Control of Synaptic Connection by Glutamate Receptor $\delta 2$ in the Adult Cerebellum

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Precise topological matching of presynaptic and postsynaptic specializations is essential for efficient synaptic transmission. Furthermore, synaptic connections are subjected to rearrangements throughout life. Here we examined the role of glutamate receptor (GluR) $\delta 2$ in the adult brain by inducible and cerebellar Purkinje cell (PC)-specific gene targeting under the pure C57BL/6 genetic background. Concomitant with the decrease of postsynaptic GluR $\delta 2$ proteins, presynaptic active zones shrank progressively and postsynaptic density (PSD) expanded, resulting in mismatching between presynaptic and postsynaptic specializations at parallel fiber–PC synapses. Furthermore, GluR $\delta 2$ and PSD-93 proteins were concentrated at the contacted portion of mismatched synapses, whereas AMPA receptors were distributed in both the contacted and dissociated portions. When GluR $\delta 2$ proteins were diminished, PC spines lost their synaptic contacts. We thus identified postsynaptic GluR $\delta 2$ as a key regulator of the presynaptic active zone and PSD organization at parallel fiber–PC synapses in the adult brain.

Key words: cerebellar Purkinje cell; conditional gene targeting; glutamate receptor $\delta 2$; postsynaptic density; synaptic connection; synaptic matching

Introduction

Brain function is based on highly complex neural networks and their dynamics. Efficient synaptic transmission requires elaborate structural specializations in both presynaptic and postsynaptic cells and precise topological matching of presynaptic transmitter release machineries and postsynaptic neurotransmitter receptor complexes. Furthermore, changes in spine morphology and number after learning are observed in several brain regions, including the cerebellum (Bailey and Kandel, 1993; Lamprecht and LeDoux, 2004). Although molecular understanding of synapse formation during development is emerging (Hering and Sheng, 2001; Goda and Davis, 2003; Scheiffele, 2003), the molecular mechanism for regulation of synaptic connections in the adult brain remains to be investigated.

The δ subfamily of glutamate receptor (GluR) was found by molecular cloning (Yamazaki et al., 1992), and its positions in

between the classical AMPA–kainate and NMDA subtypes from the amino acid sequence identity. GluR $\delta 2$, the second member of this subfamily, is selectively expressed in cerebellar Purkinje cells (PCs) (Araki et al., 1993; Lomeli et al., 1993), and within PCs, GluR $\delta 2$ proteins are localized exclusively at parallel fiber (PF)–PC synapses (Takayama et al., 1996; Landsend et al., 1997). Our previous studies showed that mutant mice lacking GluR $\delta 2$ are defective in long-term depression at PF–PC synapses, motor learning, and motor coordination (Funabiki et al., 1995; Kashiwabuchi et al., 1995; Kishimoto et al., 2001; Mishina, 2003). In addition, the number of PF–PC synapses decreased and innervation of PCs by multiple climbing fibers was sustained in GluR $\delta 2$ mutant mice (Kashiwabuchi et al., 1995; Kurihara et al., 1997; Hashimoto et al., 2001; Lalouette et al., 2001; Ichikawa et al., 2002). These studies revealed that in cerebellar PCs, in which functional NMDA receptors are absent, GluR $\delta 2$ plays a central role in synaptic plasticity, motor learning, and cerebellar wiring. However, the primary role of GluR $\delta 2$ and whether GluR $\delta 2$ regulates synaptic connections in the adult brain or only during development remained to be investigated. The NMDA receptor also plays important roles both in learning and memory (Martin et al., 2000) and in neural network formation during development (Goodman and Shatz, 1993). Furthermore, activation of NMDA receptors induces structural alterations of spines of cultured hippocampal neurons (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999). On the other hand, ablation of NMDA receptors in the adult brain exerted no detectable effects on synaptic structures (Tsien et al., 1996; Rampon et al., 2000).

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Thus, it is crucial to determine whether the diverse functional roles of GluR82 revealed by studies of conventional knock-out mice, especially regulation of cerebellar wiring, are specific for the developmental stage, although we suggested a close relationship in the molecular mechanism between synaptic plasticity at the adult stage and synapse formation during development in the cerebellum (Kashiwabuchi et al., 1995). To address this issue, we generated a target mouse line carrying the *GluR82* gene flanked by *loxP* sequences using embryonic stem (ES) cells derived from the C57BL/6 strain. Crossing the target mouse with a Cre mouse line carrying the Cre recombinase–progesterone receptor fusion protein (CrePR) gene under the control of the *GluR82* gene promoter enabled us to inductively abolish GluR82 proteins selectively in cerebellar PCs of the mature brain. Here we report the effects of inducible ablation of GluR82 proteins on the structures of PF–PC synapses in the adult cerebellum.

Materials and Methods

Generation of mice. We isolated a mouse genomic clone carrying exon 12 of the *GluR82* gene by screening a bacterial artificial chromosome library prepared from the C57BL/6 strain (Incyte Genomics, St. Louis, MO) using a mouse *GluR82* cDNA encoding putative transmembrane segments M1–M3 as a probe. The 7.9 kb *Bam*HI fragment from the genomic clone was subcloned into the *Bam*HI site of pBluescript II KS(+) (Stratagene, La Jolla, CA) to yield pGRD2B1. The 1.8 kb DNA fragment carrying the 34 bp *loxP* sequence and *Pgk-1* promoter-driven neomycin phosphotransferase gene (*neo*) flanked by two FLP recognition target (*frt*) sites was inserted into the *Hinc*II site 381 bp upstream of exon 12, and the 34 bp *loxP* sequence with 10 bp linker sequences into the *Hind*III site 122 bp downstream of exon 12. The targeting vector pTVD2V4 contained exon 12 of the *GluR82* gene flanked by *loxP* sequences, the 9.9 kb upstream and 2.3 kb downstream genomic sequences, and 4.3 kb pMC1DTPa (Taniguchi et al., 1997).

