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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-postsynaptic expression 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 GluR 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 27.2 ± 3.5% in the mutant PSD fraction. Considering a trace amount of other GluRε 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 GluRε 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 synapse 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 GluRε 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 GluRε and GluRζ1 subunits (Hirai et al., 1996; Laube et al., 1997). Third, GluRζ1 and GluRε 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; Wyszyński et al., 1997; Ehlers et al., 1998; Setou et al., 2000). Fourth, GluRε 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, GluRε 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 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 that with 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, GluRe1/NR2A and GluRe3/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 amination 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/e3 knock-out mice. A genomic DNA clone, AGRE3T1–12 (Nagasawa et al., 1996), carrying the GluRe3 gene was used to construct a targeting vector. A 0.7 kb HindIII–Not fragment containing the transmembrane segment M4 was replaced with a 1.3 kb EcoRI–BanHI fragment from pGKNeo (Yagi et al., 1993) containing the pgk-neo cassette. A 4.0 kb Sall–Not 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 GluRe3 knock-out (GluRe3–/–) 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 0.1 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 (oligo1E1), GluRe3 subunit (oligo3M4C), and GluR1 subunit (oligo1A). Oligo1E1 and oligo3M4C are complementary to the nucleotide residues 1882–1906 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 (CDKKKATRAITSTLASSFFRRSSSKDTD for the C1 and CQYHTPDITGPNLSDPSTV 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-GluR1C (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 tailing 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 1α promoter. HEK293 cells were transfected using Lipofectamine reagent (Invitrogen, Carlsbad, CA) and lysed 2 days later by the addition of hot lysis buffer (1 mm EDTA, pH 8.1, 1 mm EGTA, pH 7.9, 1% TritonX–100, and 1 mm PMSF). For control immunohistochemistry, GluR1 antibodies were preabsorbed with either C2 or C2' antigen peptides (50 μg/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 0.2 N 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 GluRe1–C2 or GluRe1–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 PGS–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 with Cy3–labeled donkey anti–rabbit IgG (1:200) and Cy5–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 fiber terminals and granule cell dendrites.

Morphological analysis. Parasagittal microslicer sections were stained with hematoxylin and used for cerebellar histology. Using the sections, J. Neurosci., August 18, 2004 • 24(33):7292–7304 • 7293
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 = 1/β × Na(1/3)/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 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 IP lab software (Nippon Roper).

Western blot analysis. For quantitative Western blot analysis, five cerebellum 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 μM pepstatin A, and 2 μM 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 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 electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Dassell, 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 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 (In-vitrogen) supplemented with 10% FBS, 5% penicillin-streptomycin, 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 μM 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 203–738). 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 [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 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 FUSIX 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 μg 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 T12 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 were genotyped by Southern blot analysis with three probes (Fig. 1B). Homozygous GluR3 mutant (GluR3-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; Kadohata 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 quantitated 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, GluR3 mRNA levels in the granule 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 tails 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-
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. 2B, E). The immunolabelings
were abolished with the use of antibodies preincubated with

immunizing peptides (Fig. 2D, 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 immunohisto-
chemical labeling and was used for immunoblot analysis only.

Hereafter, the GluR1 subunit detected by the C2, C2’, and C1
antibodies is referred to as GluR1-C2, GluR1-C2’, and
GluR1-C1, respectively.

Immunohistochemistry

Alterations at the protein level were examined by immunohisto-
chemistry at the light microscopic (Figs. 3, 4) and electron mi-

croscopic (Fig. 5) levels.

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 immuno-

ingostaining for GluR1-C2 was detected in the hippocampal CA1 region
(Fig. 3A–C, top brains). High levels were also observed in the cerebral cortex, olfac-

tory bulb, caudate-putamen, hippocam-

pal CA3 region, dentate gyrus, and cere-

bellar granular layer, whereas the level in the thalamus was low to moderate. In con-
trast, GluR1-C2’ was highest in each sub-
region of the hippocampus and also high in the cerebral cortex, thalamus, and cere-

bellar 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’ cassette mRNAs, respectively (Laurie et al., 1995). When com-
pared with the wild-type brain, GluR1-C2 in the cerebellar granular layer was reduced mildly in the GluR1-KO mouse
(Fig. 3A, bottom brain), reduced severely in the GluR3-KO mouse (Fig. 3B, bottom brain), and almost negative in the
GluR1/e3-KO mouse (Fig. 3C, bottom brain). This was also true for GluR1-C2’
(Fig. 3D, bottom brain).

