Cellular/Molecular

Membrane-Proximal Region of Glutamate Receptor δ2 Subunit Is Critical for Long-Term Depression and Interaction with Protein Interacting with C Kinase 1 in a Cerebellar Purkinje Neuron

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The glutamate receptor δ2 subunit (GluRδ2) is selectively expressed in cerebellar Purkinje neurons (PNs) and is involved in the long-term depression (LTD). However, little is known about the mechanism of its action. Acute expression of the wild-type GluRδ2 in the GluRδ2-deficient PN rescued the induction of LTD, suggesting the direct role of GluRδ2 in LTD. To identify the critical region of GluRδ2 necessary for LTD, we constructed and expressed various mutant GluRδ2 proteins in the GluRδ2-deficient PNs. The mutant GluRδ2 possessing the membrane-proximal 21 aa residues in the C-terminal cytoplasmic region rescued the induction of LTD, whereas the mutant with membrane-proximal 13 aa failed. In addition, overexpression of 865∼871 aa of GluRδ2 (corresponding to membrane-proximal 14–20 aa) fused to EGFP (enhanced green fluorescent protein) suppressed LTD in a wild-type PN. These results suggest that 865∼871 aa of GluRδ2 play an essential role in LTD. We next identified protein interacting with C kinase 1 (PICK1) as a molecule interacting with the membrane-proximal C-terminal region of GluRδ2 by yeast two-hybrid screening. PICK1 plays an essential role in LTD. It colocalized with GluRδ2 at spines of PNs, and immunoprecipitation assays showed that GluRδ2 bound to PICK1 mainly through 865–871 aa. These results indicate that 865–871 aa of GluRδ2 are essential for both LTD and interaction with PICK1, and suggest that interaction between GluRδ2 and PICK1 might be critical for the induction of LTD.

Key words: **Purkinje neuron; cerebellum; LTD; glutamate receptor; PICK1; parallel fiber**

Introduction

At most excitatory synapses in the mammalian CNS, fast neurotransmission is mediated by glutamate, which activates postsynaptic ionotropic glutamate receptor channels (GluRs) (Hollmann and Heinemann, 1994). GluRs have been classified into three major groups by pharmacological and electrophysiological properties. They are AMPA, kainate, and NMDA receptors. On the other hand, 18 GluR subunit genes have been cloned, which are classified into seven subfamilies based on the amino acid sequence homology. Among them, the δ subfamily comprised of GluRδ1 and GluRδ2 has not been related to any of AMPA, kainate, or NMDA receptors (Yamazaki et al., 1992; Araki et al., 1993; Lomeli et al., 1993). GluRδ2 is selectively expressed in a cerebellar Purkinje neuron (PN), and highly concentrated at the postsynaptic density (PSD) of parallel fiber–PN synapses, but not found at the mature climbing fiber–PN synapses (Takayama et al., 1996; Landsend et al., 1997). The functions of GluRδ2 have been elusive. The sequence similarity to other GluR subunits suggests that GluRδ2 is likely to be a GluR subunit, and GluRδ2 coimmunoprecipitated with other GluR subunits such as GluR1 and GluR6 in heterologous expression studies using human embryonic kidney 293 (HEK293) cells (Kohda et al., 2003). It is also known that the GluRδ2 with *lurcher* mutation forms a constitutively open ion channel in a PN (Zuo et al., 1997). However, so far neither glutamate binding to GluRδ2 (Araki et al., 1993; Lomeli et al., 1993) nor incorporation of GluRδ2 into any native GluRs in neurons has been demonstrated.

The first clue of GluRδ2 function was obtained in cultured PNs treated with the antisense oligonucleotides, which impaired the cerebellar long-term depression (LTD) (Hirano et al., 1994; Jeromin et al., 1996). The LTD is a type of synaptic plasticity occurring at parallel fiber–PN synapses. It is induced by conjunctive activation of parallel fibers and a climbing fiber, and has been considered as a cellular basis for motor learning (Ito, 2001). Depolarization, metabotropic glutamate receptor 1 (mGluR1) and AMPA receptor activation, and following protein kinase C (PKC) activation in a PN are required for the induction of LTD (Crepel and Krupa, 1988; Linden and Connor, 1991; Shigemoto et al.,

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1994; De Zeeuw et al., 1998). Phosphorylation of the GluR2 Ser880 residue by PKC suppresses interaction between GluR2 and glutamate receptor interacting protein (GRIP), resulting in release of GluR2 containing AMPA receptor from GRIP. GluR2 then binds to protein interacting with C kinase 1 (PICK1) and is endocytosed (Matsuda et al., 1999, 2000; Chung et al., 2000; Xia et al., 2000). The above is the current scheme for the induction of LTD. We wanted to find the role of GluRδ2 in the molecular cascade. Here, we show that GluRδ2 is directly implicated (not through developmental alteration of a PN) in LTD, and demonstrate that the membrane-proximal C-terminal region is essential for both the induction of LTD and binding to PICK1.