We isolated a subclone of ES cells derived from the C57BL/6 strain (Köntgen et al., 1993). ES cells were cultured on mitomycin C-treated neomycin-resistant fibroblasts in knock-out DMEM supplemented with 17.9% knock-out serum replacement (Invitrogen, Carlsbad, CA), 90.7 μ M nonessential amino acids, 1 mM L-glutamine, 100 μ M 2-mercaptoethanol, and 1×10^3 U/ml leukemia inhibitory factor (Chemicon International, Temecula, CA) at 37°C under humidified atmosphere containing 5% CO₂. The targeting vector was linearized by *Not*I and electroporated into ES cells using Gene Pulser (Bio-Rad, Hercules, CA) (250 V, 960 μ F, $r = \infty$). G-418 selection (150 μ g/ml) was started 36–48 h after electroporation and continued for 1 week. Recombinant clones were identified by Southern blot hybridization analysis of *Sca*I- or *Nhe*I-digested genomic DNA using the 0.6 kb *Eco*8II–*Msc*I fragment from pGRD2B1, the 0.6 kb *Pst*I fragment from pLFNeo (Takeuchi et al., 2002), and the 4.0 kb *Hind*II–*Nhe*I fragment from pD2–9 (Kashiwabuchi et al., 1995) as 5', *neo*, and 3' probes, respectively. Recombinant ES cells were injected into eight-cell stage embryos of ICR mice. The embryos were cultured to blastocysts and transferred to the pseudopregnant ICR uterus.

Resulting chimeric mice were mated to FLP66 transgenic mice of the C57BL/6 strain (Takeuchi et al., 2002), and male offspring were further crossed with C57BL/6 mice. Elimination of the *neo* gene from the genome through FLP/*frt*-mediated excision was identified by Southern blot hybridization analysis with a 3' probe. *GluR82*^{+/lox} mice were crossed with *GluR82*^{+/CrePR} mice (Kitayama et al., 2001) to yield *GluR82*^{lox/lox;CrePR} mice. Littermates derived from crossing of *GluR82*^{lox/lox} and *GluR82*^{lox/lox;CrePR} mice with the pure C57BL/6 genetic background were used for subsequent studies. Animal care was performed in accordance with institutional guidelines. Mice were fed *ad libitum* with standard laboratory chow and water in standard animal cages under a 12 h light/dark cycle.

The *GluR82*^{lox} allele was identified by PCR using primers 5'-AGCAACCTACACTCCCAAAGAAG-3' (FD2P3) and 5'-ATTCAGT-GCCAAGACAGACACAA-3' (FD2P4) or by Southern blot hybridization analysis of *Nhe*I-digested genomic DNA with a 3' probe. The

GluR82^{CrePR} allele was identified by PCR using CreP1 and CreP2 primers (Tsujita et al., 1999).

Induction of recombination. RU-486 (Sigma, St. Louis, MO) was suspended at a concentration of 50 mg/ml in water containing 0.25% (v/v) carboxymethyl cellulose and 0.5% (v/v) Tween 80. We injected 1 mg/kg body weight of RU-486 into the peritoneum of *GluR82*^{lox/lox} and *GluR82*^{lox/lox;CrePR} mice at postnatal day 42 (P42)–P45 for 2 consecutive days. *In situ* hybridization analysis of *GluR82* mRNA was performed as described (Watanabe et al., 1993) using oligonucleotide probes 5'-ACCAAGCCCCCATCATCATTCGGGTGTCAGAGTTGTGTAT-GGGA-3' and 5'-CTCTCAATGCGGGTGATAGTGAGGAAAGCGG-CAAGTTGGCTGTA-3' in mixture. Autoradiographic silver grains on PCs and the granular layer were counted to obtain the signal and noise levels, respectively. Quantitative immunoblotting analysis of GluR82 proteins in cerebellar homogenate was performed using rabbit anti-GluR82 antibody (Araki et al., 1993) and chemiluminescence (Amersham Biosciences, Piscataway, NJ). The images were acquired using a CCD camera (LAS-1000plus; Fuji Photo Film, Tokyo, Japan) and analyzed with Science Lab 98 Image Gauge (Fuji Photo Film). GluR82 signals were normalized with those of GluR1 (Watanabe et al., 1998) as an internal standard.

Histology and immunofluorescence. Under deep pentobarbital anesthesia (100 μ g/g of body weight, i.p.), mice were perfused transcardially with 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, and processed for paraffin sections (5 μ m) with a sliding microtome (SM2000R; Leica Microsystems, Nussloch, Germany). Paraffin sections were stained with hematoxylin or immunostained with rabbit anti-GluR82 antibody (Araki et al., 1993) and guinea pig anti-calbindin antibody (Nakagawa et al., 1998) followed by incubation with species-specific FITC- and Cy3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Photographs were taken by a fluorescence microscope (AX-70; Olympus, Tokyo, Japan) or a confocal laser scanning microscope (Fluoview; Olympus).

Electron microscopy. For qualitative and quantitative analyses by conventional electron microscopy, ultrathin sections cut in the parasagittal plane were prepared from the straight portion of lobules 4/5. Serial ultrathin sections were mounted on formvar-supported copper grids and stained with 2% uranyl acetate for 5 min and mixed lead solution for 2 min. In each mouse, electron micrographs were taken randomly from the neuropil of the molecular layer. Electron micrographs were taken at an original magnification of 4000 $\times$ or 10,000 $\times$ using an H-7100 electron microscope (Hitachi High-Technologies, Tokyo, Japan), and printed at a final magnification of 16,000 $\times$ or 40,000 $\times$. For measurement of active zone and postsynaptic density (PSD) lengths, electron micrographs were scanned and analyzed with MetaMorph software (Molecular Devices, Sunnyvale, CA). The two-dimensionally reconstructed images were drawn and processed for smoothing with Adobe Illustrator software (version 10; Adobe Systems, San Jose, CA). For phosphotungstic acid cytochemistry, microtome sections (300 μ m in thickness) were dehydrated in graded alcohols and incubated in 1% phosphotungstic acid in ethanol for 1 h and embedded in Epon812 resin.

