration (Fig. 3B). At 8 weeks and thereafter, mutant mice frequently displayed anatomical patterns of multiple innervation (Fig. 3C,D). For example, PC dendrites, marked as PCD-a, were mainly innervated by ascending branches of tracer-labeled CF-a (Fig. 3C) or tracer-unlabeled CF-a (Fig. 3D), respectively. These dendrites received additional innervation by tracer-unlabeled CF-b (Fig. 3C, green arrows) or tracer-labeled CF-b (Fig. 3D, red arrows), respectively, thus demonstrating the innervation by multiple CFs. Predominant monoinnervation in control mice and predominant multiple innervation in mutant mice were also confirmed using horizontal cerebellar sections at 16 weeks after RU-486 administration (supplemental Fig. S2, available at www.jneurosci.org as supplemental material). In particular, alternate innervation of single dendritic bars by tracer-labeled CF-a and tracer-unlabeled CF-b was readily captured in mutant mice (supplemental Fig. S2C,D, available at www.jneurosci.org as supplemental material). In general, terminals of additional CFs were few in number and intermingled with those formed by main CFs at 8 weeks (Fig. 3C), whereas additional CFs often covered significant portions of PC dendrites and tended to be segregated from main CFs within the same dendrites at 16 and 24 weeks after RU-486 administration (Fig. 3D; supplemental Fig. S2C,D, available at www.jneurosci.org as supplemental material). Thus, the ablation of GluR62 induces aberrant wiring of ascending CF branches to dendrites of nearby PCs, thereby causes multiple innervation, and this is exacerbated with time after RU-486 administration.

Aberrant mediolateral elongation and terminal differentiation of transverse CF branches

We also examined the transverse branch of CFs. This branch is a thin motile collateral, which originates from the parental ascending branches, extends mediolaterally in the horizontal and transverse cerebellar planes, and rarely forms conventional synapses in wild-type rodents (Rossi et al., 1991a; Sugihara et al., 1999; Nishiyama et al., 2007). In horizontal cerebellar sections, anterograde tracer BDA clearly visualized vertical ladders of ascending CF
mice, transverse branches were immuno-negative to VGluT2 at 2 weeks (Fig. 4H,I, arrowheads), but differentiated a few VGluT2-positive terminals at 8 weeks (Fig. 4J,K, arrows). These ectopic terminals were formed more around dendritic bars lacking GluRδ2 (6.1 ± 0.6 per 100 μm of transverse branches; n = 29) than around those retaining GluRδ2 (2.6 ± 0.7; n = 31; p < 0.00001, U test) (Fig. 2F,G), indicating that terminal differentiation on transverse branches was preferentially induced at around GluRδ2-ablated PC dendrites. In parallel with marked elongation of transverse branches, VGluT2-positive terminals were greatly increased in number at 16 and 24 weeks (Fig. 4L–O, arrows).

Indeed, the mean number of VGluT2-positive terminals per 100 μm of transverse branches increased strikingly from 8 weeks to 16 and 24 weeks, showing significant differences between control and mutant mice at 8 weeks (p < 0.01, U test) and at 16 and 24 weeks (p < 0.00001 for each) (Fig. 5A). We also measured the mean number of transverse branches per 100 μm of ascending branches, and found significant increase in mutant mice at 24 weeks after RU-486 administration (Fig. 5B) (p < 0.00001). Then, we plotted the number of VGluT2-positive terminals on a given transverse branch against its relative vertical height in the molecular layer (Fig. 5C–F). In both types of mice, the majority of transverse branches originated and ran in the superficial one-half of the molecular layer [control mice, 74.5% (143 of 192 branches) and 76.7% (145 of 189 branches) at 8 and 24 weeks, respectively; mutant mice, 70.0% (147 of 210 branches) and 70.4% (162 of 230 branches) at 8 and 24 weeks, respectively]. Therefore, GluRδ2 ablation has induced marked mediolateral elongation and extensive terminal differentiation in transverse CF branches, particularly, at the superficial molecular layer.