Materials and Methods

Culture. Methods of preparing primary culture of cerebellar neurons from wild-type and GluRδ2 knock-out mouse (provided by M. Mishina, Tokyo University, Tokyo, Japan) were similar to those described previously (Hirano and Kasono, 1993). Briefly, cerebella were dissected out from newborn pups, and their meninges were removed. The cerebella were incubated in Ca^{2+}- and Mg^{2+}-free HBSS containing 0.1% trypsin and 0.01% DNase for 15 min at 37°C. Neurons were dissociated by trituration and cultured on poly-D-lysine-coated coverslips in a basal medium Eagle-based medium containing 3–5% horse serum for 48 h and then for ~3 weeks in a medium without serum. One-half of the culture medium was exchanged every 4 d.

Immunocytochemistry. Cultured neurons were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature (20–25°C). After permeabilization in 0.5% Tween 20 in PBS, samples were processed for immunofluorescent staining. Primary and secondary antibodies used for staining were as follows: mouse monoclonal anti-calbindin-28 (Swant, Bellinzona, Switzerland), rabbit polyclonal anti-hemagglutinin (HA) epitope (Upstate Biotechnology, Lake Placid, NY), chick polyclonal anti-green fluorescent protein (GFP) (Chemicon, Temecula, CA), rabbit polyclonal anti-GluRδ2 (provided by M. Watanabe, Hokkaido University, Hokkaido, Japan), and Alexa 488- or Alexa 568-conjugated goat anti-rabbit, anti-mouse, or anti-chick secondary antibodies (Invitrogen, Eugene, OR). After washing, the coverslip was mounted with glycerol-based medium AntiFade (Invitrogen) and observed with a confocal microscope (CSU 10; Yokogawa Electric Corporation, Musashino, Japan) equipped on Eclipse E800 (Nikon, Tokyo, Japan).

For cell surface staining of GluRδ2 or its mutants, cultured neurons were treated with the anti-HA epitope antibody without permeabilization. Then, cells were permeabilized and treated with the anti-GFP antibody.

Electrophysiology. Whole-cell patch-clamp recording from a PN grown in culture for 3 weeks was performed in the solution containing the following (in mM): 145 NaCl, 5 KOH, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose, pH 7.3, at room temperature. It also contained 1 μM tetrodotoxin (Tocris, Bristol, UK) to suppress action potential and 20 μM bicuculline (Tocris) to suppress GABAergic IPSCs. A PN was identified as described previously (Hirano and Kasono, 1993). Patch pipettes were filled with the internal solution containing the following (in mM): 150 CsCl, 15 CsOH, 0.5 EGTA, 10 HEPES, 2 Mg-ATP (Sigma, St. Louis, MO) and 0.2 Na-GTP (Sigma), pH 7.3. The electrode resistance was 3–5 MΩ. The membrane potential of a PN was held at −70 mV unless otherwise stated. Only recording with an input resistance of >100 MΩ and series resistance of <30 MΩ was accepted, and an experiment was terminated when a change of >20% was detected. The recording was performed with an EPC-9 amplifier (HEKA, Lambrecht, Germany), and the current was filtered at 2.9 KHz. The junction potential was offset. The method for iontophoretic application of glutamate was similar to that of previous studies (Linden et al., 1991; Hirano et al., 1994). A glass pipette containing 10 mM glutamate and 10 mM HEPES, pH 7.3, was aimed at the primary or secondary dendrites of a PN. 3,5-Dihydroxyphenylglycine (DHPG) was applied from a pipette containing 2 mM DHPG and 10 mM HEPES, pH 6.5. The voltage-dependent calcium current was recorded by depolarizing a PN to −30 mV for 80 ms. The series resistance compensation was optimized. SKEDDKE or DKESDKE (control) peptide (100 μM; Invitrogen) was added to the internal solution in some experiments.

Expression vector. Expression vectors of HA-GluRδ2 and mutants were constructed as follows. The fragment containing signal peptide and HA epitope tag of pDisplay (Invitrogen) was ligated into pCXN2 (Niwa et al., 1991), and the full-length and mutant GluRδ2 [C(50–151)del, C(5–48)del, and SKEDDKE-del] cDNAs except for the signal sequence were ligated into the vector after the HA tag. The GluRδ2 cDNA was provided by M. Mishina. GluRδ2 mutants (C21 and C13) were cloned by PCR and ligated into pCAGplay (Kawaguchi and Hirano, 2006). SKEDDKE-EGFP was generated by annealing two complementary oligonucleotides and ligating into pEGFP-N1 (Clontech, Palo Alto, CA). PICK1 mutants were cloned by PCR and ligated into pEGFP-N1.

Microinjection of cDNAs. The plasmid DNA of interest (0.034 mg/ml) together with EGFP cDNA (pEGFP-N1; 0.017 mg/ml; Clontech) was microinjected into a nucleus of PN through a glass capillary (GD-1; Narishige, Tokyo, Japan) with positive pressure (Transjector 5246; Eppendorf, Hamburg, Germany) using a micromanipulator. After incubating for 16–48 h in the culture medium, injected PNs were identified by the enhanced green fluorescent protein (EGFP) fluorescence and used for electrophysiological or immunocytochemical experiments.