Postembedding immunogold. For postembedding immunogold electron microscopy, microtome sections (300 μ m in thickness) were cryoprotected with 30% glycerol in 0.1 M sodium phosphate buffer, pH 7.4, and frozen rapidly in liquid propane in a cryofixation unit (CPC; Leica Microsystems). The specimens were transferred to 0.5% uranyl acetate in methanol in a cryosubstitution unit (AFS; Leica Microsystems) and embedded in Lowicryl HM20 resin (Electron Microscopy Sciences, Hatfield, PA). Serial ultrathin sections were mounted on Formvar-supported nickel grids for GluR82 labeling, whereas for PSD-93 and AMPA receptor labeling, single sections were mounted directly to nickel grids. For the single-section labeling, sections were etched with a saturated solution of NaOH in absolute ethanol for a few seconds. After blocking with 2% human serum albumin (Wako Pure Chemicals, Osaka, Japan) in 10 mM Tris-buffered saline, pH 7.6, grids were immunoreacted with rabbit anti-GluR82 antibody (20 μ g/ml) or rabbit anti-PSD-93 antibody (20 μ g/ml) (Fukaya and Watanabe, 2000) or the mixture of rabbit anti-GluR1, -GluR2, and -GluR3 antibodies (20 μ g/ml each) (Shimuta et al., 2001) overnight and colloidal gold (10 nm)-conjugated anti-rabbit IgG (British Biocell International, Cardiff, UK) for 2 h. They were stained with 2%

Figure 1. Inducible and PC-specific *GluRδ2* gene ablation by CrePR–loxP recombination system. **A**, Schema of the exon 12 region of the *GluRδ2* gene (*GluRδ2*⁺), floxed and *neo*-inserted allele (*GluRδ2*^{lox/neo}), and floxed allele (*GluRδ2*^{lox}). Exon 12 encodes the putative transmembrane segment M3 of GluRδ2. The *GluRδ2*^{lox/neo} allele contains two *loxP* sequences flanking exon 12 of the *GluRδ2* gene and the *neo* gene flanked by two *frt* sequences. The *neo* gene was removed *in vivo* by crossing *GluRδ2*^{lox/neo};+/+ mice with FLP66 mice carrying the Flp recombinase gene under the control of the *EF1α* promoter (Takeuchi et al., 2002). N, *NheI*; S, *ScaI*. **B**, Southern blot analysis of genomic DNA from *GluRδ2*^{+/+}, *GluRδ2*^{lox/neo}, *GluRδ2*^{lox/lox}, and *GluRδ2*^{lox/neo} mice. Left, *ScaI*-digested DNA hybridized with 5' probe; middle, *NheI*-digested DNA hybridized with *neo* probe; right, *NheI*-digested DNA hybridized with 3' probe. **C**, Western blot analysis of GluRδ2 and GluRε1 proteins in cerebellar homogenates from *GluRδ2*^{+/+} and *GluRδ2*^{lox/lox} mice. **D**, Schema for induction of cerebellar PC-specific *GluRδ2* gene ablation in the adult brain. In *GluRδ2*^{lox/CrePR} mice, one allele retains the intact *GluRδ2* gene with *loxP* sequences, and the other is inactivated by insertion of the *CrePR* gene. RU-486 administration induces recombination by CrePR, leading to *GluRδ2* gene knock-out in the adult brain in a cerebellar PC-specific manner.

uranyl acetate for 5 min and mixed lead solution for 30 s. The background level was defined as the density of immunogold particles fell within 30 nm on both sides from the mitochondrial membrane (GluRδ2 and AMPA receptors) or presynaptic nonjunctional membrane (PSD-93).

Statistical analysis. Data were analyzed by Student's *t* test or one-way ANOVA followed by Tukey–Kramer *post hoc* test.

Results

Inducible and cerebellar PC-specific *GluRδ2* gene ablation

To address the functional roles of cerebellar PC-specific GluRδ2 in the adult brain by inducible Cre-mediated recombination, we constructed a targeting vector in which two *loxP* sequences were

Figure 2. Inducible and cerebellar PC-specific eliminations of *GluRδ2* mRNA and GluRδ2 proteins in *GluRδ2*^{lox/CrePR} mice. We injected RU-486 into *GluRδ2*^{lox/CrePR} mice at P42–P45 to induce CrePR recombinase activity. *GluRδ2*^{lox/lox} mice injected with the antiprogesterone served as controls. Temporal patterns of cerebellar PC-specific elimination of the *GluRδ2* mRNA and GluRδ2 proteins were assessed before and 2, 4, 8, 12, and 24 weeks after RU-486 treatment. **A**, *In situ* hybridization analysis of the *GluRδ2* mRNA in the cerebella of *GluRδ2*^{lox/lox} and *GluRδ2*^{lox/CrePR} mice before and 2, 4, and 8 weeks after drug treatment. Parasagittal sections were hybridized with ³³P-labeled oligonucleotide probes and counterstained with methyl green–pyronine. Arrowheads indicate strongly labeled PCs. **B**, Percentages of PCs labeled strongly for *GluRδ2* mRNA in the cerebella of *GluRδ2*^{lox/lox} (○) and *GluRδ2*^{lox/CrePR} (●) mice before and after drug treatment (2 mice). **C**, Western blot analysis of GluRδ2 and GluRε1 proteins in the cerebella of *GluRδ2*^{lox/lox} and *GluRδ2*^{lox/CrePR} mice before and 4, 8, 12, and 24 weeks after drug treatment (2 mice). **D**, Relative expression levels of GluRδ2 proteins in the cerebella of *GluRδ2*^{lox/lox} (○) and *GluRδ2*^{lox/CrePR} (●) mice before and 4, 8, 12, and 24 weeks after RU-486 treatment (mean ± SEM; *n* = 3). A gray circle indicates the value of mock-treated *GluRδ2*^{lox/CrePR} mice (*n* = 3). The amounts of GluRδ2 proteins in *GluRδ2*^{lox/CrePR} mice before drug treatment were taken as 100%. **E**, Immunohistochemical analysis of GluRδ2 proteins in the cerebella of *GluRδ2*^{lox/lox} and *GluRδ2*^{lox/CrePR} mice before and 4, 8, 12, and 24 weeks after drug treatment. **F**, Cerebellar histology of *GluRδ2*^{lox/lox} and *GluRδ2*^{lox/CrePR} mice 8 weeks after RU-486 treatment. Shown are hematoxylin staining and immunofluorescence for calbindin (green) and GluRδ2 (red) in the molecular layer of the cerebellum. Scale bars: **A**, 20 μm; **E**, 1 mm; **F**, 50 μm.

