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NMDA Receptor GluRε/NR2 Subunits Are Essential for Postsynaptic Localization and Protein Stability of GluRζ1/NR1 Subunit

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In NMDA receptors, GluRε/NR2 subunits strictly require the GluRζ1/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 GluRζ1 subunit in vitro. To test whether this leads to C-terminus-dependent self-postsynaptic expression in neurons in vivo, we investigated the GluRζ1 subunit in cerebellar granule cells lacking two major GluRε subunits, GluRε1/NR2A and GluRε3/NR2C. In the mutant cerebellum, synaptic labeling for the GluRζ1 subunit containing the C2 (GluRζ1-C2) or C2′ (GluRζ1-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 GluRζ1-C2 and GluRζ1-C2′. The most severe deficit was observed in the postsynaptic density (PSD) fraction: mutant levels relative to the wild-type level were 12.3 ± 3.3% for GluRζ1-C2 and 17.0 ± 4.6% for GluRζ1-C2′. The GluRζ1 subunit carrying the C1 cassette (GluRζ1-C1) was, although low in cerebellar content, also reduced to 12.7 ± 3.5% in the mutant PSD fraction. Considering a trace amount of other GluRe subunits in the mutant cerebellum, the severe reductions thus represent that the GluRζ1 subunit, by itself, is virtually unable to accumulate at postsynaptic sites, regardless of C-terminal forms. By protein turnover analysis, the degradation of the GluRζ1 subunit was accelerated in the mutant cerebellum, being particularly rapid for that carrying the C2 cassette. Therefore, accompanying expression of GluRe subunits is essential for postsynaptic localization and protein stability of the GluRζ1 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 synaptic development, synaptic plasticity, and learning and memory (Mayer and Westbrook, 1987; Bliss and Collingridge, 1993). NMDA receptors are composed of GluRζ1/NR1 and GluRε/NR2 subunits (Seeburg, 1993; Nakanishi and Masu, 1994; Mori and Mishina, 1995). The GluRζ1 subunit is encoded by a single gene but exists as several splice variants. The GluRε subunit is composed of four members (GluRε1–ε4 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 GluRe subunit is expressed together with the GluRζ1 subunit (Meguro et al., 1992; Monyer et al., 1992). Second, binding sites for agonist glutamate and coagonist glycine are shared between GluRe and GluRζ1 subunits (Hirai et al., 1996; Laube et al., 1997). Third, GluRζ1 and GluRe 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; Wyszenia-Skii et al., 1997; Ehlers et al., 1998; Setou et al., 2000). Fourth, GluRe subunits require cotransfection of the GluRζ1 subunit for their exit from the endoplasmic reticulum (ER) to the cell surface (McIlhinney et al., 1996, 1998). Indeed, when the GluRζ1 gene is deleted in hippocampal pyramidal cells in vivo, GluRe subunits...
are retained in the somatic ER and disappear from the postsynaptic site (Fukaya et al., 2003).

Four different C termini of the GluR1 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 GluR1 subunit with the C2′ tail than that with the C2 tail, and also for the GluR1 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 GluR1 subunit in neurons in vivo, by producing cerebellar granule cells lacking two major GluRe subunits, GluR1/NR2A and GluR3/ NR2C. Here, we show that, when accompanying GluRe subunits are lacking, postsynaptic localization is impaired for the GluR1 subunit with any C-terminal cassettes. Moreover, the ablation of GluRe subunits causes rapid degradation of the GluR1 subunit, particularly that carrying the C2′ cassette. Therefore, accompanying GluRe subunits are essential for postsynaptic expression and protein stability of the GluR1 subunit in neurons in vivo.

Materials and Methods

Production of the GluRe3 and GluRe1/3 knockout mice. A genomic DNA clone, AGREST1–12 (Nagasawa et al., 1996), carrying the GluRe3 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 pGKNeo (Yagi et al., 1993) containing the pgk-neo cassette. A 4.0 kb SalI–NotI fragment from pPaltDT3 (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 GluRe3 knockout (GluRe3–KO) mice were performed in the same manner as described (Sakimura et al., 1995). To produce mutant mice defective in both GluRe1 and GluRe3 subunits, GluRe3–KO mice were mated with GluRe1–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 0.1M PB, pH 7.4, for light microscopic immunohistochemistry and histology (paraffin sections, 5 μm in thickness); 4% paraformaldehyde/0.1% glutaraldehyde in 0.1 M sodium phosphate buffer (PB), pH 7.4, for light microscopic immunohistochemistry and histology (paraffin sections, 5 μm in thickness; microslicer sections, 50 μm); 4% paraformaldehyde/0.1% 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 μm). 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 cyrostat sections (20 μm).