**Multiple CF innervation is also caused by transverse CF branches**

Whether the aberrant transformation of transverse branches also caused multiple innervation was examined using mutant mice at 16 weeks after RU-486 administration. Figure 6, A and B, shows a typical example, in which a DA-594-labeled transverse branch CF-b (red) formed VGluT2-positive terminals, some of which attached to a distal dendrite PCD-a of a neighboring PC (arrowheads). This PCD-a, however, was innervated by many terminals of a DA-594-unlabeled ascending branch CF-a (green or light blue), thus representing multiple innervation. In general, such ectopic terminals of transverse CF branches were few in number and intermingled with those of main ascending CFs. In parasag-
GluR

munoelectron microscopy for VGluT2 (metal particles) and confirmed in horizontal cerebellar sections by double-labeling with DA-594 (CF-a). Multiple CF innervation was further confirmed against its relative vertical height in the molecular layer (vertical axis) in GluR

6

progressive terminal differentiation in transverse CF branches in a superficial half of the molecular layer after induction of GluR

2 flox/flox mice, and 1.18 (n = 40), and 18.1 ± 0.04 (n = 68) in GluR2

2flox/CrePR mice at 2, 8, 16, and 24 weeks, respectively (mean ± SEM; p = 0.67 at 2 weeks, 0.0066 at 8 week, p < 0.00001 at 16 and 24 weeks, U test). B, The mean number of transverse CF branches emanating from 100 μm of ascending CF branches in GluR2

2flox/fox (white bars) and GluR2

2flox/CrePR mice (black bars). Scores are 0.34 ± 0.10 (n = 45 transverse branches), 0.91 ± 0.23 (n = 39), 1.29 ± 0.30 (n = 15), and 1.14 ± 0.16 (n = 51) in GluR2

2flox/fox mice, and 0.44 ± 0.12 (n = 52), 2.37 ± 0.43 (n = 38), 13.1 ± 0.63 (n = 41), and 18.3 ± 0.64 (n = 68) in GluR2

2flox/CrePR mice at 2, 8, 16, and 24 weeks, respectively (mean ± SEM; p = 0.67 at 2 weeks, 0.0066 at 8 week, p < 0.00001 at 16 and 24 weeks, U test). Error bars indicate SEM. **p < 0.01, ***p < 0.001. C–F, Scatterplots of the number of VGluT2-positives per 100 μm of ascending branches showing distribution plots quite similar to age-matched global GluR2-knock-out mice (supplemental Fig. S3, available at www.jneurosci.org as supplemental material). The mean number of transverse branches per 100 μm of ascending branches was comparable between age-matched GluR2

2flox/CrePR and global GluR2-knock-out mice (supplemental Fig. S4B, available at www.jneurosci.org as supplemental material), although the number was significantly lower than that in GluR2

2flox/CrePR mice (p < 0.00001, U test). However, the mean number of transverse branches per 100 μm of ascending branches was comparable between age-matched GluR2

2flox/CrePR and global GluR2-knock-out mice (supplemental Fig. S4B, available at www.jneurosci.org as supplemental material). The above results indicate that the two major anatomical phenotypes of global GluR2-knock-out mice are reproduced in GluR2

2flox/CrePR mice. In addition, we confirmed in the present study that multiple CF innervation caused by both ascending and transverse branches was also observed in global GluR2-knock-out mice (supplemental Fig. S3, available at www.jneurosci.org as supplemental material).

As GluR2

2flox/CrePR mice at 24 weeks after RU-486 administration correspond to 30 weeks of age, we further compared CF phenotypes using the age-matched global GluR2-knock-out mice (supplemental Fig. S4, available at www.jneurosci.org as supplemental material). The number of VGluT2-positive terminals per 100 μm of transverse branches was abnormally high in global GluR2-knock-out mice at 30 weeks of age (supplemental Fig. S4A, available at www.jneurosci.org as supplemental material), although the number was significantly lower than that in GluR2

2flox/CrePR mice (p = 0.4250, U test). When plotting the terminal number of a given transverse branch against its relative vertical height in the molecular layer, 69.6% (135 of 194) of transverse branches were distributed in the superficial one-half of the molecular layer in global GluR2-knock-out mice at 30 weeks of age (supplemental Fig. S4C, available at www.jneurosci.org as supplemental material), showing distribution plots quite similar to age-matched GluR2

2flox/CrePR mice (Fig. 5F). From these similarities, CF phenotypes by GluR2 ablation in adult PCs mimic those by global GluR2 knock-out in many respects.

