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NMDA Receptor GluR ϵ /NR2 Subunits Are Essential for Postsynaptic Localization and Protein Stability of GluR ζ 1/NR1 Subunit

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In NMDA receptors, GluR ϵ /NR2 subunits strictly require the GluR ζ 1/NR1 subunit to exit from endoplasmic reticulum (ER) to the cell surface *in vitro* and to the postsynapse *in vivo*, whereas C terminus-dependent self-surface delivery has been demonstrated for the GluR ζ 1 subunit *in vitro*. To test whether this leads to C terminus-dependent self-postsynaptic expression in neurons *in vivo*, we investigated the GluR ζ 1 subunit in cerebellar granule cells lacking two major GluR ϵ subunits, GluR ϵ 1/NR2A and GluR ϵ 3/NR2C. In the mutant cerebellum, synaptic labeling for the GluR ζ 1 subunit containing the C2 (GluR ζ 1-C2) or C2' (GluR ζ 1-C2') cassette was reduced at mossy fiber–granule cell synapses to the extrasynaptic level. The loss was not accompanied by decreased transcription and translation levels, increased extrasynaptic labeling, or ER accumulation. Quantitative immunoblot revealed substantial reductions in the mutant cerebellum of GluR ζ 1-C2 and GluR ζ 1-C2'. The most severe deficit was observed in the postsynaptic density (PSD) fraction: mutant levels relative to the wild-type level were $12.3 \pm 3.3\%$ for GluR ζ 1-C2 and $17.0 \pm 4.6\%$ for GluR ζ 1-C2'. The GluR ζ 1 subunit carrying the C1 cassette (GluR ζ 1-C1) was, although low in cerebellar content, also reduced to $12.7 \pm 3.5\%$ in the mutant PSD fraction. Considering a trace amount of other GluR ϵ subunits in the mutant cerebellum, the severe reductions thus represent that the GluR ζ 1 subunit, by itself, is virtually unable to accumulate at postsynaptic sites, regardless of C-terminal forms. By protein turnover analysis, the degradation of the GluR ζ 1 subunit was accelerated in the mutant cerebellum, being particularly rapid for that carrying the C2 cassette. Therefore, accompanying expression of GluR ϵ subunits is essential for postsynaptic localization and protein stability of the GluR ζ 1 subunit.

Key words: NMDA receptor; mossy fiber–granule cell synapse; immunohistochemistry; immunoblot; protein turnover; cerebellum; knock-out mouse

Introduction

The NMDA-selective glutamate receptor is involved in activity-dependent changes of synaptic efficacy, which underlie synapse development, synaptic plasticity, and learning and memory (Mayer and Westbrook, 1987; Bliss and Collingridge, 1993). NMDA receptors are composed of GluR ζ 1/NR1 and GluR ϵ /NR2 subunits (Seeburg, 1993; Nakanishi and Masu, 1994; Mori and Mishina, 1995). The GluR ζ 1 subunit is encoded by a single gene

but exists as several splice variants. The GluR ϵ subunit is composed of four members (GluR ϵ 1– ϵ 4 or NR2A–2D) and determines functional and spatiotemporal diversities of NMDA receptors (Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992; Watanabe et al., 1992).

Heteromeric configuration of NMDA receptors has been evidenced from *in vitro* experiments. First, active NMDA receptors are obtained only when the GluR ϵ subunit is expressed together with the GluR ζ 1 subunit (Meguro et al., 1992; Monyer et al., 1992). Second, binding sites for agonist glutamate and coagonist glycine are shared between GluR ϵ and GluR ζ 1 subunits (Hirai et al., 1996; Laube et al., 1997). Third, GluR ζ 1 and GluR ϵ subunits interact differentially with various cytoskeletal and synaptic molecules thought to regulate receptor transport, localization, and dynamics (Kornau et al., 1995; Niethammer et al., 1996; Wyszynski et al., 1997; Ehlers et al., 1998; Setou et al., 2000). Fourth, GluR ϵ subunits require cotransfection of the GluR ζ 1 subunit for their exit from the endoplasmic reticulum (ER) to the cell surface (McIlhinney et al., 1996, 1998). Indeed, when the *GluR ζ 1* gene is deleted in hippocampal pyramidal cells *in vivo*, GluR ϵ subunits

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are retained in the somatic ER and disappear from the postsynaptic site (Fukaya et al., 2003).

Four different C termini of the GluR ζ 1 subunit are generated by optional usage of the C1 exon cassette and by alternative usage of the C2/C2' cassettes (Sugihara et al., 1992; Yamazaki et al., 1992; Hollmann et al., 1993). These variants differ in potentiation by PKC (Tingley et al., 1993), regional expression in the brain (Laurie et al., 1995), interaction with cytoskeletal filaments (Ehlers et al., 1998; Lin et al., 1998; Matsuda and Hirai, 1999), and cell surface expression (Ehlers et al., 1995). Of these variants, higher self-surface expression is shown for the GluR ζ 1 subunit with the C2' tail than that with the C2 tail, and also for the GluR ζ 1 subunit lacking the C1 cassette than that carrying it (Okabe et al., 1999). Furthermore, alternative C2/C2' usage is activity dependent and controls ER export and synaptic delivery of NMDA receptors (Mu et al., 2003).

This *in vitro* evidence prompted us to test whether this leads to C terminus-dependent self-postsynaptic expression of the GluR ζ 1 subunit in neurons *in vivo*, by producing cerebellar granule cells lacking two major GluR ϵ subunits, GluR ϵ 1/NR2A and GluR ϵ 3/NR2C. Here, we show that, when accompanying GluR ϵ subunits are lacking, postsynaptic localization is impaired for the GluR ζ 1 subunit with any C-terminal cassettes. Moreover, the ablation of GluR ϵ subunits causes rapid degradation of the GluR ζ 1 subunit, particularly that carrying the C2 cassette. Therefore, accompanying GluR ϵ subunits are essential for postsynaptic expression and protein stability of the GluR ζ 1 subunit in neurons *in vivo*.

Materials and Methods

Production of the GluR ϵ 3 and GluR ϵ 1/ ϵ 3 knock-out mice. A genomic DNA clone, λ GRE3T1–12 (Nagasawa et al., 1996), carrying the GluR ϵ 3 gene was used to construct a targeting vector. A 0.7 kb *HindIII*–*NotI* fragment containing the transmembrane segment M4 was replaced with a 1.3 kb *EcoRI*–*BamHI* fragment from pGK2Neo (Yagi et al., 1993) containing the pgk-neo cassette. A 4.0 kb *Sall*–*NotI* fragment from pPauDT3 (Yanagawa et al., 1999) was used for negative selection. TT2 embryonic stem (ES) cells were transfected by the linearized vector, and the targeted clones were identified by G418 selection, PCR, and Southern blot hybridization using the three probes shown in Figure 1A. Generation of germ-line chimeras and production of homozygous GluR ϵ 3 knock-out (GluR ϵ 3-KO) mice were performed in the same manner as described (Sakimura et al., 1995). To produce mutant mice defective in both GluR ϵ 1 and GluR ϵ 3 subunits, GluR ϵ 3-KO mice were mated with GluR ϵ 1-KO mice, as reported previously (Sakimura et al., 1995).

Sections. Under deep pentobarbital anesthesia (100 mg/kg body weight), wild-type and mutant mice at 1 month of age were perfused transcardially with the following fixatives: 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB), pH 7.4, for light microscopic immunohistochemistry and histology (paraffin sections, 5 μ m in thickness; microslizer sections, 50 μ m); 4% paraformaldehyde/0.1% glutaraldehyde in 0.1 M PB, pH 7.4, for post-embedding immunogold (Lowicryl sections, 100 nm); or 2% paraformaldehyde/2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for electron microscopy (ultrathin Epon sections, 70 nm) and granule cell count (semithin Epon sections, 1 μ m). Before Epon embedding, cerebellar slices were further postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.4, for 2 hr, stained in block with 2% uranyl acetate for 1 hr, and dehydrated in graded alcohols. Brains for *in situ* hybridization were freshly obtained for cryostat sections (20 μ m).

In situ hybridization. *In situ* hybridization analysis was performed using subunit-specific antisense oligonucleotides for the GluR ϵ 1 subunit (oligo ϵ 1E), GluR ϵ 3 subunit (oligo ϵ 3M4C), and GluR ζ 1 subunit (oligo ζ 1A). Oligo ϵ 1E and oligo ϵ 3M4C are complementary to the nucleotide residues 1862–1906 of the GluR ϵ 1 subunit cDNA (Meguro et al., 1992) and 2525–2570 of the GluR ϵ 3 subunit cDNA (Kutsuwada et al., 1992), respectively. Oligo ζ 1A was reported previously (Watanabe et al.,

1993). Probe labeling and procedures for *in situ* hybridization were done as reported previously (Watanabe et al., 1993). After washing, sections were exposed to BioMax film (Eastman Kodak, Rochester, NY) for 3 weeks. The relative gray density was obtained by scanning x-ray film autoradiograms and measuring with NIH Image software (version 1.61).

Antibody. We raised polyclonal antibodies against the C1 and C2' cassettes of the GluR ζ 1 subunit in the rabbit and guinea pig, respectively, using synthetic peptides (CDPKKKATFRAITSTLASSFKRRSSKDT for the C1 and CQYHPTDITGPNLSDPSVSTVV for the C2') conjugated to keyhole limpet hemocyanin. Procedures for immunization and antibody purification were reported previously (Watanabe et al., 1998). We also used rabbit anti-GluR ζ 1–C2 (Yamada et al., 2001), rat monoclonal anti-GluR ζ 1–N (GluR ζ 1-pan) (Yamada et al., 2001), rabbit anti-GluR ϵ 1C (Watanabe et al., 1998), rabbit anti-GluR ϵ 2C (Watanabe et al., 1998), rabbit anti-GluR ϵ 3C (Yamada et al., 2001), rabbit and guinea pig anti-postsynaptic density (PSD)-95 (Fukaya and Watanabe, 2000), guinea pig anti-vesicular glutamate transporter VGluT1 (Miyazaki et al., 2003), rabbit anti-vesicular GABA transporter VGAT (Miyazaki et al., 2003), rabbit anti-synaptophysin (Fukaya and Watanabe, 2000), and goat anti-calreticulin (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. All antibodies were used at the concentration of 0.5–1 μ g/ml, unless noted otherwise.

To check the specificity of GluR ζ 1 antibodies, the GluR ζ 1 subunit tailed with the C1–C2 cassettes (NR1–1) or with the C2' cassette only (NR1–4) was expressed under the control of the human elongation factor 1 α promoter. HEK293 cells were transfected using Lipofectamine reagent (Invitrogen, Carlsbad, CA) and lysed 2 d later by the addition of harvest buffer (1 mM EGTA, pH 8.1, 1 mM EDTA, pH 7.9, 1% TritonX-100, and 1 mM PMSF). For control immunohistochemistry, GluR ζ 1 antibodies were preabsorbed with C2 or C2' antigen peptides (50 μ g/ml each) by overnight incubation at 4°C.