inserted into the mouse *GluRδ2* gene isolated from the C57BL/6 strain. The first *loxP* sequence linked to the *neo* gene flanked by two *frt* sites was in the intron upstream of exon 12 encoding putative transmembrane segment M3, and the second one was in the intron downstream of exon 12. Using a subline of ES cells derived from the C57BL/6 strain (Köntgen et al., 1993), we obtained recombinant *GluRδ2*^{+/flox}; +/*neo* mice (Fig. 1A). By crossing to FLP66 transgenic mice of the C57BL/6 strain carrying an FLP recombinase expression vector under the control of the *EF1α* promoter (Takeuchi et al., 2002), we successfully eliminated the *neo* gene to yield *GluRδ2*^{+/flox} mice with the *GluRδ2* gene flanked by two *loxP* sequences (Fig. 1B). The expression of GluRδ2 proteins was comparable between *GluRδ2*^{+/+} and *GluRδ2*^{flox/flox} mice (Fig. 1C).

We crossed *GluRδ2*^{flox/flox} target mice with *GluRδ2*^{+/CrePR} mice carrying the *CrePR* gene inserted into the *GluRδ2* gene and thus were capable of mediating Cre–loxP recombination selectively in cerebellar PCs after induction (Kitayama et al., 2001). The resulting *GluRδ2*^{flox/CrePR} mice carrying the floxed *GluRδ2* gene on one allele and the *CrePR* gene on the other grew and mated normally. To examine the functional roles of GluRδ2 in the adult cerebellum, we induced the recombinase activity of CrePR by intraperitoneal injection of antiprogesterone RU-486 into *GluRδ2*^{flox/CrePR} mice at P42–P45 for 2 consecutive days when cerebellar wiring was well completed and fully established PF–PC synapses were functional (Fig. 1D). *GluRδ2*^{flox/flox} mice treated with RU-486 served as controls. We first examined the expression of the *GluRδ2* mRNA in cerebellar PCs by *in situ* hybridization before and at 2, 4, and 8 weeks after RU-486 injection (Fig. 2A). Before antiprogesterone administration, strong hybridization signals, defined as those with the signal-to-noise ratio >4.0, were found in almost all PCs of both *GluRδ2*^{flox/CrePR} (98%; *n* = 2) and *GluRδ2*^{flox/flox} (100%; *n* = 2) mice. RU-486 exerted little effect on the percentage of hybridization-positive PCs in *GluRδ2*^{flox/flox} mice (100% at 8 weeks after injection). In *GluRδ2*^{flox/CrePR} mice, however, the percentage of hybridization-positive PCs decreased to 23% at 4 weeks after RU-486 injection and gradually decreased thereafter with a reciprocal increase of PCs with low or no hybridization signals for the *GluRδ2* mRNA (Fig. 2B).

We then estimated the amounts of GluRδ2 proteins by immunoblot analysis, comparing those of NMDA receptor GluR1 proteins that are expressed in cerebellar granule cells but not in PCs (Yamada et al., 2001) as internal standards (Fig. 2C,D). The amounts of GluRδ2 proteins in *GluRδ2*^{flox/flox} mice were essentially unaffected by RU-486 treatment. In contrast, GluRδ2 proteins gradually decreased to 21% at 8 weeks after RU-486 injection, indicating successful *GluRδ2* gene knock-out in mature PCs by the CrePR–antiprogesterone induction system. *GluRδ2*^{flox/CrePR} mice at 8 weeks after mock treatment showed no decrease in the amount of GluRδ2 proteins (Fig. 2D). We further stained parasagittal cerebellar sections with anti-GluRδ2 antibody (Fig. 2E). Before induction, intense GluRδ2 immunoreactivity was present in the molecular layer of both *GluRδ2*^{flox/CrePR} and *GluRδ2*^{flox/flox} mice. RU-486 treatment of *GluRδ2*^{flox/flox} mice exerted little effect on GluRδ2 signals. In *GluRδ2*^{flox/CrePR} mice, however, a heterogeneous decrease of GluRδ2 immunoreactivity was discernable at 4 weeks after RU-486 injection. At 8 weeks, chimeric patterns of GluRδ2 signals became apparent with alternating strong and pale immunofluorescent bands in the molecular layer. At 12 weeks, pale or blank bands expanded in the molecular layer, being separated by thin bands with strong GluRδ2 immunoreactivity. The conditional ablation of GluRδ2 induced by RU-486 injection was not preferential in particular lobules or regions and occurred

rather evenly in the anteroposterior and mediolateral axes of the cerebellum. Multiple factors may account for the relatively slow time course of GluRδ2 ablation. Knock-down of *GluRδ2* mRNA below the detection level in 80% of PCs took 4 weeks after administration of RU-486, suggesting a time lag between drug-induced activation of CrePR and mRNA degradation. Loss of 80% of GluRδ2 proteins took an additional 4 weeks, suggesting a slow turnover rate of GluRδ2 proteins.

The cerebellum of the RU-486-treated *GluRδ2*^{flox/CrePR} mice exhibited normal foliation and laminated cortical structures (Fig. 2E). No apparent differences were detectable in the thickness of the molecular and granular layers between *GluRδ2*^{flox/CrePR} and *GluRδ2*^{flox/flox} mice until 12 weeks after RU-486 treatment. Double immunostaining for GluRδ2 and calbindin revealed that PCs extended well arborized dendrites studded with numerous spines in *GluRδ2*^{flox/CrePR} and *GluRδ2*^{flox/flox} mice at 8 weeks after RU-486 treatment (Fig. 2F), indicating that the chimeric patterns of GluRδ2 immunoreactivity were not caused by the loss of PCs or distorted arborization of dendrites.