By nuclear counterstaining with propidium

iodide (Fig. 3E, I, red) or hematoxy-

lin (Fig. 4A, E, blue), GluR1-C2 and
GluR1-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 immunoposi-
tive structures were well overlapped with
postsynaptic density protein PSD-95 (Fig.

3G, K, red), indicating that GluR1-C2 and GluR1-C2’ are accumu-
lated extensively in synaptic glomeruli. In mutants, immuno-

reactivity in synaptic glomeruli was progressively reduced in the

order of wild-type>Glur1-KO>Glur3-KO>Glur1/e3-KO

mice (Fig. 4A–D, Glur1-C2; E–H, Glur1-C2’). In the Glur1/e-
3-KO mouse, both variants were almost undetectable in synaptic

glomeruli (Fig. 3F, H, I, L). Thus, parallel with GluR subunit a-

blation, immunohistochemical visibility of Glur1-C2 and
GluR1-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 GluR1-C2 and GluR1-C2’
The subcellular localization was examined by postembedding

immunogold (Fig. 5). In the wild-type mouse, gold particles rep-

resenting GluR1-C2 or GluR1-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. Production of mutant mice defective in NMDA receptor GluR1 and/or GluR3 subunit genes. A, Schematic repre-

sentation of genomic DNA, targeting vector, and disrupted gene of the GluR3 subunit. B, Southern blot analysis of EcoRI-digested

genomic DNA using three different probes indicated in A. C, In situ hybridization analysis for Glur1, Glur3, and Glur1 cRNAs

in the wild-type, Glur1-KO, Glur3-KO, and Glur1/e3-KO brains. D, Semiquantitative evaluation of transcription levels for

GluR1 (left), Glur3 (center), and Glur1 (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, diphtheria toxin gene; Ei, EcoRI; EV, EcoRV; H, HindIII; Hi, hippocampus; N, NotI;

S, SalI. Scale bar, (in C) 1 mm.
detected at this type of synapse in the GluRe1/e3-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 0.35 ± 0.08 in the GluRe1/e3-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 GluRe1/e3-KO mice (Fig. 5E).

In our previous study using mutant mice lacking the GluRζ1 subunit, GluRe1 and GluRe2/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 GluRe1/e3-deficient granule cells by light microscopy (Fig. 3F,J) and postembedding immunogold (data not shown).

**GluRe subunits and PSD-95**

Changes of the GluRe1 subunit (Fig. 4I–L), GluRe3 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 GluRe1 subunit in the GluRe3-KO mouse (Fig. 4K) and for the GluRe3 subunit in the GluRe1-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 content, 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-
ples as a reference, the intensity of the GluRζ1-C2 or GluRζ1-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 ± 3.2% for GluRζ1-C2 and 66.0 ± 11.0% for and GluRζ1-C2’. Taking an undetectable level of GluRζ1-C1 in cerebellar homogenates, the major splice form in the cerebellum is thus the GluRζ1 subunit having the C2’ cassette (i.e., NR1–4) with that having the C2 cassette (NR1–2) in the second abundance, whereas the GluRζ1 subunit having the C1 cassette (NR1–1 and NR1–3) is considerably low in the cerebellum.

**Total GluRζ1 subunit**

Changes in cerebellar contents of the total GluRζ1 subunit were compared between the wild-type and mutant mice (Fig. 6 B, Table 1). The total GluRζ1 subunit was progressively reduced to 74.0 ± 15.8% in the GluRe1-KO mice, 69.6 ± 13.2% in the GluRe3-KO mice, and 60.3 ± 10.2% in the GluRe1/ε3-KO mice [mean ± SEM; Student’s t test; p < 0.05, compared with the wild-type level (100 ± 8.4%)] (Fig. 6 B). Taking the virtual loss of postsynaptic immunogold labeling (Fig. 5), the GluRζ1 subunit remaining in the mutant cerebella was unexpectedly high, suggesting altered intracellular distribution in the GluRe-deficient cerebellum. To address this issue, subcellular fractionation was used for immunoblot analysis on GluRζ1-C2, GluRζ1-C2’, and GluRζ1-C1.