Immunohistochemistry. Before conventional immunohistochemical incubation, paraffin sections were subjected to pepsin pretreatment, which is essential for immunohistochemical detection of NMDA receptor subunits and the PSD-95/synapse-associated protein (SAP)-90 protein family (Watanabe et al., 1998; Fukaya and Watanabe, 2000; Yamada et al., 2001; Oshima et al., 2002). After treatment with 1 mg/ml pepsin (Dako, Carpinteria, CA) in 0.2N HCl at 37°C for 10 min, sections were immunoreacted successively with rabbit or guinea pig primary antibodies overnight, biotinylated secondary antibodies for 1 hr, and streptavidin–peroxidase for 30 min, using a Histofine SAB-PO(R) kit (Nichirei, Tokyo, Japan). Immunoreaction was visualized with DAB, and photographs were taken using a light microscope (AX-70; Olympus, Tokyo, Japan).

For confocal laser-scanning microscopy (Fluoview; Olympus), pepsin-treated paraffin sections were incubated first with GluR ζ 1–C2 or GluR ζ 1–C2' antibody overnight, biotinylated goat anti-rabbit or anti-guinea pig IgG for 1 hr, and streptavidin–peroxidase for 30 min, followed by visualization using the Tyramide Signal Amplification kit [TSA Fluorescein System (green); NEN Life Science, Boston, MA]. Then, sections were incubated with guinea pig or rabbit PSD-95 antibody overnight, followed by a 2 hr incubation with Cy3-labeled donkey anti-guinea pig or anti-rabbit IgG (1:200; Jackson ImmunoResearch, West Grove, PA) or with 10 μ M propidium iodide for 10 min (Molecular Probes, Eugene, OR). Double immunofluorescence for VGluT1 and VGAT was also conducted using microslizer sections, followed by a 2 hr incubation with a mixture of Cy3- or FITC-labeled, species-specific secondary antibodies (Jackson ImmunoResearch).

Procedures for post-embedding immunogold were the same as reported previously (Fukaya and Watanabe, 2000). Ultrathin sections from three wild-type and three mutant mice were immunoreacted overnight with GluR ζ 1–C2 or GluR ζ 1–C2' antibody (10 μ g/ml) and then with colloidal gold (10 nm) conjugated with anti-rabbit or anti-guinea pig IgG (British Biocell International, Cardiff, UK) for 2 hr. Grids were stained with 2% uranyl acetate for 10 min. The number of gold particles was counted on the synaptic and extrasynaptic membranes, respectively, of asymmetrical synapses between mossy fiber terminals and granule cell dendrites.

Morphological analysis. Parasagittal microslizer sections were stained with hematoxylin and used for cerebellar histology. Using the sections,

areal measurement of the granular layer was performed by taking cerebellar images with an AX-70 light microscope equipped with a CCD camera (Sensys 1401; Nippon Roper, Tokyo, Japan) and by calculating using MetaMorph software (Nippon Roper). Semithin Epon sections stained with Mayer's hematoxylin were used for granule cell counting, as follows. The numerical density (N_V) of granule cells was obtained by the point-counting method of Weibel (1979) and using the equation: $N_V = 1/\beta \times N_A^{1.5}/V_V^{0.5}$, as reported previously (Kakizawa et al., 2000). N_A and V_V are the visible profile count of granule cell nuclei or their volume density in the granular layer, respectively, whereas β is a dimensionless shape coefficient defined here as 1.38 by assuming that granule cell nuclei are spherical.

For analysis of cerebellar synapses, electron micrographs were taken from the middle-third depth of the granular layer with an H7100 electron microscope (Hitachi, Tokyo, Japan). The mean length of PSD at mossy fiber–granule cell synapses was measured by IPLab software (Nippon Roper).

Western blot analysis. For quantitative Western blot analysis, five cerebella of each genotype were pooled for one experiment. The cerebella were homogenized in 10 volumes of sucrose buffer (0.32 M sucrose, 5 mM EDTA, 1 mM PMSF, 1 μ M pepstatin A, and 2 μ M leupeptin) and centrifuged at $700 \times g$ for 10 min to obtain the postnuclear fractions (homogenates). The synaptosomal and PSD fractions were prepared as described previously (Carlin et al., 1980). To obtain the PSD fraction, the synaptosomal fraction was further treated with 0.5% Triton X-100 for 15 min on ice and then centrifuged at $81,600 \times g$ for 1 hr. The resultant pellets were resuspended in 40 mM Tris-HCl, pH 8.0. To obtain the light membrane fraction (P3 fraction) in a separate procedure, the S2 fraction prepared from a cerebellum was centrifuged at $165,000 \times g$ for 2 hr, and the resultant pellets were resuspended with the sucrose buffer.

The determination of protein concentrations was made by the method of Lowry et al. (1951). Protein samples were fractionated by 7% SDS-PAGE and electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The blot was immunoreacted with primary antibodies and visualized using the ECL chemiluminescence detection system (Amersham, Bucks, UK). Signal intensities of immunoreaction were measured digitally. To normalize signal intensities on each blot, equal amounts of control protein samples were loaded simultaneously and defined as standard signals. Representative data from three to five experiments are shown in the figures.

Pulse labeling of cultured cerebellar cells. Cultures of cerebellar granular cells were prepared as described by Fujikawa et al. (2000), with minor modifications. Briefly, cerebella obtained from the wild-type or GluR ϵ 1-KO mice at 6–7 d of age were treated with 0.2% trypsin for 15 min at 37°C. Cells were centrifuged and suspended in MEM-Hanks (Invitrogen) supplemented with 10 mM HEPES-NaOH, pH 7.2, 1 μ M arabinosylcytosine, and 10% FCS and were plated on polyethyleneimine-coated culture plates at a density of 2.4×10^5 cells/cm². One day after plating, 20 mM KCl was added to the medium. All experiments were performed on days 7–8 *in vitro*. Northern blot analysis of GluR ϵ 1 mRNA was performed by hybridization of total RNA using a ³²P-labeled GluR ϵ 1 cDNA probe (nucleotide residues 203–758). An immunoblot was performed as described above. Pulse labeling and immunoprecipitation were performed as described by Huh and Wenthold (1999), with minor modifications. Cells were incubated for 30 min at 37°C in methionine- and cysteine-free medium supplemented with 10% FCS and then pulse labeled with 125 μ Ci/ml [³⁵S]methionine and [³⁵S]cysteine in depletion medium for 30 min. After 30 min, cells were washed immediately with cold PBS, harvested, and solubilized with buffer containing 2% SDS by incubating for 3 min at 90°C. Ten micrograms of GluR ϵ 1-N antibody were used for immunoprecipitation. Immunoprecipitated proteins were eluted and subjected to SDS-PAGE and immunoblot. After an immunoblot, radioactivity of the pulse-labeled GluR ϵ 1 subunit was analyzed by FUJIX Bioimage Analyzer (BAS2000; Fuji Film, Tokyo, Japan).

Protein synthesis inhibition. Anisomycin (Sigma, St. Louis, MO) was dissolved in 1N HCl and diluted in 0.9% saline. The pH was adjusted to 4.5 with 1N NaOH. Mice were given subcutaneous injections of 150 mg of anisomycin/kg of body weight or an equivalent volume of vehicle. At this dose, anisomycin has been shown to inhibit protein synthesis in the

brain by >90% during the first 2 hr and by >60% during the next 2 hr (Lattal and Abel, 2001). Four hours after injection, homogenate samples were prepared from the cerebellum, and an immunoblot was performed as described above.

Results

Production of GluR ϵ 1/ ϵ 3-KO mice

The *GluR ϵ 3* gene locus in murine TT2 ES cells was disrupted by homologous recombination using a targeting vector containing an 8.5 kb fragment of GluR ϵ 3 subunit genomic DNA, in which the exon encoding the transmembrane segment M4 was replaced with the neomycin phosphotransferase gene (Fig. 1A). From ES cells electroporated with the vector DNA, we selected 1210 clones in medium containing G418, from which two clones with correct targeting were identified by PCR and Southern blot hybridization analyses. Germ-line chimeric mice were generated by injecting the clone 8-88-3. Heterozygous progenies were intercrossed, and their offspring were genotyped by Southern blot analysis with three probes (Fig. 1B). Homozygous GluR ϵ 3 mutant (GluR ϵ 3-KO) mice were then crossed with the GluR ϵ 1-KO mouse (Sakimura et al., 1995) to obtain the double knock-out (GluR ϵ 1/ ϵ 3-KO) mouse. The GluR ϵ 3-KO and GluR ϵ 1/ ϵ 3-KO mice were viable, grew normally, and were fertile. However, the GluR ϵ 1/ ϵ 3-KO mouse showed mild impairment in motor coordination when tested on the rotating rod at high speeds (data not shown), as reported previously (Ebrilidze et al., 1996; Kadotani et al., 1996).

In situ hybridization

By *in situ* hybridization, the absence of GluR ϵ 1 and/or GluR ϵ 3 mRNAs in the respective mutant brains was confirmed with ³³P-labeled probes against the deleted sequences (Fig. 1C, top and middle). No genotypic differences were detected in overall distribution of GluR ϵ 1 mRNA in the brain (Fig. 1C, bottom). The relative OD in the cerebellar granular layer was quantified using a single x-ray film, to which all hybridized sections had been exposed. At the transcription level, no compensatory upregulation was seen for the GluR ϵ 1 subunit in the absence of the *GluR ϵ 3* gene, and vice versa (Fig. 1D, left and middle) ($n = 3$; Student's *t* test; $p > 0.05$ for each). Moreover, GluR ϵ 1 mRNA levels in the granular layer showed no significant differences among the four types of mice (Fig. 1D, right) ($p > 0.05$ for each). Thus, the gene knock-out of the GluR ϵ 1 subunit, GluR ϵ 3 subunit, or both does not affect the transcription levels of the remaining NMDA receptor subunits.

Specificity of GluR ϵ 1-C2, -C2', and -C1 antibodies

To examine the effect on C-terminal variants of the GluR ϵ 1 subunit, the specificity of C2, C2', and C1 cassette antibodies was assessed by immunoblot and immunohistochemical analyses (Fig. 2). In the cerebellum, all three antibodies recognized protein bands at 120 kDa. The band detected by the C2 or C2' antibody was broad and apparently consisted of multiple sub-bands in the cerebellar homogenate (Fig. 2A, Cb). In contrast, the band recognized by the C1 antibody was detected in the cerebellar PSD fraction (Fig. 2A, Cb) but not in the homogenate (data not shown), suggesting low contents of the C1 cassette in the cerebellum (see also Fig. 6A). The C2, C2', and C1 antibodies selectively recognized protein bands in HEK293 cells transfected with plasmid encoding NR1–1 (GluR ϵ 1 subunit tailed with C1–C2), NR1–4 (that tailed with C2'), or NR1–1, respectively (Fig. 2A). Preincubation of these antibodies with their immunizing peptides completely abolished these protein bands, whereas the bands were similarly detected by preincubation with the other (nonimmunizing) peptides (data not shown). These results indi-