Multiple CF innervation increases progressively after RU administration

These genotypic differences were further examined electrophysiologically. Using parasagittal cerebellar slices, PCs were recorded in the whole-cell configuration, and CIs were stimulated with a glass pipette placed in the granular layer near the recorded PCs (Konnerth et al., 1990; Hashimoto et al., 2001). Individual CF-EPSC was clearly discerned, because it was elicited in an all-or-none fashion, and showed prominent paired-pulse depression (Konnerth et al., 1990). To search CIs innervating a given PC, stimulation pipette was systematically moved by ~20 μm steps, and the stimulus intensity was gradually increased at each stimulation site (pulse width, 0.1 ms; strength, 0–90 V) (Hashimoto and Kano, 2003).

In majority of PCs from control mice, a single large CF-EPSC was elicited in an all-or-none manner at each time point exam-
ined; 75.0% (27 of 36 PCs) at 2 weeks, 78.1% (25 of 32) at 8 weeks, and 75.6% (28 of 37) at 24 weeks after RU-486 administration (Fig. 7A–C, left traces; D–F, white bars), showing no significant differences between time points examined \( p > 0.41 \) between 2 and 8 weeks, \( p = 0.90 \) between 8 and 24 weeks, \( \chi^2 \) test). Thus, most PCs in control mice are innervated by single CFs. In mutant mice, the percentage of monoinnervated PCs was 73.0% (27 of 37) at 2 weeks, but decreased to 8.3% (3 of 36) at 8 weeks, and to 2.7% (1 of 37) at 24 weeks (Fig. 7A–C, right traces; D–F, black bars). Between control and mutant mice, the frequency distribution of CF-EPSC step numbers showed no significant difference at 2 weeks \( p = 0.53, \chi^2 \) test) but differed significantly at 8 and 24 weeks \( p < 0.0001 \) for each comparison). Significant difference in the frequency distribution was also found in mutant mice between 2 and 8 weeks, and between 8 and 24 weeks \( p < 0.0001 \) for each comparison, \( \chi^2 \) test). Accordingly, the mean number of
CF-EPSC steps was progressively increased in mutant PCs (1.3 ± 0.5, 3.3 ± 1.9, and 8.2 ± 3.6 CFs at 2, 8, and 24 weeks, respectively), whereas it remained stable in control PCs (1.3 ± 0.4, 1.3 ± 0.5, and 1.3 ± 0.6 CFs at 2, 8, and 24 weeks, respectively). These results indicate that multiple CF innervation progressively increased in mutant PCs.

**Surplus CF-EPSCs with small amplitudes and slow kinetics emerge after GluRδ2 ablation**

In both types of mice, CF-EPSCs in multiply innervated PCs were composed of a single main step having the largest amplitude and one or more surplus step(s) with much smaller amplitude (supplemental Fig. S5, available at www.jneurosci.org as supplemental material). The main CF-EPSCs recorded from multiply innervated PCs and CF-EPSCs from monoinnervated PCs showed no significant differences in terms of the amplitude or kinetics (data not shown), therefore they were pooled together as “main CF-EPSCs,” and their amplitudes and kinetics were compared with those of “surplus CF-EPSCs.” These main CF-EPSCs displayed a clear paired-pulse depression to a similar extent in mutant and control mice at any time points examined (Supplemental Fig. S7A; available at www.jneurosci.org as supplemental material).