Yeast two-hybrid screening assay. Yeast two-hybrid assay was performed using the ProQuest Two-Hybrid System (Invitrogen), in which interaction of a bait fusion protein with an expressed protein results in GAL4-dependent transcription activation of *HIS3*, *URA3*, and *LacZ* reporter genes. The bait sequence (852–907 aa of GluRδ2) was fused to the yeast GAL4 DNA-binding domain in pDBLue and transfected into MaV203 yeast cells. Then, a mouse brain cDNA library comprising cDNAs fused to the GAL4 activation domain in pPC86 was subsequently introduced into these cells. Positive clones were first detected for the ability to grow on the plate lacking leucine, tryptophan, and histidine. They were selected further by their ability to grow on the plate lacking uracil, followed by the β-galactosidase assay. Plasmids were isolated from the yeast cells that were positive in all three assays, and the DNA sequence was determined.

Immunoprecipitation. Transfected HEK293T cells were suspended in NP-40 buffer [150 mM NaCl, 5 mM EDTA, 10 mM Tris, 1% (v/v) Nonidet P-40, and the protease inhibitor mixture (Nacalai Tesque, Kyoto, Japan), pH 7.5]. The insoluble fraction was removed by centrifugation at 15,000 rpm for 20 min, and the supernatant was preincubated with the precleaned protein A-Sepharose (Amersham Biosciences, Uppsala, Sweden) for 1 h at 4°C. After centrifugation, the supernatant was incubated with 2 μg of the anti-HA antibody (Upstate Biotechnology), the anti-GFP antibody (Invitrogen), the anti-PICK1 antibody (Affinity BioReagents, Golden, CO), the anti-GluRδ2 antibody, or preimmune IgG for 1 h at 4°C, and then incubated with protein A-Sepharose for 1 h at 4°C. After washing three to six times with NP-40 buffer, the immunoprecipitated fraction was boiled for 5 min in Laemmli sample buffer and subjected to Western blot analysis.

For immunoprecipitation assay using the mouse brain lysate, adult brains were homogenized in the ice-cold homogenization buffer [150 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X, 1% (v/v) deoxycholate, 10 mM Tris, pH 7.5, and the protease inhibitor mixture] and incubated for 1 h at 4°C. The insoluble fraction was removed by centrifugation, and the sample was then dialyzed with NP-40 buffer overnight and centrifuged again. The supernatant was then immunoprecipitated with the anti-PICK1 (Affinity BioReagents) or the anti-GluRδ2 antibody. Western blot of PICK1 after immunoprecipitation with the anti-GluRδ2 antibody was performed using the concentration gradient gel (2–15%; PAG mini DAIICHI; Daiichi Pure Chemicals, Tokyo, Japan). Primary and secondary antibodies used for Western blot were as follows: rabbit polyclonal anti-HA epitope antibody, mouse monoclonal anti-GFP antibody (Nacalai Tesque), rabbit polyclonal anti-GluRδ2 antibody, goat polyclonal anti-PICK1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and HRP-conjugated goat anti-rabbit, anti-mouse, or anti-goat IgG antibodies (Chemicon).

Results

GluRδ2 expression rescued LTD in GluRδ2-deficient PNs

Previous studies showed that GluRδ2 is necessary for LTD (Hirano et al., 1994, 1995; Kashiwabuchi et al., 1995; Jeromin et al., 1996). However, the impairment of LTD might be indirectly caused by the GluRδ2 deficiency through developmental alter-