In situ hybridization. In situ hybridization analysis was performed using subunit-specific antisense oligonucleotides for the GluRe1 subunit (oligoE1E), GluRe3 subunit (oligo3M4C), and GluR1 subunit (oligo1A). OligoE1E and oligo3M4C are complementary to the nucleotide residues 1862–1906 of the GluRe1 subunit cDNA (Meguro et al., 1992) and 2525–2570 of the GluRe3 subunit cDNA (Kutsuwada et al., 1992), respectively. Oligo1A 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 GluR1 subunit in the rabbit and guinea pig, respectively, using synthetic peptides (CDPKKKATRAITSTLASSFKRRRSSKDT for the C1 and CQYHEPTDITGPNLSDPSSTTV 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-GluR1–C2 (Yamada et al., 2001), rat monoclonal anti-GluR1–N (GluR1–pan) (Yamada et al., 2001), rabbit anti-GluRe1C (Watanabe et al., 1998), rabbit anti-GluRe2C (Watanabe et al., 1998), rabbit anti-GluRe3C (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-calretulin (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. All antibodies were used at the concentration of 0.5–1 μg/ml, unless noted otherwise.

To check the specificity of GluR1 antibodies, the GluR1 subunit tagged with C1–C2 cassettes (NR1–1) or with the C2′ cassette only (NR1–4) was expressed under the control of the human elongation factor 1α promoter. HEK293 cells were transfected using Lipofectamine reagent (In Vitrogen, Carlsbad, CA) and lysed 2 days after the addition of 1 mg/ml pepsin (Nagasawa et al., 2001; Oshima et al., 2002). After treatment with 1 mg/ml pepsin (Dako, Carpinteria, CA) in 0.2 N HC1 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 microscope (AX-70 Olympus, Tokyo, Japan).

For confocal laser-scanning microscopy (Fluoview; Olympus), pepsin-treated paraffin sections were incubated first with GluR1–C2 or GluR1–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 μM propidium iodide for 10 min (Molecular Probes, Eugene, OR). Double immunofluorescence for VGluT1 and VGAT was also conducted using microslicer sections, followed by a 2 hr incubation using a mouse anti-glutamate transporter (Cy3-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 GluR1–C2 or GluR1–C2′ antibody (10 μg/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 fibre terminals and granule cell dendrites.

Morphological analysis. Parasagittal microslicer 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 (Nv) of granule cells was obtained by the point-counting method of Weibel (1979) and using the equation: \( Nv = \frac{1}{\beta \times Na \times Vv^{0.5}} \), as reported previously (Kakizawa et al., 2000). Na and Vv are the visible profile count of granule cell nuclei or their volume density in the granular layer, respectively, whereas \( \beta \) 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 the point-counting method of Weibel (1979) and using the equation: \( Nv = \frac{1}{\beta \times Na \times Vv^{0.5}} \), as reported previously (Kakizawa et al., 2000). Na and Vv are the visible profile count of granule cell nuclei or their volume density in the granular layer, respectively, whereas \( \beta \) is a dimensionless shape coefficient defined here as 1.38 by assuming that granule cell nuclei are spherical.

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Western blot analysis. For quantitative Western blot analysis, five cerebellar of each genotype were pooled for one experiment. The cerebellum was homogenized in 10 volumes of sucrose buffer (0.32 M sucrose, 5 mM EDTA, 1 mM PMSF, 1 mM pepstatin A, and 2 mM leupeptin) and centrifuged at 700 × 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 × g for 1 hr. The resultant pellets were resuspended in 40 mM Tris-HCl, pH 8.0. To obtain the light membrane fraction (P5 fraction) in a separate procedure, the P2 fraction prepared from a cerebellum was centrifuged at 165,000 × 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 electrophoresed 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, cerebellum obtained from the wild-type or GluR1-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 mM arabinosylcytosine, and 10% FCS and were plated on polyethyleneimine-coated culture plates at a density of 2.4 × 10^5 cells/cm^2. 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 GluR1 mRNA was performed by hybridization of total RNA using a ^32P-labeled GluR1 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 μCi/ml[^35]S methionine and[^35]S 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 GluR1-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 GluR1 subunit was analyzed by FUJIX Bioimage Analyzer (LAS2000; 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 GluR1/e3-KO mice

The GluR3 gene locus in murine TT2 ES cells was disrupted by homologous recombination using a targeting vector containing an 8.5 kb fragment of GluR3 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 was genotyped by Southern blot analysis with three probes (Fig. 1B). Homozygous GluR3 mutant (GluR3-e3-KO) mice were then crossed with the GluR1-KO mouse (Sakimura et al., 1995) to obtain the double knock-out (GluR1/e3-KO) mouse. The GluR3-KO and GluR1/e3-KO mice were viable, grew normally, and were fertile. However, the GluR1/e3-KO mouse showed mild impairment in motor coordination when tested on the rotating rod at high speeds (data not shown), as reported previously (Ebralidze et al., 1996; Kadotani et al., 1996).