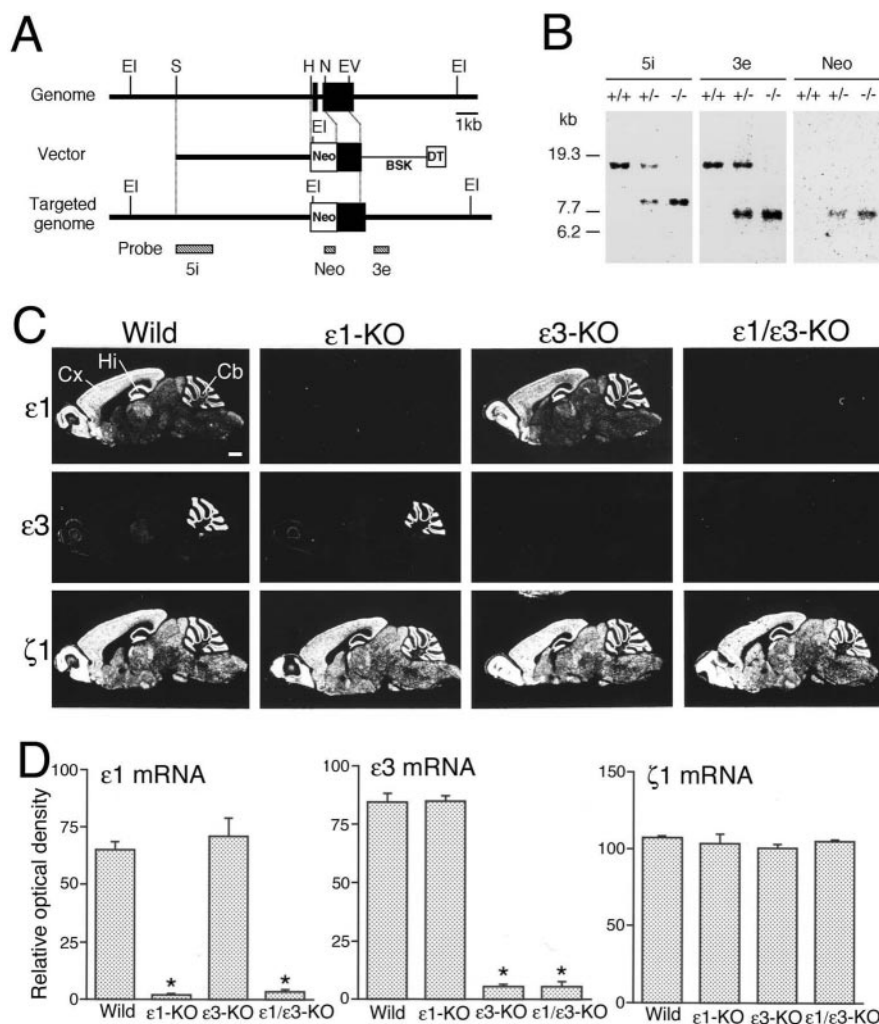


Figure 1. Production of mutant mice defective in NMDA receptor *GluRε1* and/or *GluRε3* subunit genes. *A*, Schematic representation of genomic DNA, targeting vector, and disrupted gene of the *GluRε3* subunit. *B*, Southern blot analysis of *EcoRI*-digested genomic DNA using three different probes indicated in *A*. *C*, *In situ* hybridization analysis for *GluRε1*, *GluRε3*, and *GluRζ1* mRNAs in the wild-type, *GluRε1*-KO, *GluRε3*-KO, and *GluRε1/ε3*-KO brains. *D*, Semiquantitative evaluation of transcription levels for *GluRε1* (left), *GluRε3* (center), and *GluRζ1* (right) mRNAs in the granular layer of the cerebellum (mean $\pm$ SD). The asterisks indicate statistically significant differences between wild-type and mutant mice (Student's *t* test; $p < 0.001$). BSK, Plasmid pBluescript; Cb, cerebellum; Cx, cerebral cortex; DT, diphtheria toxin gene; EI, *EcoRI*; EV, *EcoRV*; H, *HindIII*; Hi, hippocampus; N, *NotI*; S, *Sall*. Scale bar, (in *C*) 1 mm.

cate that the three antibodies are specific to the C2, C2', and C1 cassettes, respectively, and have no cross-reactivity to the others.

By immunohistochemistry, C2 and C2' antibodies yielded characteristic labelings in the adult mouse brain that were similar but not identical to each other (Fig. 2*B,E*). The immunolabelings were abolished with the use of antibodies preincubated with immunizing peptides (Fig. 2*C,G*), but not with nonimmunizing ones (Fig. 2*D,F*). Thus, the C2 and C2' antibodies are specific in use for immunohistochemistry as well as for immunoblot. However, the C1 antibody failed to produce reliable immunohistochemical labeling and was used for immunoblot analysis only.

Hereafter, the *GluRζ1* subunit detected by the C2, C2', and C1 antibodies is referred to as *GluRζ1*-C2, *GluRζ1*-C2', and *GluRζ1*-C1, respectively.

Immunohistochemistry

Alterations at the protein level were examined by immunohistochemistry at the light microscopic (Figs. 3, 4) and electron microscopic (Fig. 5) levels.

Light microscopic immunohistochemistry for *GluRζ1*-C2 and *GluRζ1*-C2'

To minimize experimental fluctuation in light microscopic immunohistochemistry, pairs of wild-type and mutant brains were embedded in single paraffin blocks, and hence all immunohistochemical procedures were applied to the section pairs under the same conditions.

In the wild-type brain, the highest immunostaining for *GluRζ1*-C2 was detected in the hippocampal CA1 region (Fig. 3*A–C*, top brains). High levels were also observed in the cerebral cortex, olfactory bulb, caudate-putamen, hippocampal CA3 region, dentate gyrus, and cerebellar granular layer, whereas the level in the thalamus was low to moderate. In contrast, *GluRζ1*-C2' was highest in each sub-region of the hippocampus and also high in the cerebral cortex, thalamus, and cerebellar granular layer, whereas it was low in the caudate-putamen (Fig. 3*D*, top brain). These patterns of immunostaining are consistent with the reported distributions of C2 and C2' cassette mRNAs, respectively (Laurie et al., 1995). When compared with the wild-type brain, *GluRζ1*-C2 in the cerebellar granular layer was reduced mildly in the *GluRε1*-KO mouse (Fig. 3*A*, bottom brain), reduced severely in the *GluRε3*-KO mouse (Fig. 3*B*, bottom brain), and almost negative in the *GluRε1/ε3*-KO mouse (Fig. 3*C*, bottom brain). This was also true for *GluRζ1*-C2' (Fig. 3*D*, bottom brain).

By nuclear counterstaining with propidium iodide (Fig. 3*E,I*, red) or hematoxylin (Fig. 4*A,E*, blue), *GluRζ1*-C2 and *GluRζ1*-C2' were shown in the wild-type mouse to be located in oval or polygonal masses between granule cells (Figs. 3*E,I*, green, 4*A,E*, brown). These immunopositive structures were well overlapped with postsynaptic density protein PSD-95 (Fig. 3*G,K*, red), indicating that *GluRζ1*-C2 and *GluRζ1*-C2' are accumulated extensively in synaptic glomeruli. In mutants, immunoreactivity in synaptic glomeruli was progressively reduced in the order of wild-type > *GluRε1*-KO > *GluRε3*-KO > *GluRε1/ε3*-KO mice (Fig. 4*A–D*, *GluRζ1*-C2; *E–H*, *GluRζ1*-C2'). In the *GluRε1/ε3*-KO mouse, both variants were almost undetectable in synaptic glomeruli (Fig. 3*F,H,J,L*). Thus, parallel with *GluRε* subunit ablation, immunohistochemical visibility of *GluRζ1*-C2 and *GluRζ1*-C2' was lost from cerebellar synaptic glomeruli, where granule cell dendrites form excitatory and inhibitory synapses with mossy fibers or Golgi cell axons, respectively.

Postembedding immunogold for *GluRζ1*-C2 and *GluRζ1*-C2'

The subcellular localization was examined by postembedding immunogold (Fig. 5). In the wild-type mouse, gold particles representing *GluRζ1*-C2 or *GluRζ1*-C2' were observed in the postsynaptic membrane at asymmetrical synapses in contact with huge mossy fiber terminals (Fig. 5*A,B*), whereas they were hardly

detected at this type of synapse in the GluR ϵ 1/ ϵ 3-KO mouse (Fig. 5C,D). We evaluated the difference by comparing the mean number of immunogold particles per micrometer of the synaptic (junctional) and extrasynaptic (nonjunctional) membranes. The mean number of gold particles for GluR ζ 1-C2 on the synaptic membrane was 5.55 ± 1.22 in the wild-type mouse (mean $\pm$ SEM; $n = 3$; 101 synapses examined), whereas it was significantly lowered to 0.35 ± 0.08 in the GluR ϵ 1/ ϵ 3-KO mouse ($n = 3$; 60 synapses; Student's t test; $p < 0.05$), the latter density being similar to the extrasynaptic density in both the wild-type and GluR ϵ 1/ ϵ 3-KO mice (Fig. 5E). As for GluR ζ 1-C2', the mean number on the synaptic membrane was 3.91 ± 0.66 in the wild-type mouse ($n = 3$; 88 synapses) and 0.60 ± 0.20 in the GluR ϵ 1/ ϵ 3-KO mouse ($n = 3$; 78 synapses), showing significant reduction ($p < 0.05$) (Fig. 5F). Thus, synaptic localization of the GluR ζ 1 subunit was significantly reduced to the extrasynaptic level in the GluR ϵ 1/ ϵ 3-KO mouse, regardless of the C2/C2' form. Despite the severe synaptic loss, no significant increase of the extrasynaptic density was evident in the GluR ϵ 1/ ϵ 3-KO mouse ($p > 0.05$ for each variant).

In our previous study using mutant mice lacking the GluR ζ 1 subunit, GluR ϵ 1 and GluR ϵ 2/NR2B subunits abnormally accumulate in perikarya of hippocampal pyramidal cells as electron-dense granules in the ER lumen (Fukaya et al., 2003). In the present study, we also searched for such somatic accumulation and intracisternal granule formation. However, we could not find any somatic accumulation of the GluR ζ 1 subunit in GluR ϵ 1/ ϵ 3-deficient granule cells by light microscopy (Fig. 3F,J) and postembedding immunogold (data not shown).

GluRe subunits and PSD-95

Changes of the GluR ϵ 1 subunit (Fig. 4I–L), GluR ϵ 3 subunit (Fig. 4M–P), and PSD-95 (Fig. 4Q–T) were examined in the granular layer. Compared with the wild-type mouse (Fig. 4I,M), glomerular staining was reduced for the GluR ϵ 1 subunit in the GluR ϵ 3-KO mouse (Fig. 4K) and for the GluR ϵ 3 subunit in the GluR ϵ 1-KO mouse (Fig. 4N), despite their normal transcription levels (Fig. 1C,D). In contrast, no differences were seen in the distribution and levels of PSD-95 among the four mouse types (Fig. 4Q–T). These results suggest that genetic ablation of given GluRe subunits has affected synaptic expression of the remaining GluRe subunits as well.