**GluRζ1-C2 and GluRζ1-C2’**

Cerebellar contents of GluRζ1-C2 and GluRζ1-C2’ were also reduced in the mutant mice (Fig. 6 B, D, G; Table 1). GluRζ1-C2 was reduced to 70.2 ± 7.1% in the GluRe1-KO mouse (p < 0.05), 66.5 ± 10.1% in the GluRe3-KO mouse (p < 0.05), and 41.4 ± 0.4% in the GluRe1/ε3-KO mouse [p < 0.01, compared with wild-type GluRζ1-C2 (100 ± 3.2%)], and GluRζ1-C2’ was reduced to 73.1 ± 6.4% in the GluRe1-KO mouse, 69.1 ± 8.6% in the GluRe3-KO mice, and 53.0 ± 5.6% in the GluRe1/ε3-KO mice [p < 0.05, compared with wild-type GluRζ1-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 GluRe1/ε3-KO mouse for GluRζ1-C2 (60.1 ± 7.4%; p < 0.05) and GluRζ1-C2’ (64.1 ± 2.7%; p < 0.05, compared with wild-type levels (100 ± 7.4% and 100 ± 12.5%, respectively) (Fig. 6 C). Because no significant changes were observed for resident ER protein calreticulin on the same blotted membrane (Fig. 6 C), this reduction was judged to be specific to the GluRζ1 subunit.

Then, the synaptic GluRζ1 subunit was assessed by comparative examination using the cerebellar synaptosomal and PSD fractions (Fig. 6 D, 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. 6 D). In the synaptosomal fraction, GluRζ1-C2 was reduced to 62.4 ± 7.6% in the GluRe1-KO mouse, 71.5 ± 12.6% in the GluRe3-KO mouse, and 34.2 ± 5.6% in the GluRe1/ε3-KO mouse [p < 0.05, compared with the wild-type synaptosomal fraction (100 ± 23.0%)]. Reduction was more pronounced in the PSD fraction: the GluRζ1-C2 level was 46.6 ± 10.5% in the GluRe1-KO mouse, 35.0 ± 17.7% in the GluRe3-KO mouse, and 12.5 ± 3.3% in the GluRe1/ε3-KO mouse [p < 0.05, compared with the wild-type PSD fraction (100 ± 23.3%)]. Similar results were obtained for GluRζ1-C2’ (Fig. 6 D). In the synaptosomal fraction, GluRζ1-C2’ was reduced to 47.9 ± 12.8% in the GluRe1-KO mouse (p < 0.05, compared with the wild-type fraction (100 ± 15.7%)).
the wild-type synaptosomal fraction (100 ± 10.5%). In the PSD fraction, the level of GluR1-C2’ was 46.2 ± 20.6% in the GluR1-KO mouse, 25.1 ± 5.3% in the GluRe3-KO mouse, and 17.0 ± 4.6% in the GluRe1/e3-KO mouse [p < 0.05, compared with the wild-type PSD fraction (100 ± 24.4%)]. Thus, synaptic GluR1-C2 and GluR1-C2’ were progressively reduced according to GluRe subunit ablation, with the most severe deficit in the PSD fraction.

GluR1–C1
The fact that GluR1-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). GluR1-C1 in the PSD fraction was also reduced severely in the mutant cerebella: 55.2 ± 4.3% in the GluRe1-KO mouse (p < 0.05), 36.6 ± 5.7% in the GluRe3-KO mouse (p < 0.05), and 12.7 ± 3.5% in the GluRe1/e3-KO mouse [p < 0.01, compared with the wild-type PSD fraction (100 ± 14.5%)]. Thus, GluR1-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 GluRe1 subunit in the GluRe3-KO mouse [67.8 ± 7.5%; p < 0.05, compared with the wild-type homogenate (100 ± 4.9%)] and for the GluRe3 subunit in the GluRe1-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 GluRe2 subunit is highly expressed in immature granule cells during the early postnatal period (Watanabe et al., 1992), we examined whether compensatory changes occurred for GluRe2 subunit expression in the mutant cerebellum at 1 month of age (Fig. 6F). In the wild-type mouse, the GluRe2 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 GluRe2 subunit between the wild-type (100.0 ± 20.6%) and GluRe1/e3-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 GluRe1-KO mouse, because the GluRe1 subunit is the major subunit at 7–8 d in vitro with the GluRe3 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 GluRe1-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 GluRe1-KO cultures (Fig. 7B), 100.0 ± 8.5% in wild-type cultures (n = 3) and 61.2 ± 15.5% in GluRe1-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) (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 a 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 GluRe 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 ± 9.9% 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 synaptoptysin (103.5 ± 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 GluRe 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 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 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. 8). No significant difference was seen in the percentage relative to the levels in vehicle-injected mice (n = 3; 100 PSDs) and 248 ± 8 mm2 in the GluR1/e3-KO mouse (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. 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

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**Figure 5.** Postembedding immunogold for GluR1-C2 (A, C, E) and GluR1/C2’ (B, D, F) at mossy fiber–granule cell synapses. Note that postynaptic 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.
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 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 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 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.