In situ hybridization

By in situ hybridization, the absence of GluR1 and/or GluR3 mRNAs in the respective mutant brains was confirmed with ^32P-labeled probes against the deleted sequences (Fig. 1C, top and middle). No genotypic differences were detected in overall distribution of GluR1 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 GluR1 subunit in the absence of the GluR3 gene, and vice versa (Fig. 1D, left and middle) (n = 3; Student’s t test; p > 0.05 for each). Moreover, GluR1 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 GluR1 subunit, GluR3 subunit, or both does not affect the transcription levels of the remaining NMDA receptor subunits.

Specificity of GluR1-C2, -C2’, and -C1 antibodies

To examine the effect on C-terminal variants of the GluR1 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 (GluR1 subunit tailing with C1–C2), NR1–4 (that tailed with C2’), or NR1–1, respectively (Fig. 2A).

Preincubation of these antibodies with their immunizing peptides (data not shown) abolished these protein bands, whereas the bands were similarly detected by preincubation with the other (nonimmunizing) peptides (data not shown). These results indi-
cated that the three antibodies are specific to the C2, C2’s, and C1 cassettes, respectively, and have no cross-reactivity to the others.

By immunohistochemistry, C2 and C2’s antibodies yielded characteristic labelings in the adult mouse brain that were similar but not identical to each other (Fig. 2B, E). The immunolabelings were abolished with the use of antibodies preincubated with immunizing peptides (Fig. 2C, G), but not with nonimmunizing ones (Fig. 2D, F). Thus, the C2 and C2’s 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’s, and C1 antibodies is referred to as GluRζ1-C2, GluRζ1-C2’s, 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. 3A–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. 3D, top brain). These patterns of immunostaining are consistent with the reported distributions of C2 and C2’s 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 GluRe1-KO mouse (Fig. 3A, bottom brain), reduced severely in the GluRe3-KO mouse (Fig. 3B, bottom brain), and almost negative in the GluRe1/e3-KO mouse (Fig. 3C, bottom brain). This was also true for GluRζ1-C2’ (Fig. 3D, bottom brain).

By nuclear counterstaining with propidium iodide (Fig. 3E, I, red) or hematoxylin (Fig. 4A, 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. 3E, I; green, 4A, E, brown). These immunopositive structures were well overlapped with post-synaptic density protein PSD-95 (Fig. 3G, 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>GluRe1-KO>GluRe3-KO>GluRe1/e3-KO mice (Fig. 4A–D, GluRζ1-C2; E–H, GluRζ1-C2’). In the GluRe1/e3-KO mouse, both variants were almost undetectable in synaptic glomeruli (Fig. 3F, H, I, J). Thus, parallel with GluRζ 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. 5A, B), whereas they were hardly

![Figure 1](https://example.com/f1.png)

**Figure 1.** Production of mutant mice defective in NMDA receptor GluRζ1 and/or GluRζ3 subunit genes. A, Schematic representation of genomic DNA, targeting vector, and disrupted gene of the GluRe3 subunit. B, Southern blot analysis of EcoRI-digested genomic DNA using three different probes indicated in A. C, In situ hybridization analysis for GluRe1, GluRe3, and GluReζ1 mRNAs in the wild-type, GluRe1-KO, GluRe3-KO, and GluReζ1/e3-KO brains. D, Semiquantitative evaluation of transcription levels for GluRe1 (left), GluRe3 (center), and GluReζ1 (right) mRNAs in the granular layer of the cerebellum (mean ± 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, diphertheria toxin gene; EI, EcoRI; EV, EcoRV; H, HindIII; Hi, hippocampus; N, Norg; S, SalI. Scale bar, (in C) 1 mm.
detected at this type of synapse in the GluR1/ε3-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 GluRζ1-C2 on the synaptic membrane was 5.55 ± 1.22 in the wild-type mouse (mean ± SEM; n = 3; 101 synapses examined), whereas it was significantly lowered to 3.91 ± 0.08 in the GluRε1/ε3-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 GluRε1/ε3-KO mice (Fig. 5E). As for GluRζ1-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 GluRε1/ε3-KO mouse (n = 3; 78 synapses), showing significant reduction (p < 0.05) (Fig. 5F). Thus, synaptic localization of the GluRζ1 subunit was significantly reduced to the extrasynaptic level in the GluRε1/ε3-KO mouse, regardless of the C2/C2’ form. Despite the severe synaptic loss, no significant increase of the extrasynaptic density was evident in the GluRε1/ε3-KO mouse (p > 0.05 for each variant).