Immunoblot

Then, we performed immunoblot analysis to biochemically characterize the effect of GluRe subunit ablation on cerebellar content and intracellular distribution of the GluR ζ 1 subunit (Fig. 6, Table 1). All immunoblotting data were quantified by three independent experiments ($n = 3$), except for GluR ζ 1-C2' in the homogenate, synaptosomal fraction, and PSD fraction of the wild-type

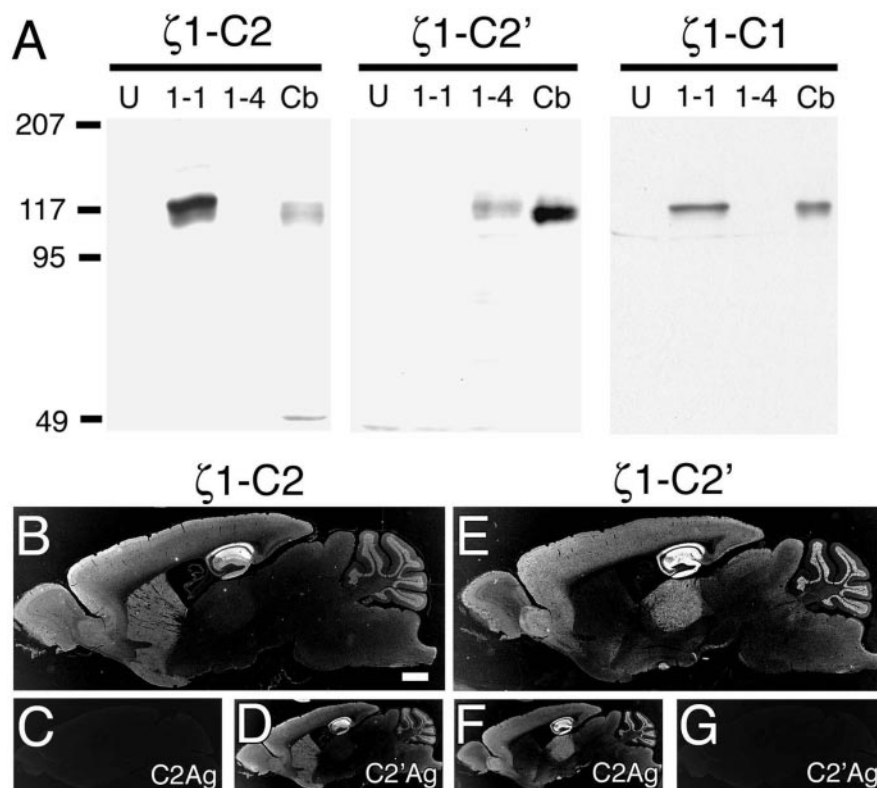


Figure 2. Specificity of GluR ζ 1-C2, GluR ζ 1-C2', and GluR ζ 1-C1 antibodies. *A*, Immunoblot using untransfected HEK293 cells (U), cells transfected with GluR ζ 1 containing the C1 and C2 cassettes (NR1-1), cells transfected with GluR ζ 1 lacking the C1 but containing the C2' cassette (NR1-4), and cerebellar proteins (Cb). Cerebellar homogenates (30 μ g) were loaded for GluR ζ 1-C2 and GluR ζ 1-C2', whereas cerebellar proteins in the PSD fraction (10 μ g) were loaded for GluR ζ 1-C1. *B–G*, Immunoperoxidase staining for GluR ζ 1-C2 (*B–D*) and GluR ζ 1-C2' (*E–G*) subunits. Antibodies were preincubated without (*B*, *E*) or with C2 (*C*, *F*) and C2' (*D*, *G*) antigen peptides. Scale bar, 1 mm.

mouse (five experiments; $n = 5$) and for GluR ζ 1-C1 in the PSD fraction of the wild-type mouse (four experiments; $n = 4$). When necessary, different amounts of protein samples were loaded in lanes for accurate comparison (see figure legends).

C-terminal exon usage in the wild-type cerebellum

The relative abundance of the GluR ζ 1 subunit was compared between the cerebrum (cerebral cortex) and cerebellum, using homogenates from the wild-type mouse (Fig. 6A). The total GluR ζ 1 content, as assessed using the GluR ζ 1-pan antibody raised against the N-terminal region (GluR ζ 1-N antibody) (Yamada et al., 2001), was three times lower in the cerebellum than in the cerebrum: the cerebellum/cerebrum ratio was calculated as $1.00 \pm 0.10/3.55 \pm 0.43$. Compared with the total GluR ζ 1 subunit, the ratio was further reduced for GluR ζ 1-C2 ($1.00 \pm 0.02/11.76 \pm 0.50$), whereas it was comparable for GluR ζ 1-C2' ($1.00 \pm 0.22/3.44 \pm 0.41$). Despite the presence of a strong band in the cerebrum, GluR ζ 1-C1 was not detected in the cerebellar homogenate, even with an increased amount of proteins loaded (Fig. 6A). These results suggest that the C-terminal form of the cerebellar GluR ζ 1 subunit is characterized by relatively higher usage of the C2' alternative cassette than the C2 cassette, and by much lower usage of the C1 optional cassette, compared with the cerebral GluR ζ 1 subunit.

The relative abundance of the C2 and C2' cassettes in the cerebellum was determined. First, NR1-1 and NR1-4 proteins expressed in HEK cells were standardized by graded dilution of these samples and by immunoblot detection with GluR ζ 1-pan antibody. Then, using the standardized NR1-1 and NR1-4 sam-

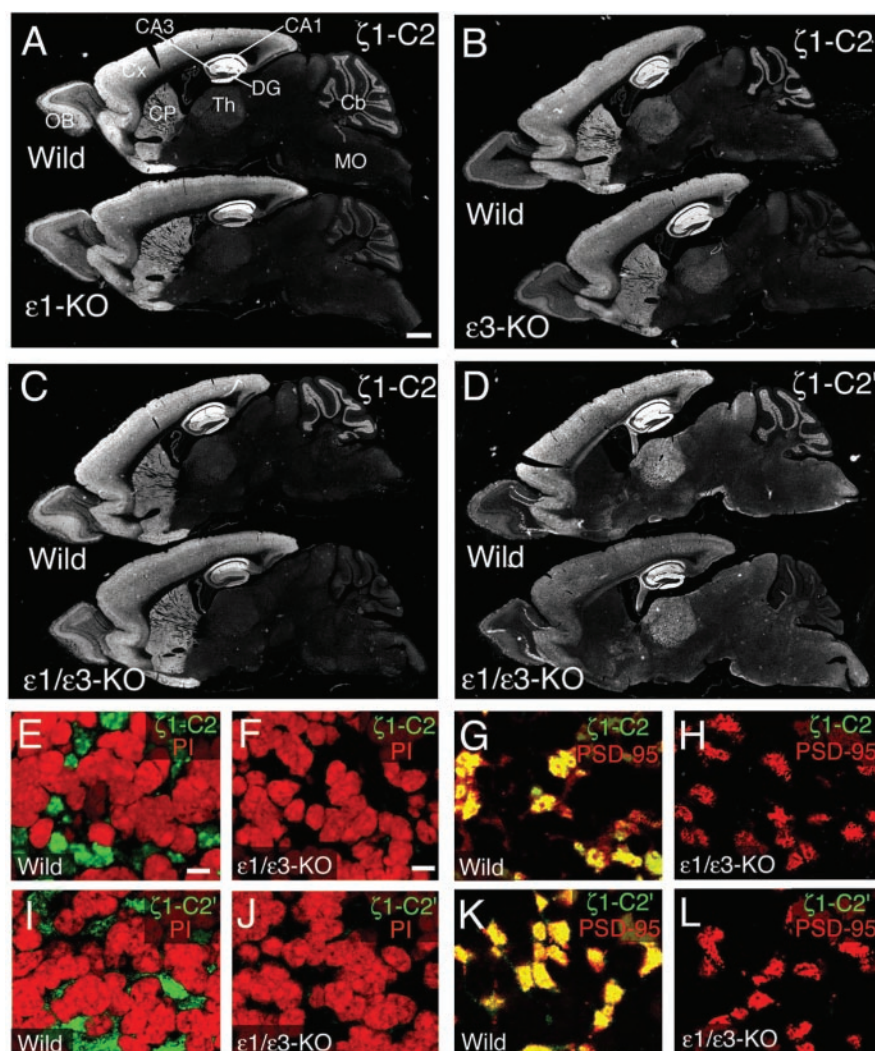


Figure 3. Immunohistochemical alterations of GluR ζ 1-C2 (A–C, E–H) and GluR ζ 1-C2' (D, I–L) in mutant mice lacking GluR ϵ subunits. A–D, Pairs of wild-type (top) and mutant (bottom; A, GluR ϵ 1-KO; B, GluR ϵ 3-KO; C, D, GluR ϵ 1/ ϵ 3-KO) brains. Photographs are negative images printed directly from preparates stained by immunoperoxidase using diaminobenzidine as a chromogen. E, F, I, J, Double fluorescence for the GluR ζ 1 subunit (green) and nuclear staining with propidium iodide (PI; red) in the cerebellar granular layer of the wild-type (E, I) and GluR ϵ 1/ ϵ 3-KO (F, J) mice. G, H, K, L, Double immunofluorescence for the GluR ζ 1 subunit (green) and PSD-95 (red) in the cerebellar granular layer of the wild-type (G, K) and GluR ϵ 1/ ϵ 3-KO (H, L) mice. CA1 and CA3, CA1 and CA3 regions of the hippocampus; Cb, cerebellum; CP, caudate–putamen; CX, cerebral cortex; DG, dentate gyrus; MO, medulla oblongata; OB, olfactory bulb; Th, thalamus. Scale bars: A, 1 mm; E, F, 5 μ m.

ples as a reference, the intensity of the GluR ζ 1-C2 or GluR ζ 1-C2' band in a given amount of cerebellar homogenates was corrected for comparison. Consequently, the relative abundance in cerebellar homogenates was estimated to be $39.1 \pm 3.2\%$ for GluR ζ 1-C2 and $66.0 \pm 11.0\%$ for and GluR ζ 1-C2'. Taking an undetectable level of GluR ζ 1-C1 in cerebellar homogenates, the major splice form in the cerebellum is thus the GluR ζ 1 subunit having the C2' cassette (i.e., NR1–4) with that having the C2 cassette (NR1–2) in the second abundance, whereas the GluR ζ 1 subunit having the C1 cassette (NR1–1 and NR1–3) is considerably low in the cerebellum.

Total GluR ζ 1 subunit

Changes in cerebellar contents of the total GluR ζ 1 subunit were compared between the wild-type and mutant mice (Fig. 6B, Table 1). The total GluR ζ 1 subunit was progressively reduced to $74.0 \pm 15.8\%$ in the GluR ϵ 1-KO mice, $69.6 \pm 13.2\%$ in the

GluR ϵ 3-KO mice, and $60.3 \pm 10.2\%$ in the GluR ϵ 1/ ϵ 3-KO mice [mean $\pm$ SEM; Student's *t* test; $p < 0.05$, compared with the wild-type level ($100 \pm 8.4\%$)] (Fig. 6B). Taking the virtual loss of postsynaptic immunogold labeling (Fig. 5), the GluR ζ 1 subunit remaining in the mutant cerebella was unexpectedly high, suggesting altered intracellular distribution in the GluR ϵ -deficient cerebella. To address this issue, subcellular fractionation was used for immunoblot analysis on GluR ζ 1-C2, GluR ζ 1-C2', and GluR ζ 1-C1.

GluR ζ 1-C2 and GluR ζ 1-C2'

Cerebellar contents of GluR ζ 1-C2 and GluR ζ 1-C2' were also reduced in the mutant mice (Fig. 6B, D, G; Table 1). GluR ζ 1-C2 was reduced to $70.2 \pm 7.1\%$ in the GluR ϵ 1-KO mice ($p < 0.05$), $66.5 \pm 10.1\%$ in the GluR ϵ 3-KO mice ($p < 0.05$), and $41.4 \pm 0.4\%$ in the GluR ϵ 1/ ϵ 3-KO mice [$p < 0.01$, compared with wild-type GluR ζ 1-C2 ($100 \pm 3.2\%$)], and GluR ζ 1-C2' was reduced to $73.1 \pm 6.4\%$ in the GluR ϵ 1-KO mice, $69.1 \pm 8.6\%$ in the GluR ϵ 3-KO mice, and $53.0 \pm 5.6\%$ in the GluR ϵ 1/ ϵ 3-KO mice [$p < 0.05$, compared with wild-type GluR ζ 1-C2' ($100 \pm 9.6\%$)].