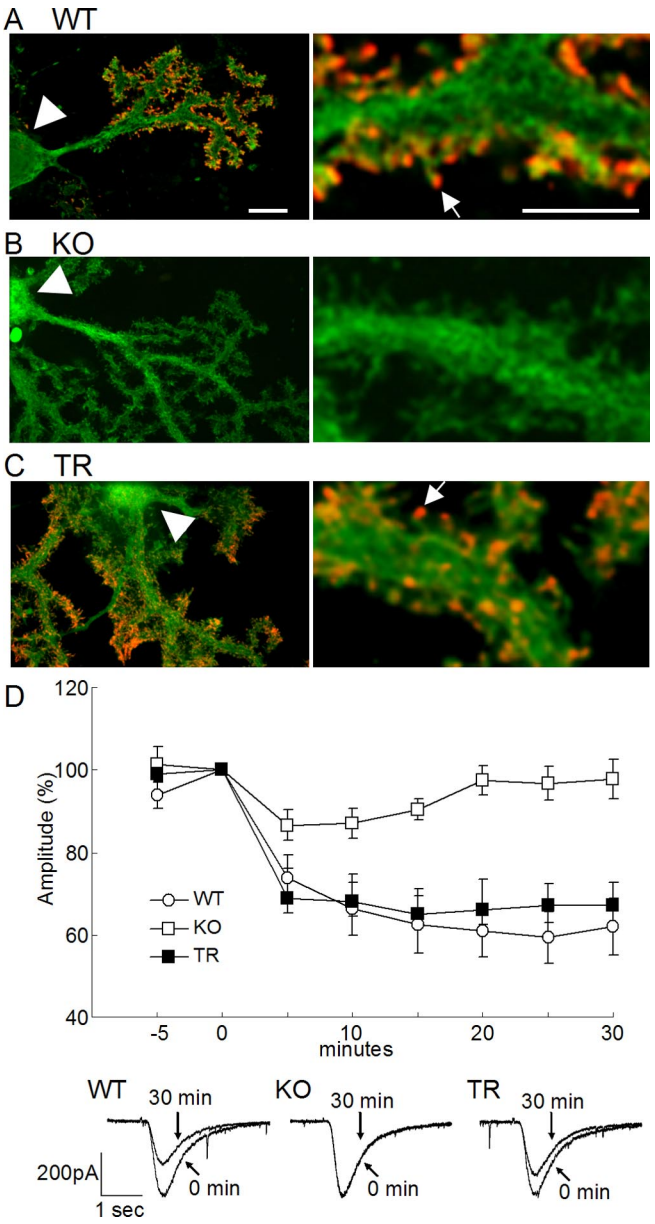

Figure 1. Rescue of LTD by overexpression of GluRδ2 in the GluRδ2-deficient PN. ***A–C***, Localization of endogenous GluRδ2 (***A***; red) and transfected GluRδ2 (***C***; red) are shown with calbindin signal (green) in PN dendrites. Both endogenous and transfected GluRδ2 are mainly localized at spines (arrows), although the transfected GluRδ2 signal was stronger than the endogenous one in most cells. The arrowheads indicate cell bodies. Scale bars: left, 10 μm; right, 5 μm. ***D***, LTD of glutamate responsiveness was not induced in PNs prepared from GluRδ2-deficient mice (KO). Acute expression of GluRδ2 in the GluRδ2-deficient PN rescued LTD (TR). LTD was induced by glutamate application coupled with depolarization of the PN at 0 min. Error bars indicate SEM. Representative traces of glutamate-induced currents before (0 min) and 30 min after the conjunctive conditioning were presented at the bottom.

ation of PN conditions such as the change in basal level of a second messenger molecule. To examine whether GluRδ2 is directly implicated in the induction of LTD, we transfected the expression construct encoding wild-type GluRδ2 by microinjection into the nucleus of GluRδ2-deficient cultured PN. The construct encoding EGFP was coinjected to identify the transfected PNs. The overexpressed GluRδ2 protein was found at dendritic spines as endogenous GluRδ2 (Fig. 1*A–C*) and rescued LTD 16–48 h after the microinjection (Fig. 1*D*). Here, LTD was induced by conjunction of direct depolarization of a PN (0 mV for 3 s; 0.05 Hz; nine times) with brief application of glutamate to primary or secondary dendrites of the PN, and LTD was monitored with the glutamate responsiveness (Linden et al., 1991; Shigemoto et al., 1994). The amplitude of glutamate response became 62.0 ± 6.9% [wild type (WT); $n = 10$], 97.8 ± 4.8% [knockout (KO); $n = 8$], and 67.0 ± 5.6% [transfected (TR); $n = 6$] 30 min after the conjunctive conditioning ($p < 0.05$ between KO and WT or TR; Student's t test). Transfection of GluRδ2 did not affect the input resistance, holding current, AMPA receptor responsiveness reflected in miniature EPSC (mEPSC) properties, mGluR responsiveness, and amplitude of voltage-dependent calcium current (Table 1). The latter three are known to be required to induce LTD. These results suggest that GluRδ2 is involved in the intracellular signal transduction for the induction of LTD.

Membrane-proximal region of GluRδ2 is essential for LTD

Next, we tried to identify the region of GluRδ2 essential for the induction of LTD. We constructed various deletion mutants of GluRδ2 and expressed them in the GluRδ2-deficient PNs (Fig. 2*A*). We focused on the cytoplasmic C-terminal region, because we thought that interaction of GluRδ2 with some cytoplasmic proteins might be essential and that several molecules have been identified to bind to that region (Hirai and Matsuda, 1999; Roche et al., 1999; Hironaka et al., 2000; Ly et al., 2002; Miyagi et al., 2002; Yue et al., 2002; Yap et al., 2003a,b; Uemura et al., 2004). All truncated mutant GluRδ2 proteins including those lacking the PSD-95/Discs large/zona occludens-1 (PDZ)-binding motif in the C-terminal tail were transported to cell surface and mainly localized in dendritic spines as wild-type GluRδ2 (Fig. 2*D*). Despite the importance of PDZ-binding motif in interaction with several postsynaptic molecules, our results suggest that PDZ interaction of GluRδ2 is dispensable for localization in dendritic spines. Transfection of the deletion mutant lacking C-terminal 50–151 aa [C(50–151)del] rescued LTD (69.3 ± 4.7%; $p < 0.05$ compared with KO; $n = 5$). In contrast, transfection of the deletion mutant lacking most of C-terminal cytoplasmic region except for membrane-proximal 5 aa (C5) and the mutant lacking C-terminal 5–48 aa [C(5–48)del] failed to rescue LTD (83.7 ± 5.0%, $n = 5$; 92.9 ± 6.2%, $n = 6$, respectively) (Fig. 2*B*). These results indicate that C(5–48) of GluRδ2 is essential for LTD. To further define the critical region for LTD, we next examined the GluRδ2 mutants lacking most of the C terminus except for the membrane-proximal 21 aa (C21) or 13 aa (C13). Transfection of C21 rescued LTD (69.3 ± 6.4%; $n = 5$; $p < 0.05$, compared with KO), whereas C13 failed (88.0 ± 2.3%; $n = 6$) (Fig. 2*C*), suggesting that the region including C(14–21) of GluRδ2 plays a critical role in the induction of LTD. Transfection of neither C21 nor C13 affected the basal properties of PNs (Table 1). One possibility is that the C(14–21) sequence (SKEDDKEI) of GluRδ2 binds to an intracellular signaling molecule implicated in LTD. To test this, PNs were transfected with the EGFP-conjugated C(14–20) peptide (SKEDDKE-EGFP) which might interfere binding of GluRδ2 with the postulated signaling molecule (Fig. 3*A*). Nonpolar isoleucine at C21 was not included in the peptide. The transfection suppressed LTD (102.2 ± 8.4%; $n = 7$), whereas that of EGFP did not (62.8 ± 7%; $n = 6$; $p < 0.05$) (Fig. 3*B*). The transfection did not affect the basal properties of PNs (Table 1). Furthermore, intracellular application of SKEDDKE peptide (100 μM) suppressed LTD (84.8 ± 5.5%; $n = 7$; $p < 0.05$), whereas application of control peptide (DKESDKE) did not (68.4 ± 3.8%; $n = 7$) (Fig. 3*C*). These results suggest that GluRδ2 might interact with a certain molecule through C(14–20), which might be essential for the induction of LTD.