In our previous study using mutant mice lacking the GluRζ1 subunit, GluRε1 and GluRε2/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 GluRζ1 subunit in GluRε1/ε3-deficient granule cells by light microscopy (Fig. 3F,J) and postembedding immunogold (data not shown).

GluRe subunits and PSD-95
Changes of the GluRε1 subunit (Fig. 4I–L), GluRε3 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 GluRε1 subunit in the GluRε3-KO mouse (Fig. 4K) and for the GluRε3 subunit in the GluRε1-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 GluRζ1 subunit (Fig. 6, Table 1). All immunoblotting data were quantified by three independent experiments (n = 3), except for GluRζ1-C2’ in the homogenate, synaptosomal fraction, and PSD fraction of the wild-type mouse (five experiments; n = 5) and for GluRζ1-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 GluRζ1 subunit was compared between the cerebrum (cerebral cortex) and cerebellum, using homogenates from the wild-type mouse (Fig. 6A). The total GluRζ1 content, as assessed using the GluRζ1-pan antibody raised against the N-terminal region (GluRζ1-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 ± 0.10/3.55 ± 0.43. Compared with the total GluRζ1 subunit, the ratio was further reduced for GluRζ1-C2 (1.00 ± 0.02/11.76 ± 0.50), whereas it was comparable for GluRζ1-C2’ (1.00 ± 0.22/3.44 ± 0.41). Despite the presence of a strong band in the cerebrum, GluRζ1-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 GluRζ1 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 GluRζ1 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 GluRζ1-pan antibody. Then, using the standardized NR1–1 and NR1–4 sam-
the second abundance, whereas the GluRc cassette (i.e., NR1 for comparison. Consequently, the relative abundance in cerebellar homogenates was corrected as a reference, the intensity of the GluRc subunits.

A–D, Pairs of wild-type (top) and mutant (bottom; A, GluRc1-KO; B, GluRc3-KO; C, D, GluRc1/-3-KO) brains. Photographs are negative images printed directly from preparations stained by immunoperoxidase using diaminobenzidine as a chromagen. E, F, I, J, Double fluorescence for the GluRc1 subunit (green) and nuclear staining with propidium iodide (PI; red) in the cerebellar granular layer of the wild-type (E, I) and GluRc1/-3-KO (F, J) mice. G, H, K, L, Double immunofluorescence for the GluRc1 subunit (green) and PSD-95 (red) in the cerebellar granular layer of the wild-type (G, K) and GluRc1/-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 μm.

Figure 3. Immunohistochemical alterations of GluRc1-C2 (A–C, E–H) and GluRc1-C2’ (D, I–L) in mutant mice lacking GluRe subunits. A–D, Pairs of wild-type (top) and mutant (bottom; A, GluRc1-KO; B, GluRc3-KO; C, D, GluRc1/-3-KO) brains. Photographs are negative images printed directly from preparations stained by immunoperoxidase using diaminobenzidine as a chromagen. E, F, I, J, Double fluorescence for the GluRc1 subunit (green) and nuclear staining with propidium iodide (PI; red) in the cerebellar granular layer of the wild-type (E, I) and GluRc1/-3-KO (F, J) mice. G, H, K, L, Double immunofluorescence for the GluRc1 subunit (green) and PSD-95 (red) in the cerebellar granular layer of the wild-type (G, K) and GluRc1/-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 μm.

GluRc3-KO mice, and 60.3 ± 10.2% in the GluRc1/e3-KO mice [mean ± SEM; Student’s t test; p < 0.05, compared with the wild-type level (100 ± 8.4%)] (Fig. 6B). Taking the virtual loss of postsynaptic immunogold labeling (Fig. 5), the GluRc1 subunit remaining in the mutant cerebella was unexpectedly high, suggesting altered intracellular distribution in the GluRc-deficient cerebellum. To address this issue, subcellular fractionation was used for immunoblot analysis on GluRc1-C2, GluRc1-C2’, and GluRc1-C1.