Analysis of the P3 or microsomal fraction, which contains organelles including the ER, Golgi apparatus, and synaptic mitochondria, revealed a significant decrease in the GluR ϵ 1/ ϵ 3-KO mouse for GluR ζ 1-C2 ($60.1 \pm 7.4\%$; $p < 0.05$) and GluR ζ 1-C2' [$64.1 \pm 2.7\%$; $p < 0.05$, compared with wild-type levels ($100 \pm 7.4\%$ and $100 \pm 12.5\%$, respectively)] (Fig. 6C). Because no significant changes were observed for resident ER protein calreticulin on the same blotted membrane (Fig. 6C), this reduction was judged to be specific to the GluR ζ 1 subunit.

Then, the synaptic GluR ζ 1 subunit was assessed by comparative examination using the cerebellar synaptosomal and PSD

fractions (Fig. 6D, G; Table 1). The quality of these fractions was confirmed in all four genotypes, by the enrichment of the presynaptic protein synaptophysin in the synaptosomal fraction, the enrichment of postsynaptic protein PSD-95 in the PSD fraction, and the lack of synaptophysin in the PSD fraction (Fig. 6D). In the synaptosomal fraction, GluR ζ 1-C2 was reduced to $62.4 \pm 7.6\%$ in the GluR ϵ 1-KO mouse, $71.5 \pm 12.6\%$ in the GluR ϵ 3-KO mouse, and $34.2 \pm 5.6\%$ in the GluR ϵ 1/ ϵ 3-KO mouse [$p < 0.05$, compared with the wild-type synaptosomal fraction ($100 \pm 23.0\%$)]. Reduction was more pronounced in the PSD fraction: the GluR ζ 1-C2 level was $46.6 \pm 10.5\%$ in the GluR ϵ 1-KO mouse, $35.0 \pm 17.7\%$ in the GluR ϵ 3-KO mouse, and $12.3 \pm 3.3\%$ in the GluR ϵ 1/ ϵ 3-KO mouse [$p < 0.05$, compared with the wild-type PSD fraction ($100 \pm 23.3\%$)]. Similar results were obtained for GluR ζ 1-C2' (Fig. 6D). In the synaptosomal fraction, GluR ζ 1-C2' was reduced to $47.9 \pm 12.8\%$ in the GluR ϵ 1-KO mouse ($p <$

0.05), $60.5 \pm 17.2\%$ in the GluR ϵ 3-KO mouse, and $26.3 \pm 4.3\%$ in the GluR ϵ 1/ ϵ 3-KO mouse [$p < 0.01$, compared with the wild-type synaptosomal fraction ($100 \pm 10.5\%$)]. In the PSD fraction, the level of GluR ζ 1-C2' was $46.2 \pm 20.6\%$ in the GluR ϵ 1-KO mouse, $25.1 \pm 5.3\%$ in the GluR ϵ 3-KO mouse, and $17.0 \pm 4.6\%$ in the GluR ϵ 1/ ϵ 3-KO mouse [$p < 0.05$, compared with the wild-type PSD fraction ($100 \pm 24.4\%$)]. Thus, synaptic GluR ζ 1-C2 and GluR ζ 1-C2' were progressively reduced according to GluRe subunit ablation, with the most severe deficit in the PSD fraction

GluR ζ 1-C1

The fact that GluR ζ 1-C1 was detectable in the PSD fraction but not in homogenates suggests its low contents in the cerebellum and its enriched localization in the postsynapse (Figs. 2A, 6E; Table 1). GluR ζ 1-C1 in the PSD fraction was also reduced severely in the mutant cerebella: $55.2 \pm 4.3\%$ in the GluR ϵ 1-KO mouse ($p < 0.05$), $36.6 \pm 5.7\%$ in the GluR ϵ 3-KO mouse ($p < 0.05$), and $12.7 \pm 3.5\%$ in the GluR ϵ 1/ ϵ 3-KO mouse [$p < 0.01$, compared with the wild-type PSD fraction ($100 \pm 14.5\%$)] (Fig. 6E,G). Therefore, GluR ζ 1-C1 also requires the presence of GluRe subunits for its successful postsynaptic expression.

GluRe subunits and other synaptic molecules

Consistent with immunohistochemical results, a significant reduction was also observed for cerebellar contents of the GluR ϵ 1 subunit in the GluR ϵ 3-KO mouse [$67.8 \pm 7.5\%$; $p < 0.05$, compared with the wild-type homogenate ($100 \pm 4.9\%$)] and for the GluR ϵ 3 subunit in the GluR ϵ 1-KO mouse [$67.9 \pm 7.0\%$; $p < 0.05$, compared with the wild-type homogenate ($100 \pm 2.9\%$)] (Fig. 6B). Therefore, the ablation of one GluRe subunit has also reduced cerebellar contents of the other endogenous GluRe subunit.

Because the GluR ϵ 2 subunit is highly expressed in immature granule cells during the early postnatal period (Watanabe et al., 1992), we examined whether compensatory changes occurred for GluR ϵ 2 subunit expression in the mutant cerebellum at 1 month of age (Fig. 6F). In the wild-type mouse, the GluR ϵ 2 subunit was prominently expressed in the cerebral cortex, whereas it was low but still detectable in the cerebellum, as reported previously (Takahashi et al., 1996). No significant difference was observed in the cerebellar level of the GluR ϵ 2 subunit between the wild-type ($100.0 \pm 20.6\%$) and GluR ϵ 1/ ϵ 3-KO mice ($107.9 \pm 33.2\%$; $p > 0.05$).

Regardless of the substantial reduction of the GluR ζ 1 subunit, no significant changes were seen for PSD-95 and synaptophysin in the whole cerebellar homogenate, synaptosome fraction, and PSD fraction (Fig. 6B,D). Rather, levels of PSD-95 showed an increasing tendency in mutants, but the changes were not statistically significant.

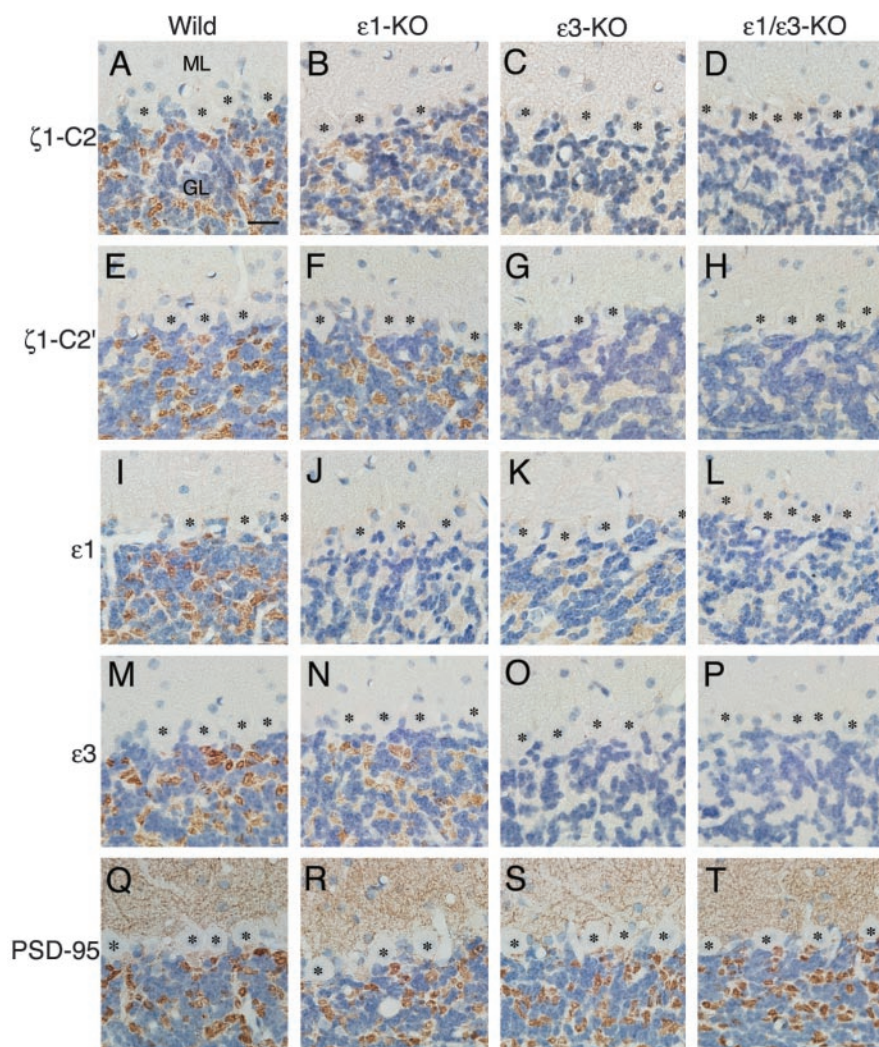


Figure 4. Immunoperoxidase for the cerebellar cortex using antibodies specific to GluR ζ 1-C2 (A–D), GluR ζ 1-C2' (E–H), GluR ϵ 1 (I–L), GluR ϵ 3 (M–P), and PSD-95 (Q–T). Note a gradual reduction of immunoreactivities for the GluR ζ 1 subunit from A to D or from E to H. All sections are counterstained with hematoxylin. Cell bodies of Purkinje cells are indicated by asterisks. GL, Granular layer; ML, molecular layer. Scale bar, 20 μ m.

GluR ζ 1 synthesis and turnover

The reduced cerebellar contents suggest lowered synthesis or accelerated degradation of GluR ζ 1 proteins in the absence of GluRe subunits. To address this, transcription, translation, and turnover of the GluR ζ 1 subunit were compared between wild-type and mutant cerebella.

Transcription and translation levels

Cultured granule cells were prepared from wild-type and mutant cerebella. In this study, mutant granule cells were obtained from the GluR ϵ 1-KO mouse, because the GluR ϵ 1 subunit is the major subunit at 7–8 d *in vitro* with the GluR ϵ 3 subunit at lower abundance (Huh and Wenthold, 1999). Northern blot analysis showed no significant changes in the transcription levels of GluR ζ 1 mRNA in the wild-type culture ($100.0 \pm 16.3\%$; $n = 3$) and GluR ϵ 1-KO culture ($117.4 \pm 41.6\%$; $n = 3$; $p > 0.05$) (Fig. 7A).