In control mice, both main and surplus CF-EPSCs were distributed as a single population with a peak around 0.5 ms, and their amplitude and 10–90% rise time were similar among various time points after RU-486 administration (Fig. 8A, C, E; supplemental Fig. S5A, C, F; available at www.jneurosci.org as supplemental material). In mutant mice at 2 weeks, the distributions of amplitude and rise time of CF-EPSCs were comparable with those in control mice (Fig. 8B; supplemental Fig. S5B; available at www.jneurosci.org as supplemental material). In mutant mice at 8 and 24 weeks, however, the rise time segregated into two distinct populations (Fig. 8D, F; supplemental Fig. S5D, F; available at www.jneurosci.org as supplemental material). The fast population (<0.7 ms, mean SD for CF-EPSCs from control mice) consisted mostly of the main CFs and centered around 0.5 ms (Fig. 8D, F; supplemental Fig. S5D, F; available at www.jneurosci.org as supplemental material). However, the slow population (>0.7 ms) consisted entirely of surplus CFs peaked around 2–3 ms (3.0 ± 1.6 ms at 8 weeks; n = 75) (Fig. 8D; supplemental Fig. S5D, available at www.jneurosci.org as supplemental material) (2.2 ± 1.0 ms at 24 weeks; n = 246) (Fig. 8F; supplemental Fig. S5F; available at www.jneurosci.org as supplemental material). Importantly, when those slow CF-EPSCs were omitted, the frequency distribution of fast CF-EPSCs was comparable between control and mutant mice at each time point (Fig. 8G–I), indicating that the CF input generating slow EPSCs was selectively recruited to mutant PCs. Surplus slow CF-EPSCs were also increased in amplitude between 8 and 24 weeks (19.7 ± 14.7 pA, n = 69; 61.0 ± 86.5 pA, n = 259; mean ± SD at 8 and 24 weeks, respectively; p < 0.0001, U test) (supplemental Fig. S6; available at www.jneurosci.org as supplemental material). At 24 weeks, in addition to slow CF-EPSCs with small amplitude, those with large amplitude (>200 pA) were occasionally observed (supplemental Fig. S6B; available at www.jneurosci.org as supplemental material). At each time point, there was no significant correlation between the rise time and amplitude of slow CF-EPSCs [Pearson’s coefficients (r²) = 0.04 and 0.03 at 8 and 24 weeks, respectively] (supplemental Fig. S6; available at www.jneurosci.org as supplemental material).

In parallel with the increase of surplus slow CF-EPSC, the 10–90% rise time and decay time constant of the main CF-EPSCs became slower. At 2 weeks, the 10–90% rise time of the main CF from mutant mice was similar to that from control mice (0.4 ± 0.1 ms, n = 30; 0.4 ± 0.1 ms, n = 27; mean ± SD from control and mutant mice, respectively; p = 0.71, U test) (Fig. 8A, B; supplemental Figs. S5A, B, S7B; available at www.jneurosci.org as supplemental material), and 8 weeks (0.5 ± 0.1 ms, n = 31; 0.5 ± 0.1 ms, n = 31; from control and mutant mice, respectively; p = 0.71) (Fig. 8C, D; supplemental Figs. S5C, D, S7B; available at www.jneurosci.org as supplemental material).
However, it was significantly slower in mutant mice than in control mice at 24 weeks (0.5 \pm 0.1 ms, n = 38; 0.6 \pm 0.2 ms, n = 33; from control and mutant mice, respectively; \(p < 0.001\)) (Fig. 8E,F; supplemental Figs. S5E,F, S7B, available at www.jneurosci.org as supplemental material). The decay time constant of the mutant main CF was similar to that of control at 2 weeks (5.6 \pm 1.6 ms, n = 30; 6.1 \pm 1.4 ms, n = 28; mean \pm SD from control and mutant mice, respectively; \(p = 0.25\), U test); however, it became significantly slower than that of control one thereafter (5.5 \pm 1.1 ms, n = 29; 7.4 \pm 2.3 ms, n = 31; at 8 weeks, from control and mutant mice, respectively; 4.8 \pm 1.1 ms, n = 29; 6.8 \pm 2.3 ms, n = 32; at 24 weeks, from control and mutant mice, respectively; \(p < 0.0001\) for each comparison) (supplemental Fig. S7C, available at www.jneurosci.org as supplemental material).