GluRc1-C2 and GluRc1-C2’ Cerebellar contents of GluRc1-C2 and GluRc1-C2’ were also reduced in the mutant mice (Fig. 6B, D, G; Table 1). GluRc1-C2 was reduced to 70.2 ± 7.1% in the GluRc1-KO mouse (p < 0.05), 66.5 ± 10.1% in the GluRc3-KO mouse (p < 0.05), and 41.4 ± 0.4% in the GluRc1/e3-KO mouse [p < 0.01, compared with wild-type GluRc1-C2 (100 ± 3.2%)], and GluRc1-C2’ was reduced to 73.1 ± 6.4% in the GluRc1-KO mouse, 69.1 ± 8.6% in the GluRc3-KO mouse, and 53.0 ± 5.6% in the GluRc1/e3-KO mouse [p < 0.05, compared with wild-type GluRc1-C2’ (100 ± 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 GluRc1/e3-KO mouse for GluRc1-C2 (60.1 ± 7.4%; p < 0.05) and GluRc1-C2’ [64.1 ± 2.7%; p < 0.05, compared with wild-type levels (100 ± 7.4% and 100 ± 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 GluRc1 subunit.

Then, the synaptic GluRc1 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, GluRc1-C2 was reduced to 62.4 ± 7.6% in the GluRc1-KO mouse, 71.5 ± 12.6% in the GluRc3-KO mouse, and 34.2 ± 5.6% in the GluRc1/e3-KO mouse [p < 0.05, compared with the wild-type synaptosomal fraction (100 ± 23.0%)]. Reduction was more pronounced in the PSD fraction: the GluRc1-C2 level was 46.6 ± 10.5% in the GluRc1-KO mouse, 35.0 ± 17.7% in the GluRc3-KO mouse, and 12.3 ± 3.3% in the GluRc1/e3-KO mouse [p < 0.05, compared with the wild-type PSD fraction (100 ± 23.3%)]. Similar results were obtained for GluRc1-C2’ (Fig. 6D). In the synaptosomal fraction, GluRc1-C2’ was reduced to 47.9 ± 12.8% in the GluRc1-KO mouse (p < 0.05, compared with the wild-type PSD fraction (100 ± 23.3%).

Changes in cerebellar contents of the total GluRc1 subunit were compared between the wild-type and mutant mice (Fig. 6B, Table 1). The total GluRc1 subunit was progressively reduced to 74.0 ± 15.8% in the GluRc1-KO mouse, 69.6 ± 13.2% in the GluRc3-KO mouse, and 60.3 ± 10.2% in the GluRc1/e3-KO mouse [mean ± SEM; Student’s t test; p < 0.05, compared with the wild-type level (100 ± 8.4%)].
0.05), 60.5 ± 17.2% in the GluR3-KO mouse, and 26.3 ± 4.3% in the GluRε/ε3-KO mouse [p < 0.01, compared with the wild-type synaptosomal fraction (100 ± 10.9%)]. In the PSD fraction, the level of GluRζ-C2’ was 46.2 ± 20.6% in the GluRε1-KO mouse, 25.1 ± 5.3% in the GluRε3-KO mouse, and 17.0 ± 4.6% in the GluRε/ε3-KO mouse [p < 0.05, compared with the wild-type PSD fraction (100 ± 24.4%)]. Thus, synaptic GluRζ-C2 and GluRζ-C2’ were progressively reduced according to GluRe subunit ablation, with the most severe deficit in the PSD fraction.

GluRζ-C1
The fact that GluRζ-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). GluRζ1-C1 in the PSD fraction was also reduced severely in the mutant cerebella: 55.2 ± 4.3% in the GluRε1-KO mouse (p < 0.05), 36.6 ± 4.7% in the GluRε3-KO mouse (p < 0.05), and 12.7 ± 3.5% in the GluRε/ε3-KO mouse [p < 0.01, compared with the wild-type PSD fraction (100 ± 14.5%)] (Fig. 6E, G). Therefore, GluRζ1-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 GluRε1 subunit in the GluRε3-KO mouse (67.8 ± 7.5%; p < 0.05, compared with the wild-type homogenate [100 ± 4.9%]) and for the GluRε3 subunit in the GluRε1-KO mouse [67.9 ± 7.0%; p < 0.05, compared with the wild-type homogenate (100 ± 2.9%)] (Fig. 6B). Therefore, the ablation of one GluRe subunit has also reduced cerebellar contents of the other endogenous GluRe subunit.