Appearance of synaptic mismatching and free spines

By electron microscopy, we inspected structures of PF–PC synapses in *GluRδ2*^{flox/CrePR} and *GluRδ2*^{flox/flox} mice at 8 weeks after RU-486 treatment; treated *GluRδ2*^{flox/CrePR} and *GluRδ2*^{flox/flox} mice were hereafter called mutant and control mice, respectively. Asymmetrical synapses were the most frequent type of synapses in the molecular layer of the cerebellum, and most of them were identified as PF–PC synapses on the basis of their characteristic morphology (Miyazaki et al., 2004). On single electron micrographs, all PC spines having PSD formed synaptic contacts with PFs in control mice (Fig. 3A). In mutant mice, however, some PC spines possessing PSD lacked presynaptic counterparts, suggesting the emergence of free spines (Fig. 3B, f). Furthermore, we noted that PSD in some contacted spines extended over the apposed active zone (Fig. 3B, arrows). In contrast, active zone and PSD were well matched in control mice (Fig. 3A).

We then examined randomly sampled PC spines by serial electron microscopy. In control mice, all PC spines had synaptic contacts with PF terminals (Fig. 3C). The analysis convincingly demonstrated the emergence of PC spines free from synaptic contacts in mutant mice, and they were thoroughly surrounded by cytoplasmic sheets of Bergmann glia (Fig. 3D). In such free spines, PSD-like condensation was present but was apparently smaller in size. These free spines were reminiscent of those found in *GluRδ2* null mutant mice (Kashiwabuchi et al., 1995; Kurihara et al., 1997).

Serial electron microscopy further enabled us to precisely classify PC spines of mutant mice as those forming matched contact, those forming mismatched contact, and free spines. For quantitative comparison, we defined a synapse as mismatched when the edges of the active zone and PSD were >100 nm apart in any of its serial sections. Under this criterion, almost all PC spines in control mice were classified as normal matched synapses [99.2 ± 0.6% (mean ± SEM); *n* = 359; three mice] (Fig. 3E). In contrast, 17.1 ± 3.3% of PC spines in mutant mice (*n* = 358; three mice) were free from synaptic contacts and 13.1 ± 2.7% had mismatched contacts with PF terminals (Fig. 3G,H). Such mismatching between the active zone and PSD was further confirmed by phosphotungstic acid cytochemistry that selectively visualizes electron-opaque materials in the active zone, synaptic cleft, and PSD (Bloom and Aghajanian, 1966) (Fig. 3I–L). Two-dimensional extents of the active zone and PSD were reconstructed from serial sections (Fig. 3M–P); when superimposed,

Figure 3. Emergence of mismatched PF–PC synapses and free PC spines. RU-486-treated *GluRδ2*^{flox/flox} mice served as controls. **A**, Normal synapses (n) in the cerebellum of a control mouse (RU-486-treated *GluRδ2*^{flox/flox} mouse). Active zone and PSD are matched at all PF–PC synapses. **B**, Mismatched synapses (m) and free spines (f) in the cerebellum of a mutant mouse (RU-486-treated *GluRδ2*^{flox/CrePR} mouse). Arrows indicate the mismatch between active zone and PSD at PF–PC synapses. **C**, **D**, Serial images of a normal synapse in the control cerebellum (**C**) and of a free spine in the mutant cerebellum (**D**). Asterisks indicate dendritic spines of PCs. **E–H**, Serial images through normal (**E**), matched (**F**), and mismatched (**G**, **H**) synapses. White and black arrowheads indicate the edges of active zone and PSD, respectively. **I–L**, Phosphotungstic acid cytochemistry of a normal synapse in the control cerebellum (**I**) and matched (**J**) and mismatched (**K**) synapses and a free spine (**L**) in the mutant cerebellum. White and black arrowheads indicate the edges of active zone and PSD, respectively. **M–P**, Two-dimensionally reconstructed images of active zone (gray) and PSD (black) at normal (**M**), matched (**N**), and mismatched synapses (**O**), and free spines (**P**). Reconstruction was accomplished by stacking and smoothing images of three to six serial sections in the vertical axis. Linear outlines in the top and bottom sides are caused by the omission of sections through the margin of synapses, which were inappropriate for structural identification and reconstruction. Scale bars: **A**, **C**, 500 nm; **E**, **I**, **M**, 200 nm.

these extents of the active zone and PSD overlapped at all synapses in control mice, thus being normal synapses (Fig. 3M). At mismatched synapses in mutant mice, PSD was irregularly expanded over the apposed active zone (Fig. 3O). Interestingly, some synapses classified as matched in mutant mice showed a dissociating tendency between active zone and PSD (Fig. 3F,N). Free spines had PSD, but the areas were smaller than those at normal, matched, and mismatched synapses (Fig. 3P). Free spines and mismatched synapses were hardly detectable in *GluR δ 2^{flox/CrePR}* mice at 8 weeks after mock treatment ($n = 300$; three mice).

Ablation of GluR δ 2 proteins and structural alterations at PF–PC synapses

We applied postembedding immunogold to examine whether these structural alterations at PC synapses correlate with GluR δ 2 protein levels at individual synapses. The labeling density per 1 μm of PSD length was determined by measuring the total number of gold particles and the total length of PSD appearing on serial sections through a given synapse (Fig. 4). The mean labeling density at normal synapses in control mice was 14.8 ± 0.9 ($n = 46$; three mice) (Fig. 4A,E). In mutant mice, the mean GluR δ 2-labeling density decreased in the order of matched synapses (9.2 ± 0.7 ; $n = 65$; three mice) (Fig. 4B,F) > mismatched synapses (5.4 ± 0.6 ; $n = 44$) (Fig. 4C,G) > free spines (0.2 ± 0.2 ; $n = 18$) (Fig. 4D,H); the background density was 0.2 ± 0.1 ($n = 56$; total measured length, 116.4 μm). There were significant differences in the GluR δ 2 labeling among matched synapses, mismatched synapses, and free spines in mutant mice (ANOVA, $F_{(2,124)} = 26.9$, $p < 0.0001$; *post hoc* test, $p < 0.01$). Furthermore, the GluR δ 2-labeling density in the dissociated portion of PSD at mismatched synapses (1.2 ± 0.7 ; $n = 44$; three mice) was very low and close to that of free spines, whereas the density in the contacted portion at mismatched synapses (6.8 ± 0.7) was close to that of matched synapses. Thus, the density in the dissociated portion was much smaller than that in the contacted portion (*t* test; $p < 0.0001$).