The slow kinetics of CF-EPSCs might be attributable to the distal extension of CF territory (Hashimoto et al., 2001; Uemura et al., 2007); however, EPSC waveforms can also be affected by other factors, including the passive membrane properties and dendritic arborization (Hashimoto et al., 2001; Roth and Hausser, 2001). Therefore, we next examined the passive membrane properties of PCs. Both time constants for the fast (\(\tau_1\)) and slow (\(\tau_2\)) components were not significantly different between control and mutant PCs at any time points examined (supplemental Table S1, available at www.jneurosci.org as supplemental material). Then we calculated several parameters representing passive membrane properties and found that there was no significant difference between the two strains at 2 and 8 weeks. However, in mutant mice at 24 weeks, the lumped dendritic capacitance (C2) became smaller, and lumped resistance (R3) became larger than control mice (supplemental Table S1, available at www.jneurosci.org as supplemental material). These results suggest that, at 24 weeks, the average total membrane area of the mutant PC dendrites is considerably smaller than that of control. This is consistent with the morphological data that the thickness of the mutant molecular layer decreases progressively (Fig. 1G). Therefore, the slow rise and decay time constants in mutant PCs cannot be explained by the altered passive membrane properties. Together, these results collectively demonstrate that GluR2 ablation in adult PCs induces aberrant innervation of distal dendrites by both main and surplus CFs, which may lead to the generation of numerous slow EPSCs with small amplitude and the slowing of main CF-EPSC kinetics.
GluR2 suppresses distal extension and innervation by ascending CF branch

In GluR2\textsuperscript{2flox/CrePR} mice, relative height of CF terminals in the molecular layer started to increase at 8 weeks after RU-486 administration and further progressed until 24 weeks, when numerous VGluT2-positive CF terminals were distributed along atrophied distal dendrites (Fig. 1G,H). In horizontal cerebellar sections, this change was reflected as extended ladders of ascending CF branches along straight dendritic bars (Fig. 4). Importantly, distal extension of ascending branches occurred selectively along GluR2\textsuperscript{-}-ablated dendrites (Fig. 2A–E), and VGluT2-positive terminals were differentiated on them (Fig. 1E,F). Therefore, GluR2 suppresses aberrant distal extension and innervation by ascending CF branches in adult cerebellum.

This suppressive action to CF innervation should be an indirect role of GluR2. First, CF synapses in adult PCs lack GluR2 (Takayama et al., 1995, 1996; Landsend et al., 1997; Zhao et al., 1998). Second, spines on distal dendrites are innervated exclusively by PFs in adult wild-type animals (Palay and Chan-Palay, 1974; Napper and Harvey, 1988), and the loss of GluR2 generates free spines on distal dendrites (Ichikawa et al., 2002). Third, viral transfer of GluR2 to adult GluR2-knock-out mice rapidly restores PF synapse formation in transfected PCs (Kakegawa et al., 2008, 2009). These results suggest that the primary role of GluR2 is to control and consolidate the connectivity of PF–PC synapses, which then suppresses aberrant extension and innervation by CFs. The present finding eventually highlights that GluR2 actively fuels heterosynaptic competition in adult cerebellum and gives PFs a competitive advantage over CFs. This molecular function will underlie rapid reinnervation of PC spines by sprouting PFs after surgical lesion to PFs (Chen and Hillman, 1982) and may also account for proximal expansion of PF innervation after surgical denervation of CFs (Sotelo et al., 1975) or CF regression after activity blockade by TTX (Bravin et al., 1999; Morando et al., 2001; Cesa et al., 2003, 2007).