Because the GluRε2 subunit is highly expressed in immature granule cells during the early postnatal period (Watanabe et al., 1992), we examined whether compensatory changes occurred for GluRε2 subunit expression in the mutant cerebellum at 1 month of age (Fig. 6F). In the wild-type mouse, the GluRε2 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 GluRε2 subunit between the wild-type (100.0 ± 20.6%) and GluRε1/ε3-KO mice (107.9 ± 33.2%; p > 0.05).

Regardless of the substantial reduction of the GluRζ1 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.

GluRζ1 synthesis and turnover
The reduced cerebellar contents suggest lowered synthesis or accelerated degradation of GluRζ1 proteins in the absence of GluRe subunits. To address this, transcription, translation, and turnover of the GluRζ1 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 GluRε1-KO mouse, because the GluRε1 subunit is the major subunit at 7–8 d in vitro with the GluRε3 subunit at lower abundance (Huh and Wenthold, 1999). Northern blot analysis showed no significant changes in the transcription levels of GluRζ1 mRNA in the wild-type culture (100.0 ± 16.3%; n = 3) and GluRε1-KO culture (117.4 ± 41.6%; n = 3; p > 0.05) (Fig. 7A).

Translation activities were compared by a pulse-labeling experiment with [35S]methionine and [35S]cysteine. First, the total GluRζ1 subunit was compared using GluRζ1-pan antibody, and a significant reduction was observed in GluRε1-KO cultures (Fig. 7B), 100.0 ± 8.5% in wild-type cultures (n = 3) and 61.2 ± 15.5% in GluRε1-KO cultures (n = 4; p < 0.05). In the same
cultures, no significant difference was observed for PSD-95 [100.0 ± 3.9% in wild-type cultures and 92.2 ± 4.3% in GluR1-KO cultures (p > 0.05)] (Fig. 7B), indicating selective reduction of the GluR1 subunit in GluR1-KO cultures. Then, we immunoprecipitated the GluR1 subunit from culture lysates. Immunoblot with the GluR1-pan antibody showed that similar amounts of the GluR1 subunit were immunoprecipitated from the wild-type (100 ± 18.9%) and GluR1-KO (102 ± 11.7%) cultures (Fig. 7C, top), indicating successful immunoprecipitation by nonsaturable amount of GluR1-pan antibody, as expected. In the immunoprecipitated GluR1 subunit, the content of the radioactive (pulse-labeled) GluR1 subunit was significantly increased in GluR1-KO cultures, 100.0 ± 14.9% in wild type and 179.1 ± 73.2% in GluR1-KO (p < 0.05) (Fig. 7C, bottom). Because the relative increase of the pulse-labeled GluR1 subunit was inversely proportional to the reduction of the total GluR1 subunit, the translation of the GluR1 subunit in GluR1-KO cultures was judged to be comparable with that in wild-type cultures. Therefore, the reduced GluR1 subunit in the absence of the GluR subunit is unlikely to result from lowered transcription and translation levels.

Protein turnover analysis
The stability of the GluR1 subunit was compared 4 hr after administration of the protein synthesis inhibitor anisomycin (Fig. 7D). 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 GluR1 subunit was reduced mildly to 87.0 ± 17.3% in the wild-type cerebellum but severely to 35.0 ± 0.9% in GluR1/e3-KO cerebellum (p < 0.05) (Fig. 7D,E). In contrast, no significant genotypic differences were observed for PSD-95 (109.7 ± 23.9% in the wild type and 88.8 ± 10.9% in the GluR1/e3-KO; p > 0.05) or synaptophysin (103.3 ± 1.4% in the wild type and 103.8 ± 3.8% in the GluR1/e3-KO; p > 0.05) (Fig. 7D). These results suggest that reduced cerebellar contents of the GluR1 subunit thus arise from its accelerated degradation by the ablation of GluR1 subunits.