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 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 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-

...
injection, the total GluR subunit concentration was severely reduced in the GluR1/C2 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 (McIlnnhy 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–proteosomal degradation in the cytoplasm (Johnston et al., 1998; Fukaya et al., 2003).

### C-terminal-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 Seeberg, 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/C2/C3-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-terminal-dependent self-ER export mechanisms. When transfected to heterologous cells in vitro, the GluR1 subunit ended with a C1–C2 tail (i.e.,

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

<table>
<thead>
<tr>
<th>Protein</th>
<th>Genotype</th>
<th>Homogenate</th>
<th>Synaptosome</th>
<th>PSD fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>GluR1-pan</td>
<td>Wild type</td>
<td>100.0 ± 8.4</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>e1-kO</td>
<td>74.0 ± 15.8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>e3-KO</td>
<td>69.6 ± 13.2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>e1/e3-KO</td>
<td>60.3 ± 10.2*</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>GluR1-C2</td>
<td>Wild type</td>
<td>100.0 ± 3.2</td>
<td>100 ± 23.0</td>
<td>100 ± 23.3</td>
</tr>
<tr>
<td></td>
<td>e1-kO</td>
<td>70.2 ± 7.1*</td>
<td>62.4 ± 7.6</td>
<td>46.6 ± 10.5</td>
</tr>
<tr>
<td></td>
<td>e3-KO</td>
<td>66.5 ± 10.1*</td>
<td>71.5 ± 12.6</td>
<td>35.0 ± 17.7</td>
</tr>
<tr>
<td></td>
<td>e1/e3-KO</td>
<td>41.4 ± 0.4**</td>
<td>34.2 ± 5.6*</td>
<td>12.3 ± 3.3*</td>
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<tr>
<td>GluR1-C2’</td>
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<td>100.0 ± 9.6</td>
<td>100 ± 10.5</td>
<td>100 ± 24.4</td>
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<tr>
<td></td>
<td>e1-kO</td>
<td>73.1 ± 6.4</td>
<td>47.9 ± 12.8*</td>
<td>46.2 ± 20.6</td>
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<tr>
<td></td>
<td>e3-KO</td>
<td>69.1 ± 8.6</td>
<td>60.5 ± 17.2</td>
<td>25.1 ± 5.3</td>
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<tr>
<td></td>
<td>e1/e3-KO</td>
<td>53.0 ± 5.6*</td>
<td>26.3 ± 4.3**</td>
<td>17.0 ± 4.6*</td>
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<tr>
<td>GluR1-C1</td>
<td>Wild type</td>
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<td>100 ± 14.5</td>
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<tr>
<td></td>
<td>e1-kO</td>
<td>ND</td>
<td>55.2 ± 4.3*</td>
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</tr>
<tr>
<td></td>
<td>e1/e3-KO</td>
<td>ND</td>
<td>12.7 ± 3.5**</td>
<td>—</td>
</tr>
</tbody>
</table>

Each value was calculated from the signals of immunoblot bands and is relative to the wild-type cerebellum in each fraction. All data are given as the mean ± SEM for three experiments, except for GluR1-C2’ proteins of the wild-type mouse (n = 5) and the GluR1-C1 protein of the wild-type PSD fraction (n = 4). Genotypic differences relative to wild-type cerebella in each fraction were statistically determined by Student’s t test (*p < 0.05; **p < 0.01). Not tested; ND, not detectable.
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 RXR residing in the C1 cassette (Stanley 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{zeta}1–C2' than GluR{zeta}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 GluRe subunits are lacking. Considering the fact that most central neurons coexpress GluR{zeta}1 and one or more GluRe subunits (Watanabe et al., 1993, 1994), the C terminus-dependent self-ER export mechanisms for GluR{zeta}1 subunit would be suppressed or masked by the strong action of GluRe-dependent cell surface delivery and synaptic localization for the GluR{zeta}1 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 GluR{gamma}1 subunit, determines the total number of functional NMDA receptors in cultured cerebellar granule cells (Przybyslawski 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{zeta}1 and GluRe subunits in a different but cooperative manner. In the absence of GluRe subunits, the GluR{zeta}1 subunit is unable to be localized on the postsynapse, regardless of its C-terminal forms. Without the GluR{gamma}1 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.

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