Then, the countermechanisms should be working in adult cerebellum to balance the heterosynaptic competition. In this regard, P/Q-type Ca\textsuperscript{2+} channel may be one of the likely candidates, because it exerts such action during cerebellar development (Miyazaki et al., 2004). Whatever the counter mechanisms, it will ensure rapid reinnervation of adult PCs by surviving CFs after subtotal lesion of the inferior olive (Rossi et al., 1991a,b).

GluR2 suppresses aberrant mediolateral wiring by transverse CF branch

GluR2\textsuperscript{2} ablation also drastically changed transverse CF branches, i.e., aberrant mediolateral elongation and progressive terminal differentiation (Figs. 4, 5). Ectopic terminals on transverse branches contacted PC dendrites at variable distances from parental ascending branches (Fig. 4F–O). Because transverse branches in wild-type rodents rarely form synapses on PCs (Rossi et al., 1991a; Sugihara et al., 1999; Nishiyama et al., 2007), our finding indicates that transverse branches retain synapse-forming ability, but this ability is potently suppressed by mechanisms, including GluR2. Because these aberrant transverse branches were preferentially distributed in the superficial molecular layer (Fig. 5E,F), where PF–PC synapses are the main synaptic constituent, and also because ectopic CF terminals did innervate distal dendrites of GluR2\textsuperscript{-}-ablated PCs (Figs. 1F, 6), the emergence of free spines on distal dendrites likely induce aberrant mediolateral and distal extension of CF innervation.

Mediolateral extension of CF wiring will have another impact on the cerebellar physiology. Longitudinal olivocerebellar micro-
zones have been identified in the cerebellar cortex, based on high
synchrony of complex spike activity (Llínás and Sasaki, 1989;
Sasaki et al., 1989; Sugihara et al., 1993; Lang et al., 1999; Fukuda
et al., 2001) and Ca²⁺ spikes (Ozden et al., 2008; Mukamel et al.,
2009; Schultz et al., 2009). Each microzone is ~500 μm in width,
stable across behavioral states, and has sharp boundary with the
neighboring microzones (Mukamel et al., 2009). This synchrony
is based on electrical coupling of nearby olivary neurons through
dendrodendritic gap junction (Llínás et al., 1974; Sotelo et al.,
1986; Angaut and Sotelo, 1989; de Zeeuw et al., 1990) and topo-
graphical projection from given subnuclei of the inferior olive to
specific longitudinal cortical zones (Sugihara et al., 1999, 2001).
Axons derived from single olivary neurons project rostrocaudally
to a narrow band (8 μm in width) within a single lobe or across
multiple lobules, but do not project mediolaterally (Sugihara et
al., 1999, 2001). Therefore, very frequent mediolateral wiring by
transverse branches will broaden and even disrupt functional or-
organization of the olivocerebellar microzone system in GluR₂-
ablated cerebellum. Considering that GluR₂-deficient PCs produce
abnormal oscillating action potentials at ~10 Hz be-
cause of enhanced CF activities and are thought to relate to in-
voluntary spontaneous eye movement with characteristic 10 Hz
oscillation (Yoshida et al., 2004), the aberrant mediolateral wir-
ing could be one such anatomical basis for anomalous oscillatory
activities and severe motor deficits in this mutant.

GluR₂ is essential to maintain CF monoinnervation
Aberrant distal extension and mediolateral wiring eventually
produced an enormous number of surplus branches that inner-
vated many PCs around the main target, leading to frequent oc-
currence of multiple innervation (Figs. 3, 6). This anatomical
phenotype was in good agreement with electrophysiological data.
The number of CF-EPSC steps in GluR₂^<sup>−/−</sup> mice at 2
weeks after RU-486 administration was comparable with that in
GluR₂<sup>+/+</sup> mice (Fig. 7D). However, the number of CF-EPSC
steps was significantly increased at 8 weeks (Fig. 7E), and ~90% of
PCs exhibited more than four steps at 24 weeks (Fig. 7F). Most
surplus CF-EPSCs had small amplitude and slow kinetics (Figs. 7,
8; supplemental Fig. S5, available at www.jneurosci.org as sup-
plemental material). These surplus CF-EPSCs with slow kinetics
increased in both number and amplitude, whereas those with fast
kinetics were unchanged (Fig. S6–F, supplemental Figs. S5, S6,
available at www.jneurosci.org as supplemental material). Con-
idering that CF-EPSCs are strongly attenuated and slowed by
dendritic filtering (Hashimoto et al., 2001; Roth and Hauser,
2001), slow CF-EPSCs with small amplitude likely correspond to
aberrant wiring formed by ascending and transverse CF branches
onto distal dendrites.