Changes in sizes of the active zone and PSD

From serial profiles of a given classified synapse, we chose a single profile having the largest PSD to compare the length of active zone and PSD (45 synapses from three control mice; 43 matched and 39 mismatched synapses and 28 free spines from three mutant mice). As shown in Figure 5A by cumulative frequency plots, the length of active zone decreased significantly in the order of normal synapses in control mice (348 ± 12 nm) > matched synapses in mutant mice (296 ± 10 nm) > mismatched synapses in mutant mice (252 ± 10 nm) (ANOVA, $F_{(2,124)} = 19.1$, $p < 0.0001$; *post hoc* test, $p < 0.01$ for normal vs matched or mismatched; $p < 0.05$ for matched vs mismatched). Thus, the sizes of active zone corresponded well with the densities of GluR δ 2 immunogold particles. The length of PSD decreased significantly in the order of normal synapses in control mice (379 ± 13 nm) > matched synapses in mutant mice (332 ± 10 nm) > free spines in mutant mice (226 ± 11 nm), but increased at mismatched synapses in mutant mice (424 ± 12 nm) (ANOVA, $F_{(3,151)} = 43.1$, $p < 0.0001$; *post hoc* test, $p < 0.01$ for normal vs free, matched vs mismatched or free, and mismatched vs free; $p < 0.05$ for normal vs matched or mismatched) (Fig. 5B). Consequently, the ratio of active zone to PSD lengths at mismatched synapses (0.59 ± 0.01) was significantly smaller than that at normal (0.92 ± 0.01) and matched synapses (0.89 ± 0.01) (ANOVA, $F_{(2,124)} = 167$, $p < 0.0001$; *post hoc* test, $p < 0.01$) (Fig. 5C).

Figure 4. Immunogold electron microscopic analysis of GluR δ 2 proteins. **A–D**, Immunogold labeling for GluR δ 2 proteins at a normal synapse in a control mouse (**A**) and at a matched synapse (**B**), a mismatched synapse (**C**), and a free spine (**D**) in a mutant mouse. Arrowheads indicate the edge of PSD. Scale bar, 200 nm. **E–H**, Histograms for GluR δ 2-labeling densities per 1 μm of PSD length at normal (**E**), matched synapses (**F**), mismatched synapses (**G**), and free spines (**H**). The number of labeled synapses was 46 of 46 for normal synapses, 60 of 65 for matched synapses, 41 of 44 for mismatched synapses, and 1 of 18 for free spines. Of the 41 labeled mismatched synapses, immunogold labeling at the contacted and dissociated portions was detected at 41 or 4 synapses, respectively.

Effect of GluR δ 2 ablation on postsynaptic distributions of PSD-93 and AMPA receptor proteins

We further examined the effect of GluR δ 2 ablation after the distribution of PSD-93 and AMPA receptors at PF–PC synapses

Figure 5. Lengths of the active zone and PSD. *A, B*, Cumulative frequency plots of active zone length (*A*) and PSD length (*B*) at normal synapses in control mice (open circles) and at matched synapses (filled circles), mismatched synapses (triangles), and free spines (squares) in mutant mice. *C*, Scattergram plot between active zone and PSD lengths at normal (open circles), matched (filled circles), and mismatched synapses (triangles).

(Zhao et al., 1998; Roche et al., 1999; Fukaya and Watanabe, 2000) by postembedding immunogold. Because the labeling density for PSD-93 and AMPA receptors was much lower than that for GluR δ 2, immunogold was applied to single sections, in which both sides of the sections are immunoreacted, thus leading to an increase in the sensitivity and accuracy of detection.

In mutant mice, the mean PSD-93-labeling density decreased in the order of matched synapses > mismatched synapses > free spines (Fig. 6*A*). There were significant differences in the PSD-93 labeling among matched synapses, mismatched synapses, and free spines in mutant mice (ANOVA, $F_{(2,429)} = 9.1$, $p = 0.0001$; *post hoc* test, $p < 0.05$ for matched vs mismatched; $p < 0.01$ for matched vs free). Furthermore, at mismatched synapses, the PSD-93-labeling density was higher in the contacted portion of PSD than in the dissociated portion (Fig. 6*B*), showing a significant difference (*t* test; $p = 0.03$).

PCs express high levels of AMPA receptor subunits, GluR1, GluR2, and GluR3 (Keinänen et al., 1990), and all of them are localized at PF synapses (Zhao et al., 1998). To further increase the labeling density of AMPA receptors, we used a mixture of antibodies against the GluR1, GluR2, and GluR3 subunits. In mutant mice, the mean AMPA receptor-labeling density decreased in the order of matched synapses > mismatched synapses > free spines (Fig. 6*C*). There were significant differences in the AMPA receptor labeling among matched synapses, mismatched synapses, and free spines in mutant mice (ANOVA, $F_{(2,407)} = 9.6$, $p < 0.0001$; *post hoc* test, $p < 0.01$ for matched vs mismatched or free). There were no significant differences in the AMPA receptor-labeling density between the contacted and dissociated portions of PSD at mismatched synapses (*t* test; $p = 0.10$) (Fig. 6*D*).