Translation activities were compared by a pulse-labeling experiment with [35 S]methionine and [35 S]cysteine. First, the total GluR ζ 1 subunit was compared using GluR ζ 1-pan antibody, and a significant reduction was observed in GluR ϵ 1-KO cultures (Fig. 7B), $100.0 \pm 8.5\%$ in wild-type cultures ($n = 3$) and $61.2 \pm 15.5\%$ in GluR ϵ 1-KO cultures ($n = 4$; $p < 0.05$). In the same

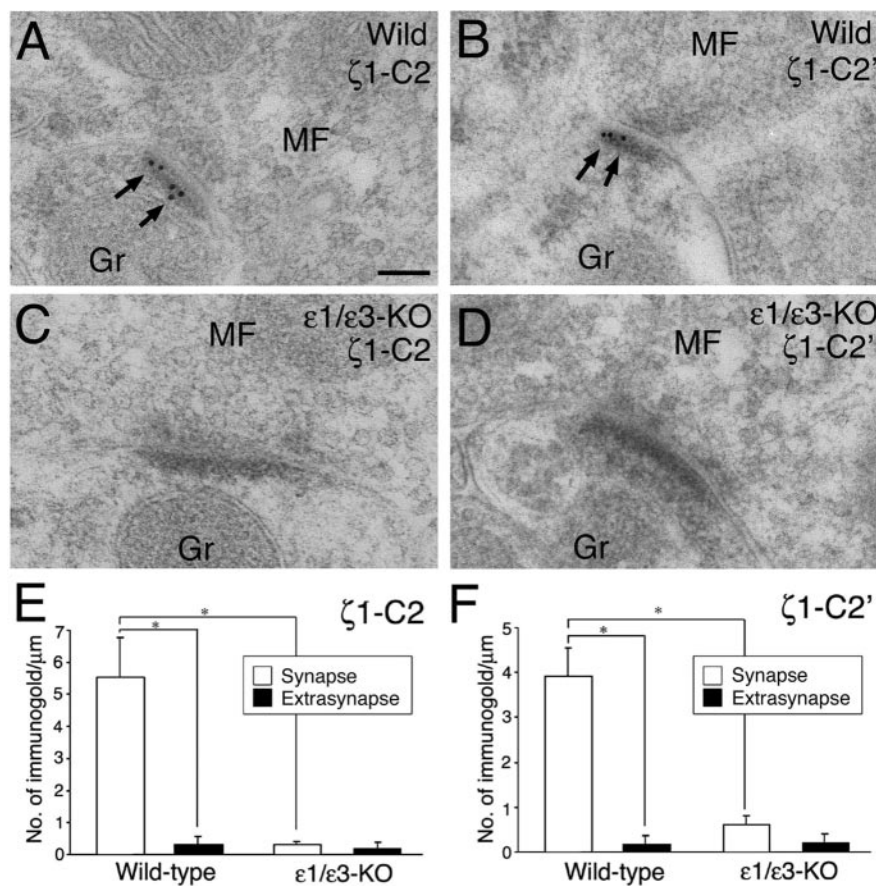


Figure 5. Postembedding immunogold for GluR ζ 1-C2 (*A, C, E*) and GluR ζ 1-C2' (*B, D, F*) at mossy fiber–granule cell synapses. Note that postsynaptic labeling for both variants is severely reduced in the GluR ϵ 1/ ϵ 3-KO mouse (*C, D*), in contrast to dense labeling in the wild-type mouse (*A, B*, arrows). *E, F*, The mean number of immunogold particles per micrometer of synaptic (white columns) and extrasynaptic (black columns) membranes of asymmetrical synapses in the wild-type and GluR ϵ 1/ ϵ 3-KO mice. Bars on the columns represent the SEM. The asterisks indicate statistically significant differences (Student's *t* test; $p < 0.05$). Gr, Granule cell dendrite; MF, mossy fiber terminal. Scale bar, 100 nm.

cultures, no significant difference was observed for PSD-95 [$100.0 \pm 3.9\%$ in wild-type cultures and $92.2 \pm 4.3\%$ in GluR ϵ 1-KO cultures ($p > 0.05$)] (Fig. 7*B*), indicating selective reduction of the GluR ζ 1 subunit in GluR ϵ 1-KO cultures. Then, we immunoprecipitated the GluR ζ 1 subunit from culture lysates. Immunoblot with the GluR ζ 1-pan antibody showed that similar amounts of the GluR ζ 1 subunit were immunoprecipitated from the wild-type ($100 \pm 18.9\%$) and GluR ϵ 1-KO ($102 \pm 11.7\%$) cultures (Fig. 7*C*, top), indicating successful immunoprecipitation by a nonsaturable amount of GluR ζ 1-pan antibody, as expected. In the immunoprecipitated GluR ζ 1 subunit, the content of the radioactive (pulse-labeled) GluR ζ 1 subunit was significantly increased in GluR ϵ 1-KO cultures, $100.0 \pm 14.9\%$ in wild type and $179.1 \pm 73.2\%$ in GluR ϵ 1-KO ($p < 0.05$) (Fig. 7*C*, bottom). Because the relative increase of the pulse-labeled GluR ζ 1 subunit was inversely proportional to the reduction of the total GluR ζ 1 subunit, the translation of the GluR ζ 1 subunit in GluR ϵ 1-KO cultures was judged to be comparable with that in wild-type cultures. Therefore, the reduced GluR ζ 1 subunit in the absence of the GluRe subunit is unlikely to result from lowered transcription and translation levels.

Protein turnover analysis

The stability of the GluR ζ 1 subunit was compared 4 hr after administration of the protein synthesis inhibitor anisomycin

(Fig. 7*D*). The reduction in anisomycin-injected mice ($n = 3$) was evaluated as the percentage relative to the levels in vehicle-injected mice ($n = 3$). The total GluR ζ 1 subunit was reduced mildly to $87.0 \pm 17.3\%$ in the wild-type cerebellum but severely to $35.0 \pm 0.9\%$ in GluR ϵ 1/ ϵ 3-KO cerebellum ($p < 0.05$) (Fig. 7*D, E*). In contrast, no significant genotypic differences were observed for PSD-95 ($109.7 \pm 23.9\%$ in the wild type and $88.8 \pm 10.9\%$ in the GluR ϵ 1/ ϵ 3-KO; $p > 0.05$) or synaptophysin ($103.3 \pm 1.4\%$ in the wild type and $103.8 \pm 3.8\%$ in the GluR ϵ 1/ ϵ 3-KO; $p > 0.05$) (Fig. 7*D*). These results suggest that reduced cerebellar contents of the GluR ζ 1 subunit thus arise from its accelerated degradation by the ablation of GluRe subunits.

Moreover, different stabilities among GluR ζ 1 variants were discerned in the GluR ϵ 1/ ϵ 3-KO mouse. At 4 hr after anisomycin injection, GluR ζ 1-C2 was reduced severely and significantly to $27.6 \pm 8.4\%$ in the mutant mouse compared with $89.8 \pm 16.3\%$ in the wild-type mouse ($p < 0.05$) (Fig. 7*D, E*). In contrast, the reduction of GluR ζ 1-C2' was moderate [$66.5 \pm 14.7\%$ in the GluR ϵ 1/ ϵ 3-KO mouse and $82.1 \pm 30.0\%$ in the wild-type mouse ($p > 0.05$)] (Fig. 7*D, E*). Therefore, GluR ζ 1-C2 is subjected to more rapid degradation than GluR ζ 1-C2', when GluRe subunits are lacking.

Cerebellar anatomy

To finally address whether these alterations arose, at least in part, from abnormal cellular and synaptic differentiation in granule cells, the anatomy of the GluR ϵ 1/ ϵ 3-KO cerebellum ($n = 3$) was compared with that of the wild-type cerebellum ($n = 3$) (Fig. 8). No histological differences were noted in size, lobule formation, or laminated structure of the cerebellum (Fig. 8*A, B*, insets). This was confirmed by measurement of the mean area of the granular layer, using midsagittal cerebellar sections [$2.45 \pm 0.08 \text{ mm}^2$ in the wild-type mouse and $2.56 \pm 0.12 \text{ mm}^2$ in the GluR ϵ 1/ ϵ 3-KO mouse, showing no significant difference (mean $\pm$ SEM; Student's *t* test; $p > 0.05$)]. By morphometric measurement for granule cell nuclei (Fig. 8*A, B*), the density of granule cells was estimated to be 3.86 ± 0.56 in the wild-type mouse and 3.77 ± 0.30 ($\times 10^6/\text{mm}^3$ of the granular layer) in the GluR ϵ 1/ ϵ 3-KO mouse, showing no significant difference ($p > 0.05$). Electron microscopy revealed that asymmetrical synapses between huge mossy fiber terminals and digits of granule cell dendrites were normally formed and organized into synaptic glomeruli (Fig. 8*C, D*). No significant difference was seen in the length of PSD at mossy fiber–granule cell synapses, being $243 \pm 33 \text{ nm}$ in the wild-type mouse ($n = 3$; 100 PSDs) and $248 \pm 8 \text{ nm}$ in the GluR ϵ 1/ ϵ 3-KO mouse ($n = 3$; 120 PSDs; $p > 0.05$). Double immunofluorescence for vesicular glutamate transporter VGLUT1 and vesicular GABA transporter VGAT demonstrated normal organization of excitatory and inhibitory afferents into synaptic glomeruli (Fig. 8*E, F*): in both mice, VGLUT1-positive

terminals (i.e., mossy fiber terminals, red) were located centrally, whereas VGAT-positive terminals (Golgi cell terminals, green) were located peripherally and surrounded the former.

Therefore, impaired synaptic localization and accelerated turnover of the GluR ζ 1 subunit is unlikely to result from developmental defects in the cerebellar anatomy and circuitry.

Discussion

In the present study, we produced animal models with cerebellar granule cells lacking major GluR ϵ subunits, GluR ϵ 1 and GluR ϵ 3, and examined changes in the expression, distribution, and localization of the GluR ζ 1 subunit. The most notable change emerged in the immunohistochemical visibility of the GluR ζ 1 subunit in the cerebellar granular layer. The order of immunohistochemical loss is grossly parallel with that in a previous electrophysiological study in which NMDA receptor-mediated currents are significantly reduced in granule cells lacking the GluR ϵ 1 or GluR ϵ 3 subunit, and almost absent in those lacking both subunits (Kadotani et al., 1996). The loss of the GluR ζ 1 subunit is attributable to neither transcriptional and translational downregulations nor to abnormal cellular and synaptic differentiations of granule cells. This results from impaired synaptic localization and protein stability of the GluR ζ 1 subunit in the absence of GluR ϵ subunits, as follows.