Moreover, different stabilities among GluR1 variants were discerned in the GluR1/e3-KO mouse. At 4 hr after anisomycin injection, GluR1-C2 was reduced severely and significantly to 27.6 ± 8.4% in the mutant mouse compared with 89.8 ± 16.3% in the wild-type mouse (p < 0.05) (Fig. 7D,E). In contrast, the reduction of GluR1-C2’ was moderate [66.5 ± 14.7% in the GluR1/e3-KO mouse and 82.1 ± 30.0% in the wild-type mouse (p > 0.05)] (Fig. 7D,E). Therefore, GluR1-C2 is subjected to more rapid degradation than GluR1-C2’, when GluR1 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 GluR1/e3-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. 8A,B, insets). This was confirmed by measurement of the mean area of synaptic glomeruli (Fig. 8D). No significant difference was seen in the granular layer, using midsagittal cerebellar sections [2.45 ± 0.08 mm² in the wild-type mouse and 2.56 ± 0.12 mm² in the GluR1/e3-KO mouse, showing no significant difference (mean ± SEM; Student’s t test; p > 0.05)]. By morphometric measurement for granule cell nuclei (Fig. 8A,B), the density of granule cells was estimated to be 3.86 ± 0.56 in the wild-type mouse and 3.77 ± 0.30 (× 10⁷/mm² of the granular layer) in the GluR1/e3-KO mouse, showing no significant difference (p > 0.05). Electron microscopy revealed that asymmetrical synapses between huge mossy fiber terminals and digit of granule cell dendrites were normally formed and organized into synaptic glomeruli (Fig. 8C,D). No significant difference was seen in the length of PSD at mossy fiber–granule cell synapses, being 243 ± 33 nm in the wild-type mouse (n = 3; 100 PSDs) and 248 ± 8 nm in the GluR1/e3-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. 8E,F): in both mice, VGlut1-positive

Figure 5. Postembedding immunogold for GluR1-C2 (A, C, E) and GluR1-C2’ (B, D, F) at mossy fiber–granule cell synapses. Note that postsynaptic labeling for both variants is severely reduced in the GluR1/e3-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 GluR1/e3-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.
and were progressively reduced in the GluR1 concentrated in synaptic glomeruli in the wild-type cerebellum. This notion is compatible with our previous observation on immunohistochemical invisibility of the GluR1 subunit in adult wild-type Purkinje cells (Yamada et al., 2001), in which GluR1 mRNA is highly expressed but none of the GluR subunits are transcribed (Watanabe et al., 1994). Thus, GluR-dependent synaptic localization of the GluR1 subunit seems general to various neurons.

The C terminus of GluR 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; Wysszn et al., 1997; Ehlers et al., 1998). C-terminal truncation of the GluR1 or GluR2 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 subunits with multiple members of the PSD protein family is a likely mechanism for the GluR-dependent postsynaptic localization of the GluR1 subunit.

GluR subunits increase protein stability of the GluR1 subunit

There is a large cytoplasmic pool for the GluR1 subunit (Hall and Soderling, 1997). This pool, being obtained as Triton X-100-

**Discussion**

In the present study, we produced animal models with cerebellar granule cells lacking major GluR subunits, GluR1 and GluR3, and examined changes in the expression, distribution, and localization of the GluR1 subunit. The most notable change emerged in the immunohistochemical visibility of the GluR1 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 GluR1 or GluR3 subunit, and almost absent in those lacking both subunits (Kadotani et al., 1996). The loss of the GluR1 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 GluR1 subunit in the absence of GluR subunits, as follows.

**GluR subunits are essential for postsynaptic localization of the GluR1 subunit**

In light microscopic immunohistochemistry, GluR1-C2 and GluR1-C2' were concentrated in synaptic glomeruli in the wild-type cerebellum and were progressively reduced in the GluR-deficient cerebellum. By postembedding immunogold, both variants were localized selectively on the postsynaptic membrane at mossy fiber–granule cell synapses and disappeared almost completely in the GluR1/ε3-KO mouse. By immunoblot with fractionated protein samples, the most severe deficit of the GluR1 subunit was observed in the PSD fraction of the GluR1/ε3-KO mouse, with the level relative to the wild-type PSD fraction being 12.3% for GluR1-C2, 17.0% for GluR1-C2', and 12.7% for GluR1-C1. Considering that the cerebellum at 1 month of age contains trace or low levels of the GluR2 (Fig. 6C) and GluR4 (our unpublished data) subunits as well, the remaining GluR1 subunit in the PSD fraction would have reduced more severely, if all four GluR subunits are thoroughly ablated. Based on these findings, the first conclusion of the present study is that GluR subunits are the major molecular determinant for postsynaptic localization of the GluR1 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 GluR1 subunit in adult wild-type Purkinje cells (Yamada et al., 2001), in which GluR1 mRNA is highly expressed but none of the GluR subunits are transcribed (Watanabe et al., 1994). Thus, GluR-dependent synaptic localization of the GluR1 subunit seems general to various neurons.