Temporal patterns of structural alterations at PF–PC synapses

We then examined the appearance of mismatched synapses and free spines in *GluR δ 2^{flax/flax}* and *GluR δ 2^{flax/CrePR}* mice before and at 4, 8, 12, and 24 weeks after RU-486 administration. Before antiprogesterone treatment, there were very few, if any, free spines and mismatched synapses with PF terminals in both types of mice (Fig. 7*A, B*). After RU-486 administration, a significant number of mismatched synapses and free spines appeared at 4 weeks in *GluR δ 2^{flax/CrePR}* mice, and their fractions increased progressively toward 24 weeks (Fig. 7*E*). At 24 weeks, $42.0 \pm 2.7\%$ of PC spines ($n = 300$; three mice) were free from synaptic contacts with PFs and $28.3 \pm 3.4\%$ of spines had mismatched synapses, whereas matched synapses occupied only $29.7 \pm 5.9\%$ (Fig. 7*D*). On the other hand, free spines and mismatched synapses were very rare

in antiprogesterone-treated *GluR δ 2^{flax/flax}* mice even at 24 weeks after treatment (Fig. 7*C*).

Discussion

Neurobiological and behavioral traits are fairly complex and are most probably influenced by a large number of genes as well as environmental factors. There is ample evidence for large genetic differences between inbred mouse strains at the neurobiological and behavioral levels (Plomin, 1990; Wolfer et al., 1997). The inducible and neuron-specific gene targeting system developed in this study will be useful for dissecting the molecular basis of the structure and function of the adult brain under

the pure C57BL/6 genetic background. Here we show that inducible ablation of GluR δ 2 in the adult cerebellum resulted in mismatching between the active zone and PSD at PF–PC synapses. Furthermore, a significant number of PC spines became free from PF terminals. We thus identified GluR δ 2 as a key molecule that regulates presynaptic and postsynaptic matching and synaptic connection in the adult brain. These results provide evidence for the notion that there is a common molecular mechanism underlying synaptic plasticity and synapse formation in the cerebellum (Kashiwabuchi et al., 1995). A number of molecules, including GluRs, receptor tyrosine kinases, and cell adhesion molecules, have been implicated in synapse formation and morphological maturation of dendritic spines during development (Hering and Sheng, 2001; Goda and Davis, 2003; Scheiffele, 2003), but the molecular basis of synaptic connection and its modulation in the adult brain are poorly understood. There is ample evidence for the involvement of NMDA receptors in synapse refinement *in vivo* during development and in structural modification of dendritic spines in cultured neurons (Goodman and Shatz, 1993; Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999); however, no structural alterations were found at synapses by conditional ablation of NMDA receptors in the adult brain (Tsien et al., 1996; Rampon et al., 2000). In cultured hippocampal neurons, the AMPA receptor GluR2 subunit was important for morphogenesis of dendritic spines (Passafaro et al., 2003). On the other hand, no abnormalities in synaptic structures were detectable in mutant mice lacking the AMPA receptor GluR2 subunit (Sans et al., 2003; Petralia et al., 2004). Additional studies will be required to examine whether molecular determinants of synaptic structures during development are also important for modifications of synaptic structures in the adult brain. The present investigation provides direct evidence that GluR δ 2 does regulate presynaptic and postsynaptic matching and synaptic connections in the adult cerebellum.

Immunogold labeling of GluR δ 2 proteins under electron microscopy revealed that in mutant mice, the density of gold particles was reduced more at mismatched synapses than at matched synapses. Furthermore, the density of GluR δ 2-labeling particles at mismatched synapses in mutant mice was much lower than that at normal synapses of control mice. Thus, certain amounts of GluR δ 2 proteins in PSD are critical for proper matching of presynaptic and postsynaptic specializations. Consistently, GluR δ 2-labeling particles were scarcely detectable in free PC spines of mutant mice. In addition, the fractions of mismatched synapses and free spines in total PC spines progressively increased concomitantly with the gradual decrease of GluR δ 2 proteins after

Figure 6. Immunogold electron microscopic analysis of PSD-93 and AMPA receptor proteins. **A**, PSD-93-labeling densities per 1 μ m of PSD length at normal synapses in three control mice and at matched synapses, mismatched synapses, and free spines in three mutant mice. The number of labeled synapses was 208 of 320 for normal synapses, 173 of 357 for matched synapses, 18 of 53 for mismatched synapses, and 0 of 22 for free spines. The background density was 0.2 ± 0.1 ($n = 53$; total measured length, 58.7 μ m). **B**, PSD-93-labeling densities in the contacted and dissociated portions of PSD at mismatched synapses in three mutant mice. Of the 18 labeled mismatched synapses, immunogold labeling at the contacted and dissociated portions was detected at 16 or 2 synapses, respectively. **C**, AMPA receptor-labeling densities per 1 μ m of PSD length at normal synapses in three control mice and at matched synapses, mismatched synapses, and free spines in three mutant mice. The number of labeled synapses was 98 of 225 for normal synapses, 121 of 231 for matched synapses, 64 of 123 for mismatched synapses, and 9 of 56 for free spines. The background density was 0.2 ± 0.1 ($n = 37$; total measured length, 36.8 μ m). **D**, AMPA receptor-labeling densities in the contacted and dissociated portions of PSD at mismatched synapses in three mutant mice. Of the 64 labeled mismatched synapses, immunogold labeling at the contacted and dissociated portions was detected at 53 or 15 synapses, respectively. Error bars represent SEM. Dotted lines indicate background densities. * $p < 0.05$; ** $p < 0.01$.