Table 1. Effects of transfection on the basal properties of PNs

			mEPSC			
	Ca^{2+} current (nA)	DHPG (%)	Amplitude (pA)	Frequency (Hz)	Rise time (ms)	Half-width (ms)
WT	5.7 ± 3.3 (13)	100 ± 42 (24)	17 ± 6	5.9 ± 2.5	2.6 ± 0.8	7.8 ± 2.3 (7)
EGFP	5.7 ± 1.4 (11)	110 ± 43 (10)	16 ± 5	7.5 ± 6.5	2.4 ± 0.7	7.2 ± 2.0 (10)
SKEDDKE-EGFP	5.9 ± 3.0 (11)	106 ± 33 (10)	20 ± 6	6.8 ± 7.4	2.5 ± 0.6	7.9 ± 1.4 (9)
SKEDDKE peptide	4.9 ± 2.4 (11)	95 ± 32 (9)	16 ± 5	10.0 ± 3.7	2.2 ± 0.3	8.0 ± 1.1 (8)
Control peptide	5.3 ± 2.5 (10)	88 ± 37 (8)	19 ± 4	8.5 ± 5.0	2.3 ± 0.6	7.4 ± 1.4 (10)
KO	5.7 ± 2.8 (11)	100 ± 33 (40)	21 ± 4	12.6 ± 12.1	2.1 ± 0.5	7.6 ± 1.6 (9)
δ2	6.3 ± 3.0 (11)	89 ± 20 (9)	18 ± 6	7.8 ± 5.0	2.4 ± 0.6	8.0 ± 1.2 (10)
C21	5.3 ± 3.4 (11)	112 ± 30 (7)	16 ± 5	11.8 ± 9.6	2.5 ± 0.4	8.0 ± 1.8 (10)
C13	5.3 ± 3.7 (11)	87 ± 23 (9)	19 ± 9	9.8 ± 7.6	2.1 ± 0.6	6.8 ± 1.2 (10)

The peak voltage-dependent calcium currents were measured during 80 ms depolarization of a PN to −30 mV. DHPG responses were measured by iontophoretic application of DHPG to primary or secondary dendrites. The amplitudes of DHPG-induced currents in the transfected PNs were compared with the average amplitude of 8–10 nontransfected PNs in the same culture. The amplitude, frequency, 10–90% rise time, and half-height width of mEPSCs are presented. *N* is presented in the parentheses.

PICK1 interacts with GluRδ2

To identify proteins interacting with the membrane-proximal region of GluRδ2 C terminus, we performed yeast two-hybrid screening using GluRδ2 C(1–56) as bait. After screening of over 10^6 clones in a mouse brain cDNA library, six candidates were obtained. Among them, three were encoding PICK1, which is a PSD protein originally isolated as a PKCα binding protein (Staudinger et al., 1995). It has a PDZ domain, a Bin/Amphiphysin/Rvs (BAR) domain containing a coiled-coil region, and an acidic region (Staudinger et al., 1997; Xia et al., 1999; Boudin and Craig, 2001; Peter et al., 2004).

To examine interaction between GluRδ2 and PICK1 further, we stained the wild-type PNs expressing PICK1 fused with EGFP (PICK1-EGFP) with the anti-GluRδ2 antibody and the anti-GFP antibody. As shown in Figure 4*A*, PICK1-EGFP colocalized with GluRδ2 at dendritic spines of PNs. Next, we performed immunoprecipitation experiments. We expressed PICK1-EGFP fusion protein together with HA-GluRδ2 in HEK293T cells. The anti-GFP antibody coimmunoprecipitated HA-GluRδ2, and the anti-HA antibody coimmunoprecipitated PICK1-EGFP in the cell lysates (Fig. 4*B*,*C*). Next, to examine the *in vivo* interaction, a coimmunoprecipitation experiment was performed using the cell lysate prepared from the mouse cerebellum. GluRδ2 was coimmunoprecipitated by the anti-PICK1 antibody, and PICK1 was coimmunoprecipitated by the anti-GluRδ2 antibody (Fig. 4*D*,*E*). These results indicate that PICK1 binds to GluRδ2 both in HEK293T cells and in the cerebellum.