In contrast to GluR₂<sup>−/−</sup> mice, a substantial fraction of
surplus CF-EPSCs in global GluR₂ knockout mice displays fast
as well as slow kinetics (Hashimoto et al., 2001). It is during the
second postnatal week that PF synapses are formed enormously
onto spines of growing PC dendrites in wild-type rodents (Woodward et al., 1971; Sotelo, 1978; Takács and Hámori, 1994),
and that free spines increase drastically in global GluR₂ knockout
mice (Kurihara et al., 1997). Furthermore, it is also during this
period that a single “winner” CF translocates to PC dendrites
(Hashimoto et al., 2009). Since PF synapse activity is required to
eliminate surplus CFs in normal cerebellar development (Bravin et al., 1995; Kazikawa et al., 2000), severe impairment during the
period of active PF–PC synaptogenesis in global GluR₂ knockout
mice may allow surplus CFs with fast kinetics to survive into
adolescence. Furthermore, free spines generated on growing PC

dendrites might help surplus CFs to translocate from soma to proximal dendrites. By contrast, the elimination of surplus CFs
and dendritic translocation of a single winner CF have completed
well before the onset of GluR₂ ablation in GluR₂<sup><sup>−/−</sup></sup> mice. Therefore, unoccupied postsynaptic substrates are only available
at distal dendrites in GluR₂<sup>−/−</sup> mice, resulting in more purely
distal type of multiple innervation than global GluR₂ knockout
mice. Considering that the density of VGluT2-positive terminals on transverse branches was significantly higher
in GluR₂<sup>−/−</sup> mice than global GluR₂ knockout mice (supplemental Fig. S4A, available at www.jneurosci.org as sup-
plemental material), some compensatory mechanisms might
work less in adulthood than during development.

Temporal relationship of molecular, synaptic, and
behavioral deficits
Together with our previous study using GluR₂<sup>−/−</sup> mice
(Takeuchi et al., 2005), temporal relationships of molecular ab-
lution, synaptic wiring abnormality, and behavioral deficit can be
discussed. After RU-486 administration, the percentage of PCs
expressing GluR₂ mRNA was reduced to one-half level at 2
weeks and 20% at 4 weeks. Cerebellar contents of GluR₂ protein
were reduced to one-half level at 4 weeks, 21% at 8 weeks, and
<10% by 24 weeks (Takeuchi et al., 2005). Considering that RU-
486 was only administered at 2 consecutive days during P42–P45,
this protracted time course should represent high stability of
GluR₂ mRNA and protein rather than low efficiency of gene
recombination.

Synaptic wiring abnormalities, including mismatched PF syn-
apses, free spines, extended CF territory, and multiple CF innerva-
tion, all became evident by 8 weeks after RU-486 administration,
when cerebellar GluR₂ contents were reduced to 21% of the control level. The abnormalities of PF–PC synapses correlated inversely with synaptic GluR₂ protein densities (Takeuchi et al., 2005) and the aberrant CF innervation occurred against GluR₂ ablating PC dendrites. These results indicate that defects of synaptic wiring proceed in a manner highly sensitive to
GluR₂ loss. In comparison, motor discoordination was not ap-
parent at 8 weeks and gradually deteriorated thereafter (Fig. 9).
Considering severe abnormalities in synaptic wiring at 16–24
weeks after RU-486 administration, our findings suggest that an-
imals come to manifest cerebellar symptoms and disorders after
considerable accumulation of wiring defects.

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