GluR ϵ subunits are essential for postsynaptic localization of the GluR ζ 1 subunit

In light microscopic immunohistochemistry, GluR ζ 1-C2 and GluR ζ 1-C2' were concentrated in synaptic glomeruli in the wild-type cerebellum and were progressively reduced in the GluR ϵ -deficient cerebella. By postembedding immunogold, both variants were localized selectively on the postsynaptic membrane at mossy fiber–granule cell synapses and disappeared almost completely in the GluR ϵ 1/ ϵ 3-KO mouse. By immunoblot with fractionated protein samples, the most severe deficit of the GluR ζ 1 subunit was observed in the PSD fraction of the GluR ϵ 1/ ϵ 3-KO mouse, with the level relative to the wild-type PSD fraction being 12.3% for GluR ζ 1-C2, 17.0% for GluR ζ 1-C2', and 12.7% for GluR ζ 1-C1. Considering that the cerebellum at 1 month of age contains trace or low levels of the GluR ϵ 2 (Fig. 6C) and GluR ϵ 4 (our unpublished data) subunits as well, the remaining GluR ζ 1 subunit in the PSD fraction would have reduced more severely, if all four GluR ϵ subunits are thoroughly ablated. Based on these findings, the first conclusion of the present study is that GluR ϵ subunits are the major molecular determinant for postsynaptic localization of the GluR ζ 1 subunit regardless of its C-terminal forms, at least, in cerebellar granule cells. This notion is compatible with our previous observation on immunohistochemical invisibility of the GluR ζ 1 subunit in adult wild-type Purkinje cells (Yamada et al.,

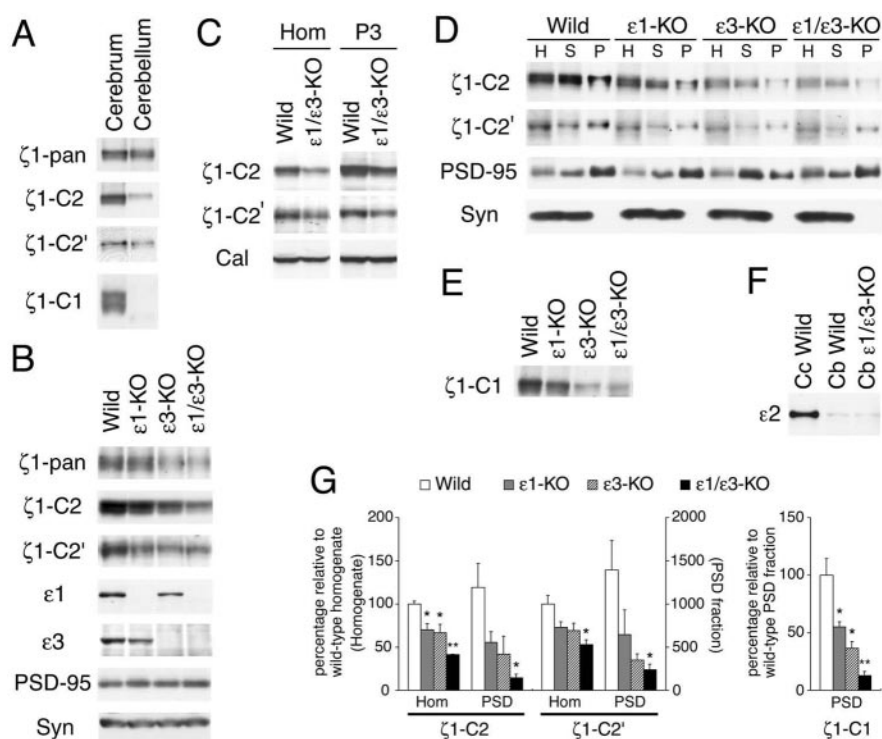


Figure 6. Immunoblot analysis. *A*, GluR ζ 1 subunit in wild-type cerebral and cerebellar homogenates. The proteins loaded in lanes for total GluR ζ 1 (ζ 1-pan), GluR ζ 1-C2, and GluR ζ 1-C2' are 10 μ g for the cerebellum and 30 μ g for the cerebellum, and those for GluR ζ 1-C1 are 30 and 50 μ g, respectively. *B*, Cerebellar contents of GluR ζ 1, GluR ϵ 1, GluR ϵ 3, PSD-95, and synaptophysin in the wild-type, GluR ϵ 1-KO, GluR ϵ 3-KO, and GluR ϵ 1/ ϵ 3-KO mice. Each lane contains 30 μ g of proteins. *C*, GluR ζ 1-C2, GluR ζ 1-C2', or calreticulin levels in homogenates (Hom) and P3 fraction (P3) prepared from the wild-type and GluR ϵ 1/ ϵ 3-KO cerebella. Each lane contains 30 μ g of proteins. *D*, GluR ζ 1-C2, GluR ζ 1-C2', PSD-95, or synaptophysin levels in homogenates (H), synaptosomal fraction (S), and PSD fraction (P) in the wild-type, GluR ϵ 1-KO, GluR ϵ 3-KO, and GluR ϵ 1/ ϵ 3-KO mice. The proteins loaded in each lane are 30 μ g for homogenates, 10 μ g for the synaptosomal fraction, and 3 μ g for the PSD fraction. *E*, GluR ζ 1-C1 levels in the PSD fraction. Each lane contains 10 μ g of PSD fraction proteins. *F*, GluR ϵ 2 levels in homogenates prepared from the wild-type cerebral cortex (Cc, Wild) and from the cerebellum of the wild-type (Cb, Wild) or GluR ϵ 1/ ϵ 3-KO (Cb, ϵ 1/ ϵ 3-KO) mouse. The proteins loaded in each lane are 10 μ g for the cerebral cortex and 30 μ g for the cerebellum. *G*, Histograms showing progressive loss of GluR ζ 1 proteins in mutant cerebella. The left histograms show the percentage of GluR ζ 1-C2 and GluR ζ 1-C2' levels in the mutant cerebellar homogenates (see left ordinate) and PSD fraction (see right ordinate) relative to the levels in wild-type cerebellar homogenates (mean $\pm$ SEM). The right histograms show the percentage of GluR ζ 1-C1 levels in the PSD fraction of mutant cerebella relative to the level in wild-type PSD fraction (mean $\pm$ SEM). The statistical difference relative to the wild-type levels in each fraction was determined by a Student's unpaired *t* test (**p* < 0.05; ***p* < 0.01).

2001), in which GluR ζ 1 mRNA is highly expressed but none of the GluR ϵ subunits are transcribed (Watanabe et al., 1994). Thus, GluR ϵ -dependent synaptic localization of the GluR ζ 1 subunit seems general to various neurons.

The C terminus of GluR ϵ subunits interacts strongly and specifically with PDZ domain proteins in the PSD, such as PSD-95, SAP-102, and Chapsyn-110 (Kornau et al., 1995; Niethammer et al., 1996; Wyszynski et al., 1997; Ehlers et al., 1998). C-terminal truncation of the GluR ϵ 1 or GluR ϵ 2 subunit has reduced the amount of synaptic NMDA receptors in hippocampal pyramidal cells (Mori et al., 1998; Steigerwald et al., 2000). Although synaptic localization of NMDA receptors is not impaired in mutant mice lacking PSD-95 (Migaud et al., 1998), the interaction of C termini of GluR ϵ subunits with multiple members of the PSD protein family is a likely mechanism for the GluR ϵ -dependent postsynaptic localization of the GluR ζ 1 subunit.

GluR ϵ subunits increase protein stability of the GluR ζ 1 subunit

There is a large cytoplasmic pool for the GluR ζ 1 subunit (Hall and Soderling, 1997). This pool, being obtained as Triton X-100-

Table 1. Relative concentrations of GluR ζ 1 proteins in subcellular fractions of the wild-type and mutant cerebella

Protein	Genotype	Homogenate	Synaptosome	PSD fraction
GluR ζ 1-pan	Wild type	100.0 $\pm$ 8.4	—	—
	ϵ 1-KO	74.0 $\pm$ 15.8	—	—
	ϵ 3-KO	69.6 $\pm$ 13.2	—	—
	ϵ 1/ ϵ 3-KO	60.3 $\pm$ 10.2*	—	—
GluR ζ 1-C2	Wild type	100.0 $\pm$ 3.2	100 $\pm$ 23.0	100 $\pm$ 23.3
	ϵ 1-KO	70.2 $\pm$ 7.1*	62.4 $\pm$ 7.6	46.6 $\pm$ 10.5
	ϵ 3-KO	66.5 $\pm$ 10.1*	71.5 $\pm$ 12.6	35.0 $\pm$ 17.7
	ϵ 1/ ϵ 3-KO	41.4 $\pm$ 0.4**	34.2 $\pm$ 5.6*	12.3 $\pm$ 3.3*
GluR ζ 1-C2'	Wild type	100.0 $\pm$ 9.6	100 $\pm$ 10.5	100 $\pm$ 24.4
	ϵ 1-KO	73.1 $\pm$ 6.4	47.9 $\pm$ 12.8*	46.2 $\pm$ 20.6
	ϵ 3-KO	69.1 $\pm$ 8.6	60.5 $\pm$ 17.2	25.1 $\pm$ 5.3
	ϵ 1/ ϵ 3-KO	53.0 $\pm$ 5.6*	26.3 $\pm$ 4.3**	17.0 $\pm$ 4.6*
GluR ζ 1-C1	Wild type	ND	—	100 $\pm$ 14.5
	ϵ 1-KO	ND	—	55.2 $\pm$ 4.3*
	ϵ 3-KO	ND	—	36.6 $\pm$ 5.7*
	ϵ 1/ ϵ 3-KO	ND	—	12.7 $\pm$ 3.5**

Each value was calculated from the signals of immunoblot bands and is relative to the value of wild-type cerebellum in each fraction. All data are given as the mean $\pm$ SEM for three experiments, except for GluR ζ 1-C2' proteins of the wild-type mouse ($n = 5$) and the GluR ζ 1-C1 protein of the wild-type PSD fraction ($n = 4$). Genotypic differences relative to wild-type scores within respective sample fractions were statistically determined by Student's t test. * $p < 0.05$; ** $p < 0.01$. —, Not tested; ND, not detectable.

solubilized P3 fraction, represents an unassembled, monomeric GluR ζ 1 subunit (Chazot and Stephenson, 1997). Turnover analysis has further demonstrated biphasic decay of the [35 S]methionine-labeled GluR ζ 1 subunit with half-lives of 2 and 34 hr (Huh and Wenthold, 1999). Of the two, the slowly degraded (or stable) pool represents the GluR ζ 1 subunit on the cell surface, whereas the rapidly degraded pool represents an unassembled GluR ζ 1 subunit in the cytoplasm. From this *in vitro* evidence, we had supposed that the cytoplasmic pool would increase with reciprocal decrease of the surface/synaptic pool in the GluR ϵ -deficient cerebella.

Contrary to our presumption, GluR ζ 1-C2 and GluR ζ 1-C2' in the GluR ϵ 1/ ϵ 3-KO cerebellum were reduced not only in the synaptosomal and PSD fractions but also in the P3 fraction to 60–64%. This result led us to assume that before becoming the cytoplasmic and surface/synaptic pools, a fraction of the GluR ζ 1 subunit undergoes more rapid, premature degradation and that this might have increased in the GluR ϵ -deficient cerebella. To test this, we used protein turnover analysis. At 4 hr after anisomycin injection, the total GluR ζ 1 subunit relative to the level after vehicle injection was severely reduced in the GluR ϵ 1/ ϵ 3-KO cerebellum (35.0%), whereas it decreased mildly in the wild-type cerebellum (87.9%). Therefore, the second conclusion of the present study is that accompanying GluR ϵ subunits enhance protein stability of the GluR ζ 1 subunit, leading to increases of both the cytoplasmic and synaptic pools. The enhanced protein stability appears to be linked, at least in part, with the formation of heteromeric NMDA receptors in the ER because coexpression of GluR ζ 1 and GluR ϵ subunits greatly promotes the export of the GluR ζ 1 subunit from the ER to the cell surface (McIlhinney et al., 1996, 1998) and to the postsynaptic site (the present study). The premature degradation of the GluR ζ 1 subunit is probably conducted as either ER-associated degradation in the ER or ubiquitin–proteasomal degradation in the cytoplasm (Johnston et al., 1998; Fukaya et al., 2003).