Interacting regions of GluRδ2 and PICK1

To identify the interacting region of GluRδ2 with PICK1, we expressed PICK1 and the various C-terminal regions of GluRδ2 fused to enhanced yellow fluorescent protein (EYFP) in HEK293T cells, and performed coimmunoprecipitation experiments using the anti-PICK1 antibody. The fusion protein containing the 20 aa in GluRδ2 membrane-proximal C terminus [C(1–20)] bound to PICK1; however, that containing C(1–18) failed (Fig. 5*A*). Next, we performed coimmunoprecipitation experiments with the mutant GluRδ2 lacking C(14–20) (SKEDDKE-del). Little PICK-EGFP was immunoprecipitated with the anti-HA antibody recognizing HA-SEKDDKE-del (Fig. 5*B*). We also examined interaction between SKEDDKE-EGFP and PICK1. SKEDDKE-EGFP was immunoprecipitated with the anti-PICK1 antibody (Fig. 5*C*). Next, we examined whether SKEDDKE-EGFP interferes binding of GluRδ2 to PICK1. PICK1-EGFP and HA-GluRδ2 were cotransfected with either SKEDDKE-EGFP or EGFP in HEK293T, and the cell lysate was immunoprecipitated with the anti-HA antibody and immunoblotted with the anti-GFP antibody. Expression of SKEDDKE-EGFP decreased the amount of PICK1-EGFP in the precipitate (46.3 ± 15.6%; $n = 5$; $p < 0.01$; Mann–Whitney *U* test) (Fig. 5*D*). These results suggest that GluRδ2 bound to PICK1 mainly through C(14–20) (SKEDDKE). Thus, the membrane-proximal region C(14–20) is necessary for both LTD and interaction with PICK1.

Next, we tried to identify the region of PICK1 essential for interaction with GluRδ2. We constructed numbers of PICK1 deletion mutants (Fig. 6*A*) and performed coimmunoprecipitation experiments. As shown in Figure 6*B*, 245–278 aa of PICK1 fused to EGFP [PICK(245–278)] was clearly immunoprecipitated by the anti-HA antibody recognizing HA-GluRδ2, whereas PICK(1–120) and PICK(274–416) were only slightly immunoprecipitated. Thus, the major interacting region of PICK1 with GluRδ2 seems to be located in 245–278 aa, although involvement of other regions cannot be excluded. Then, we examined whether PICK(245–278) interferes with interaction between GluRδ2 and PICK1. Coexpression of GluRδ2, PICK1, and PICK(245–278) did not decrease PICK1 precipitates by the anti-HA antibody, but rather increased (data not shown). Thus, PICK(245–278) could not be used as a molecular tool interfering with interaction between PICK1 and GluRδ2. The 245–278 aa of PICK1 might be involved in multimerization of PICK1 (Peter et al., 2004; Lu and Ziff, 2005). Together, it is suggested that GluRδ2 binds to PICK1 mainly through interaction of GluRδ2 C(14–20) and PICK1(245–278).

Discussion

The LTD is not induced in a PN in which GluRδ2 is knocked out or down (Hirano et al., 1994, 1995; Kashiwabuchi et al., 1995; Jeromin et al., 1996). Transgenic expression of GluRδ2 in the GluRδ2-deficient PN rescues the induction of LTD (Hirai et al., 2005), supporting involvement of GluRδ2 in LTD. However, the possibility that impairment of LTD in the GluRδ2-deficient PNs might be caused by developmental alteration of PN properties can not be excluded. To examine this issue, we expressed GluRδ2 acutely in the GluRδ2-deficient PN by microinjection of cDNA into the nucleus and examined LTD. We showed that the transfected GluRδ2 was located at dendritic spines as endogenous GluRδ2, and rescued LTD within 16 h. These results support the idea that GluRδ2 is directly involved in the induction of LTD.

Here, we identified the critical region of GluRδ2 required for LTD by expressing various GluRδ2 mutants in the GluRδ2-deficient PNs. We focused on the C-terminal cytoplasmic region, because most GluR subunits have a PDZ binding motif at the extreme C terminus to which numbers of PSD proteins bind