antiprogesterone induction of CrePR-mediated *GluR δ 2* gene knock-out in *GluR δ 2^{lox/CrePR}* mice. Thus, structural changes at PF–PC synapses appear to proceed from normal contacts via mismatched contacts to free spines according to the reduction of synaptic contents of GluR δ 2 protein. As the density of GluR δ 2 immunolabeling decreased, the length of the active zone became shorter in the order of normal, matched, and mismatched synapses. Furthermore, the dissociated portion of PSD at mismatched synapses showed a very low density of GluR δ 2 immunogold particles such as free spines, whereas the density in the contacted portion at mismatched synapses was much higher and closer to that of matched synapses. The direct relationship between GluR δ 2 density and synaptic contact suggests that postsynaptic GluR δ 2 is essential for the maintenance of the presynaptic active zone and acts as the coordinator for presynaptic and postsynaptic differentiations. Thus, our results provide evidence for the postsynaptic control of the presynaptic active zone at mature synapses. Possibly, the postsynaptic GluR δ 2 complex makes a physical linkage between the active zone and PSD to ensure the presynaptic and postsynaptic matching. There could be three potential mechanisms for this (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). First, GluR δ 2 may regulate the presynaptic active zone by direct interaction with an active zone component through its N-terminal domain. Interestingly, studies using cultured hippocampal neurons showed that an extracellular domain of the AMPA receptor GluR2 subunit was important for induction of dendritic spines (Passafaro et al., 2003). In addition, analysis of mutant mice showed that the laminin α 4 chain that can bind to presynaptic Ca^{2+} channels was required for the apposition of active zones and junctional folds at the neuromuscular junction (Patton et al., 2001). Second, GluR δ 2 may regulate the presynaptic active zone through the interaction between a postsynaptic GluR δ 2-interacting molecule and an active zone component. Interestingly, NMDA-type GluRs interact with Eph receptor tyrosine kinases capable of *trans*-synaptic signaling (Dalva et al., 2000). In *Caenorhabditis elegans*, ionotropic glutamate receptor GLR-1 is colocalized at the cell surface with SOL-1 (suppressor of *lurcher-1*) containing four extracellular β -barrel-forming domains known as CUB (complement subcomponents C1r/C1s, Vegf, Bmp1) domains (Zheng et al., 2004). Third, GluR δ 2 may regulate the presynaptic active zone more indirectly through a postsynaptic scaffold protein that binds to a molecule interacting with an active zone component. The C terminal of GluR δ 2 associates with PSD-93 and PSD-95, which bind to the postsynaptic membrane protein neuroligin that is capable of interacting *trans*-synaptically with presynaptic β -neurexin (Roche et al., 1999; Kim and Sheng, 2004; Uemura et al., 2004); however, there were no detectable alterations in structures at PF–PC synapses in PSD-93 mutant mice (McGee et al., 2001). Multiple scaffold proteins might be functionally redundant. Thus, elucidation of the GluR δ 2 mechanism will provide a key for understanding the regulation of synaptic connections in the adult brain and the precise topological matching of presynaptic and postsynaptic specializations that are essential for efficient synaptic transmission.

The length of PSD was significantly shorter at matched synapses in mutant mice than at normal synapses in control mice according to the significant decrease in the density of GluR δ 2 immunogold particles at matched synapses in mutant mice. A further decrease in GluR δ 2 proteins, however, resulted in enlargement of PSD at mismatched synapses compared with matched synapses in mutant mice. Namely, a strong decrease in GluR δ 2 proteins at the postsynaptic site of PF–PC synapses

Figure 7. Temporal pattern of appearance of mismatched synapses and free spines in the cerebellum of *GluRδ2*^{flox/CrePR} mice after CrePR induction by RU-486 treatment. RU-486-treated *GluRδ2*^{flox/flox} mice served as controls. **A–D**, Electron micrographs of PF–PC synapses in the cerebella of *GluRδ2*^{flox/flox} (**A, C**) and *GluRδ2*^{flox/CrePR} (**B, D**) mice before (**A, B**) and 24 weeks after (**C, D**) RU-486 treatment. Arrows indicate the mismatch between active zone and PSD at PF–PC synapses. An arrowhead indicates a rarely encountered abnormal spine, which possesses a PSD but contacts the nonterminal portion of PFs. f, Free spines; m, mismatched synapses; n, normal or matched synapses. Scale bar, 500 nm. **E**, Percentages of mismatched synapses (triangles) and free spines (circles) in *GluRδ2*^{flox/flox} and *GluRδ2*^{flox/CrePR} mice before and 4, 8, 12, and 24 weeks after RU-486 treatment. Data from *GluRδ2*^{flox/flox} and *GluRδ2*^{flox/CrePR} mice are indicated by open and filled marks, respectively. Error bars represent SEM.

shrunk the active zone but increased the PSD. Thus, the opposite effect of GluRδ2 ablation on the sizes of the active zone and PSD caused mismatching between the presynaptic and postsynaptic differentiations. The C terminal of GluRδ2 associates with the PDZ (PSD-95/Discs large/zona occludens-1) domain-containing proteins, including PSD-93, protein tyrosine phosphatase PTP-MEG, and Delphilin (Roche et al., 1999; Hironaka et al., 2000; Miyagi et al., 2002). Recently, we found that GluRδ2 bound to the Shank family of scaffold proteins through an internal motif in the C-terminal region, and GluRδ2–Shank complexes associated with the metabotropic GluR1α, the AMPA-type GluR, and the inositol 1,4,5-trisphosphate receptor, which are essential for cerebellar long-term depression (Uemura et al., 2004). Thus, it is reasonable to assume that GluRδ2 regulates the organization of PSD through the interaction with multiple postsynaptic scaffold proteins. In fact, PSD-93 protein density decreased in parallel with the decrease of GluRδ2 proteins at PSD. Similar to GluRδ2

proteins, the PSD-93-labeling density was higher in the contacted portion of PSD than in the dissociated portion at mismatched synapses. On the other hand, there were no significant differences in the AMPA receptor-labeling density between the contacted and dissociated portions of PSD at mismatched synapses. In addition, AMPA receptor labeling was found in free spines without GluRδ2. The GluRδ2-linked distribution of PSD-93 and the relatively independent distribution of AMPA receptors at PSD suggest that GluRδ2 regulates the PSD organization at PF–PC synapses. Because ablation of PSD-93 alone appeared not to affect the synaptic structures (McGee et al., 2001), orchestration of multiple scaffold proteins by GluRδ2 may be essential. The decrease in GluRδ2 proteins at mismatched synapses thus caused the loosening of PSD organization, resulting in the enlargement of PSD. Loosening of PSD organization by GluRδ2 ablation may trigger the subsequent postsynaptic atrophy leading to free spines.

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