C terminus-dependent self-ER export mechanisms in neurons *in vivo*

Relatively higher uses of the C2' cassette in the cerebellum and of the C1 and C2 cassettes in the cerebral cortex are generally con-

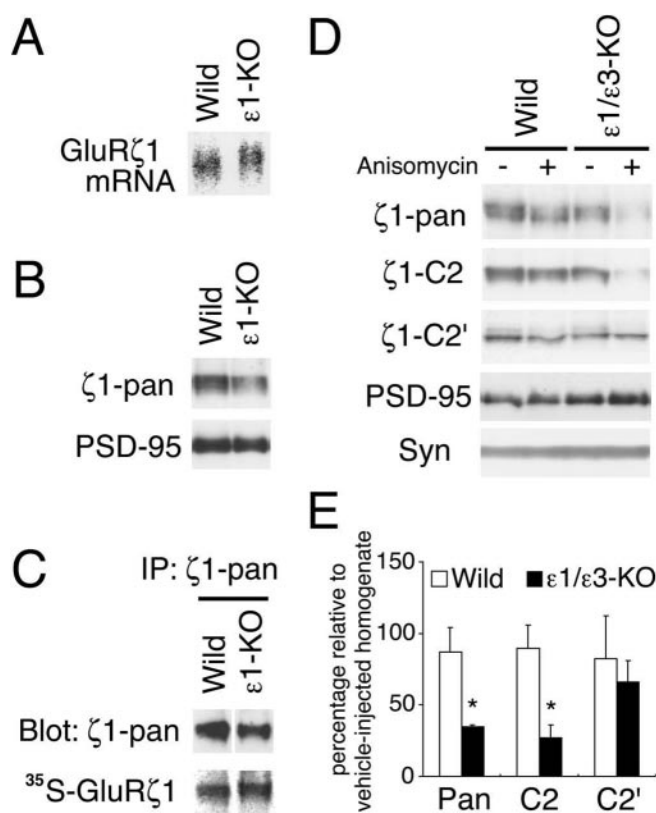


Figure 7. Transcription, translation, and protein turnover analyses for the GluR ζ 1 subunit. *A*, Northern blot analysis for GluR ζ 1 mRNA in the wild-type and GluR ϵ 1-KO cerebellar cultures. The total RNA loaded in each lane is 5 μ g. *B*, Immunoblot with GluR ζ 1-pan and PSD-95 antibodies for cell lysates from wild-type and GluR ϵ 1-KO cerebellar cultures. Each lane contains 30 μ g of proteins. *C*, Pulse-labeling experiment. After pulse-labeling with [35 S]Met/Cys for 30 min, cell lysates were immunoprecipitated with GluR ζ 1-pan antibody. Immunoprecipitates were then analyzed to compare the amount of precipitated GluR ζ 1 subunit by immunoblot (Blot: ζ 1-pan) and to compare the radioactivity of pulse-labeled GluR ζ 1 subunit (35 S-GluR ζ 1). *D*, Protein stability experiment at 4 hr after anisomycin injection to the wild-type and GluR ϵ 1/ ϵ 3-KO mice. Cerebellar homogenates from vehicle-injected (–) and anisomycin-injected (+) mice were immunoreacted with GluR ζ 1-pan, GluR ζ 1-C2, GluR ζ 1-C2', PSD-95, or synaptophysin antibody. *E*, Histograms showing different stabilities among total GluR ζ 1 subunit (pan), GluR ζ 1-C2, and GluR ζ 1-C2' in anisomycin-injected cerebellar homogenates. Values are expressed as the percentage of the protein levels relative to those from vehicle-injected homogenates in each mouse type (mean $\pm$ SEM). The statistical difference in the reduction between the wild-type and GluR ϵ 1/ ϵ 3-KO mice was determined by a Student's unpaired t test (* $p < 0.05$).

sistent with previous studies by *in situ* hybridization and immunoblot analyses (Laurie and Seeburg, 1994; Al-Hallaq et al., 2001). In the present study, C-terminal cassette-dependent differences of the GluR ζ 1 subunit were also appreciated in the brain *in vivo*. A notable difference was seen in protein stability between GluR ζ 1-C2 and GluR ζ 1-C2' when GluR ϵ subunits were lacking. Four hours after anisomycin injection, GluR ζ 1-C2 was reduced severely to 27.6%, whereas GluR ζ 1-C2' was mildly reduced to 66.5% in the GluR ϵ 1/ ϵ 3-KO mouse, suggesting a different turnover rate depending on the C2/C2' form. Unfortunately, how GluR ζ 1-C1 is stable in the absence of GluR ϵ subunits was not addressed in the present study because of its particularly low contents in the cerebellum.

The C-terminal cassette-related difference in the protein stability appears to be concerned with C terminus-dependent self-ER export mechanisms. When transfected to heterologous cells *in vitro*, the GluR ζ 1 subunit ended with a C1–C2 tail (i.e.,

NR1–1 or NR1 $_{X11}$) is lowest in the cell surface expression and extensively accumulates intracellularly, whereas the splice variant lacking the C1 cassette and ended with a C2' tail (NR1–4 or NR1 $_{X00}$) displays the highest surface expression (McIlhinney et al., 1996; Okabe et al., 1999). As the underlying mechanisms, particular domains in the C2' tail have been shown to facilitate forward ER trafficking to the cell surface. One is by the PDZ-interacting domain in the C2' tail, which mediates self-suppression of the ER retention motif RRR residing in the C1 cassette (Standley et al., 2000; Scott et al., 2001), and the other is by the TVV export motif in the C2' tail, which recruits NMDA receptors to ER exit sites (Mu et al., 2003). Taking this evidence from *in vitro* studies into our observations, the higher protein stability of GluR ζ 1–C2' than GluR ζ 1–C2 can be interpreted that more efficient ER export for the former might have prolonged its turnover rate. Therefore, the C terminus-dependent self-ER export system seems functional in neurons *in vivo*, when accompanying GluR ϵ subunits are lacking. Considering the fact that most central neurons coexpress GluR ζ 1 and one or more GluR ϵ subunits (Watanabe et al., 1993, 1994), the C terminus-dependent self-ER export mechanisms for GluR ζ 1 subunit would be suppressed or masked by the strong action of GluR ϵ -dependent cell surface delivery and synaptic localization for the GluR ζ 1 subunit.

Facilitative interaction between GluR ϵ subunits

We noticed reduced immunohistochemical and immunoblot levels for the GluR ϵ 1 or GluR ϵ 3 subunit in the absence of the other. This suggests that facilitative interaction also works among GluR ϵ subunits, which would lead to the increase of receptor complex formation or protein stability. This notion is in line with a finding that the availability of GluR ϵ subunits, but not the GluR ζ 1 subunit, determines the total number of functional NMDA receptors in cultured cerebellar granule cells (Prybylowski et al., 2002).

Through our present and previous analyses using the *in vivo* system, it is evident that synaptic expression of NMDA receptors is regulated by GluR ζ 1 and GluR ϵ subunits in a different but cooperative manner. In the absence of GluR ϵ subunits, the GluR ζ 1 subunit is unable to be localized on the postsynapse, regardless of its C-terminal forms. Without the GluR ζ 1 subunit, GluR ϵ subunits are obliged to undergo ER retention (Fukaya et al., 2003). These dual mechanisms will constitute strict quality control mechanisms by which heteromeric NMDA receptors constructed properly are only permitted to be expressed on the postsynapse.

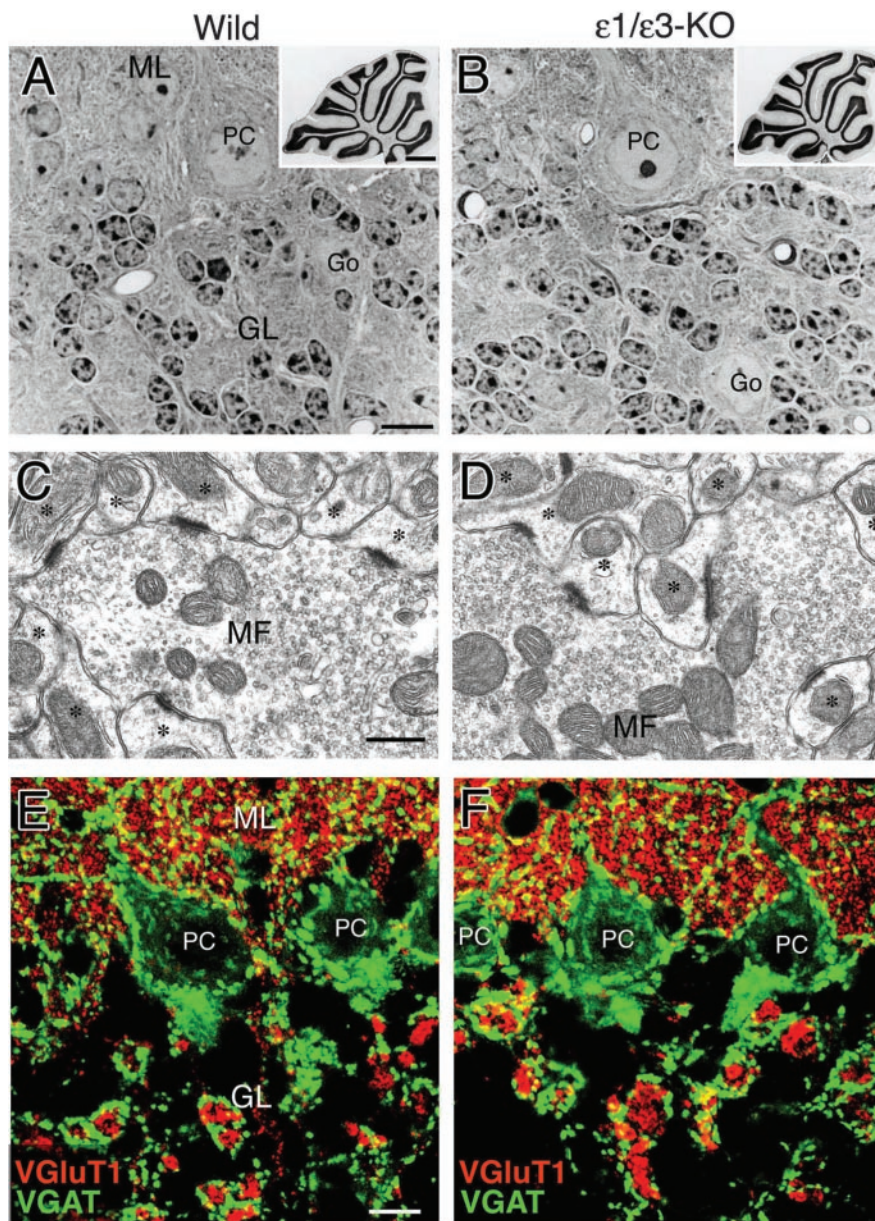


Figure 8. Anatomical and synaptic organization of the wild-type (*A, C, E*) and GluR ϵ 1/ ϵ 3-KO (*B, D, F*) cerebella. *A, B*, Normal cytoarchitecture of the cerebellar cortex is shown by hematoxylin-stained Epon semithin sections. Normal cerebellar histoarchitecture is also shown by hematoxylin-stained microtome sections (insets). *C, D*, Electron micrographs showing huge mossy fiber terminals (MF) forming asymmetrical synapses with granule cell dendrites (asterisk). *E, F*, Double immunofluorescence for vesicular glutamate transporter VGLUT1 (red) and vesicular GABA transporter VGAT (green) in the cerebellar cortex. GL, Granular layer; Go, Golgi cell; ML, molecular layer; PC, Purkinje cell. Scale bars: *A*, 10 μ m (inset, 1 mm); *C, D*, 0.2 μ m; *E, F*, 10 μ m.

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