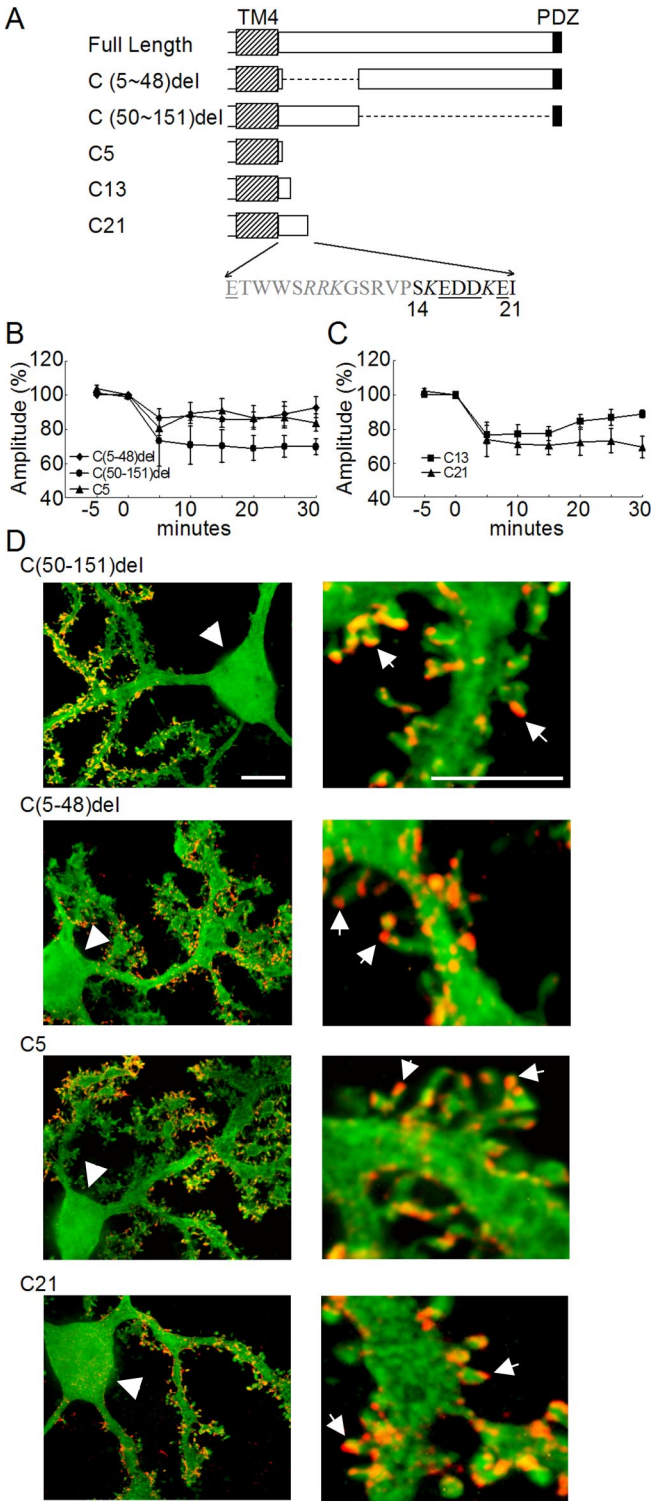

Figure 2. The membrane-proximal C-terminal residues of GluRδ2 are essential for LTD. ***A***, GluRδ2 mutant constructs with deletions in the C terminus. All of these proteins have HA tag in the amino terminus. The residue number is counted from the end of last transmembrane segment (TM4). PDZ-binding motif (PDZ) is located at the extreme C terminus. Acidic amino acids in C(14–21) are underlined, and basic amino acids are written in italic characters. ***B***, ***C***, LTD in the PNs transfected with one of GluRδ2 mutant cDNAs. Error bars indicate SEM. ***D***, Cell surface staining of the transfected mutant GluRδ2 proteins. The anti-HA antibody (red) recognized the mutant GluRδ2 proteins on the cell surface. Intracellular EGFP (green) was also stained. The arrowheads indicate cell bodies, and the arrows indicate HA-GluRδ2 signals on dendritic spines. Two serial images focused on different planes are superimposed. Scale bars: left, 10 μm; right, 5 μm.

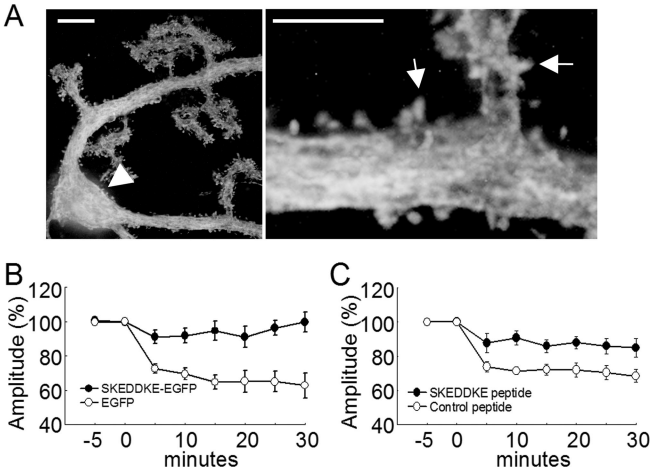

Figure 3. ***A***, Localization of the SKEDDKE-EGFP, which was immunostained with the anti-GFP antibody. SKEDDKE-EGFP was distributed throughout the cytoplasm including dendritic spines in a PN. The arrowhead indicates a cell body, and the arrows indicate dendritic spines. Three serial images focused on different planes are superimposed. Scale bars: left, 10 μm; right, 5 μm. ***B***, LTD in the wild-type PNs transfected with either EGFP or SKEDDKE-EGFP. SKEDDKE-EGFP expression suppressed LTD. ***C***, Impairment of LTD in the wild-type PNs perfused with SKEDDKE peptide. Error bars indicate SEM.