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**GluR subunits increase protein stability of the GluR1 subunit**

There is a large cytoplasmic pool for the GluR1 subunit (Hall and Soderling, 1997). This pool, being obtained as Triton X-100-

**Immunoblot analysis.** A, GluR1 subunit in wild-type cerebral and cerebellar homogenates. The proteins loaded in lanes for total GluR1 (C1-pan), GluR1-C2, and GluR1-C2' are 10 μg for the cerebrum and 30 μg for the cerebellum, and those for GluR1-C1 are 30 and 50 μg, respectively. B, Cerebellar contents of GluR1, GluR1, PSD-95, and synaptophysin in the wild-type, GluR1-KO, GluR3-KO, and GluR1/ε3-KO mice. Each lane contains 30 μg of proteins. C, GluR1-C2, GluR1-C2', or calreticulin levels in homogenates (Hom) and P3 fraction (P3) prepared from the wild-type and GluR1/ε3-KO cerebellum. Each lane contains 30 μg of proteins. D, GluR1-C2, GluR1-C2', PSD-95, or synaptophysin levels in homogenates (H), synaptosomal fraction (S), and PSD fraction (P) in the wild-type, GluR1-KO, GluR3-KO, and GluR1/ε3-KO mice. The proteins loaded in each lane are 30 μg for homogenates, 10 μg for the synaptosomal fraction, and 3 μg for the PSD fraction. E, GluR1-C1 levels in the PSD fraction. Each lane contains 10 μg of PSD fraction proteins. F, GluR2 levels in homogenates prepared from the wild-type cerebral cortex (Cb, Wild) and from the cerebellum of the wild-type (Cb, Wild) or GluR1/ε3-KO (Cb, ε1/ε3-KO) mouse. The proteins loaded in each lane are 10 μg for the cerebral cortex and 30 μg for the cerebellum. G, Histograms showing progressive loss of GluR1 proteins in mutant cerebella. The left histograms show the percentage of GluR1-C2 and GluR1-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 ± SEM). The right histograms show the percentage of GluR1-C1 levels in the PSD fraction of mutant cerebella relative to the level in wild-type PSD fraction (mean ± 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).
injection, the total GluR subunit undergoes more rapid, premature degradation and that the C1 and C2 cassettes in the cerebral cortex are generally conducted as either ER-associated degradation in the ER or ubiquitin–proteasomal degradation in the cytoplasm (Johnston et al., 1999). Of the two, the slowly degraded (stable) pool represents the GluR1 subunit on the cell surface, whereas the rapidly degraded pool represents an unassembled GluR1 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 deficient cerebellum.

Contrary to our presumption, GluR1-C2 and GluR1-C2′ in the GluR1/e3-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 GluR1 subunit undergoes more rapid, premature degradation and that this might have increased in the GluR-deficient cerebellum. To test this, we used protein turnover analysis. At 4 hr after anisomycin injection, the total GluR1 subunit relative to the level after vehicle injection was severely reduced in the GluR1/e3-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 subunits enhance protein stability of the GluR1 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 GluR1 and GluR subunits greatly promotes the export of the GluR1 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 GluR1 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 consistent 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 GluR1 subunit were also appreciated in the brain in vivo. A notable difference was seen in protein stability between GluR1-C2 and GluR1-C2′ when GluR subunits were lacking. Four hours after anisomycin injection, GluR1-C2 was reduced severely to 27.6%, whereas GluR1-C2′ was mildly reduced to 66.5% in the GluR1/e3-KO mouse, suggesting a different turnover rate depending on the C2/C2′ form. Unfortunately, how GluR1-C1 is stable in the absence of GluR 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 GluR1 subunit ended with a C1–C2 tail (i.e.,
NR1–1 or NR1X11) 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 NR1X100) 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 RXR 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 GluRζ1–C2′ than GluRζ1–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 GluRe subunits are lacking. Considering the fact that most central neurons coexpress GluRζ1 and one or more GluRe subunits (Watanabe et al., 1993, 1994), the C-terminus-dependent self-ER export mechanisms for GluRζ1 subunit would be suppressed or masked by the strong action of GluRe-dependent cell surface delivery and synaptic localization for the GluRζ1 subunit.

Facilitative interaction between GluRe subunits

We noticed reduced immunohistochemical and immunoblot levels for the GluRe1 or GluRe3 subunit in the absence of the other. This suggests that facilitative interaction also works among GluRe 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 GluRe subunits, but not the GluRζ1 subunit, determines the total number of functional NMDA receptors in cultured cerebellar granule cells (Przybylewski 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 GluRζ1 and GluRe subunits in a different but cooperative manner. In the absence of GluRe subunits, the GluRζ1 subunit is unable to be localized on the postsynapse, regardless of its C-terminal forms. Without the GluRζ1 subunit, GluRe 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 GluRe1/ζ3-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 microslicer 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 µm (inset, 1 mm); C, 0.2 µm; E, 10 µm.

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