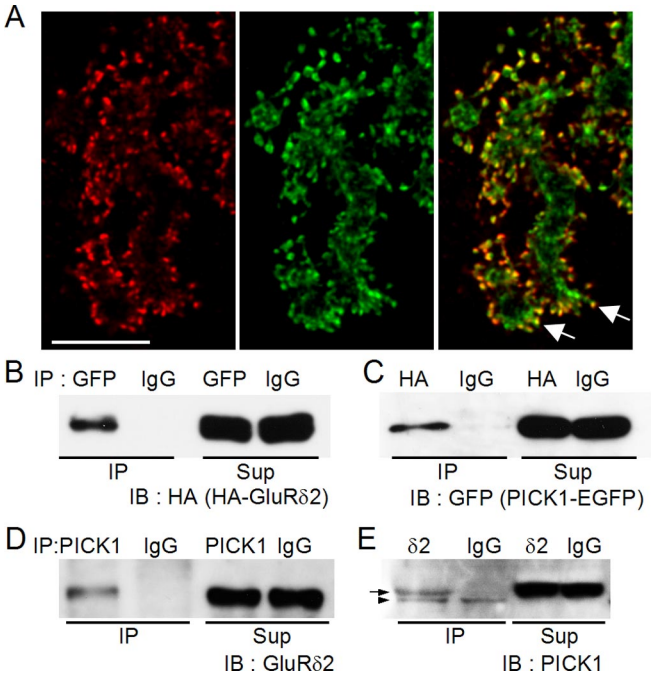

Figure 4. Interaction of GluRδ2 with PICK1. ***A***, Localization of PICK1. The PICK1-EGFP signal (green) and the endogenous GluRδ2 signal (red) are shown. PICK1 and GluRδ2 were colocalized (arrows). Scale bar, 10 μm. ***B***, ***C***, HA-GluRδ2 (***B***) or PICK1-EGFP (***C***) was immunoprecipitated with the anti-GFP or the anti-HA antibody, respectively, in the cell lysate prepared from HEK293T cells expressing HA-GluRδ2 and PICK1-EGFP. ***D***, ***E***, GluRδ2 (***D***) or PICK1 (***E***) was immunoprecipitated with the anti-PICK1 or the anti-GluRδ2 antibody in the brain lysate prepared from the mouse cerebellum. The immunoprecipitates were immunoblotted with the anti-GluRδ2 or the anti-PICK1 antibody. The arrow indicates PICK1 signal, and the arrowhead indicates the band corresponding to the anti-GluRδ2 antibody used for immunoprecipitation. IP, Immunoprecipitated; IB, immunoblot; Sup, supernatant.

(Kim and Sheng, 2004). It is known that these interactions are important for receptor localization and function. GluRδ2 C terminus is also known to interact with several proteins via the PDZ binding motif (Roche et al., 1999; Hironaka et al., 2000; Miyagi et

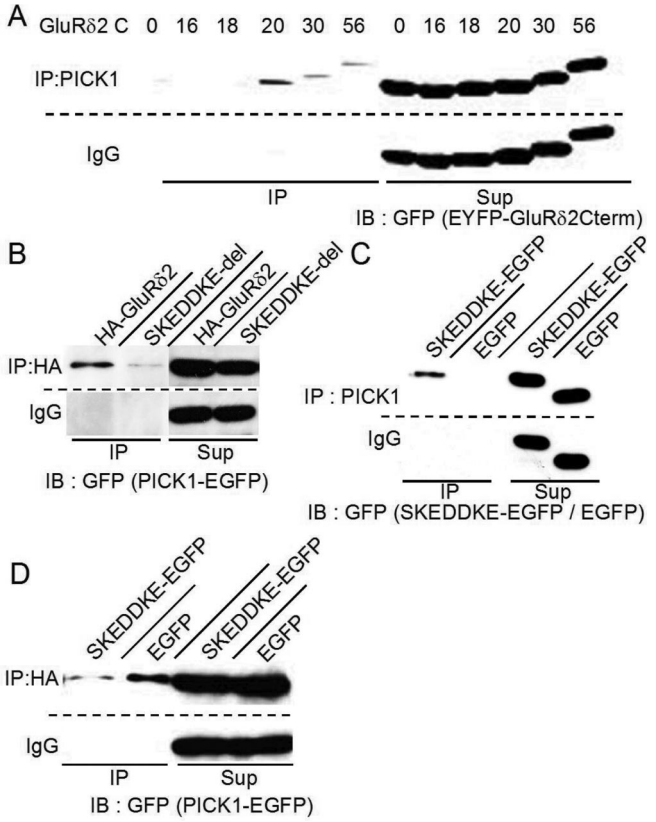

Figure 5. SKEDDKE of GluRδ2 is involved in binding to PICK1. ***A***, The GluRδ2 partial peptides fused to EYFP were immunoprecipitated with the anti-PICK1 antibody. The membrane-proximal peptides longer than 20 were precipitated (IP 20, 30, 56). ***B***, PICK-EGFP was only slightly immunoprecipitated with HA-SKEDDKE-del by the anti-HA antibody. ***C***, SKEDDKE-EGFP but not EGFP was precipitated by the anti-PICK1 antibody in the cell lysate prepared from HEK293T cells expressing PICK1 and SKEDDKE-EGFP or EGFP. ***D***, Suppression of GluRδ2-PICK1 binding by SKEDDKE-EGFP. PICK1 was immunoprecipitated with the anti-HA antibody and immunoblotted with the anti-GFP antibody recognizing PICK1-EGFP. Coexpression of SKEDDKE-EGFP reduced PICK1 in the precipitate. IP, Immunoprecipitated; IB, immunoblot; Sup, supernatant.

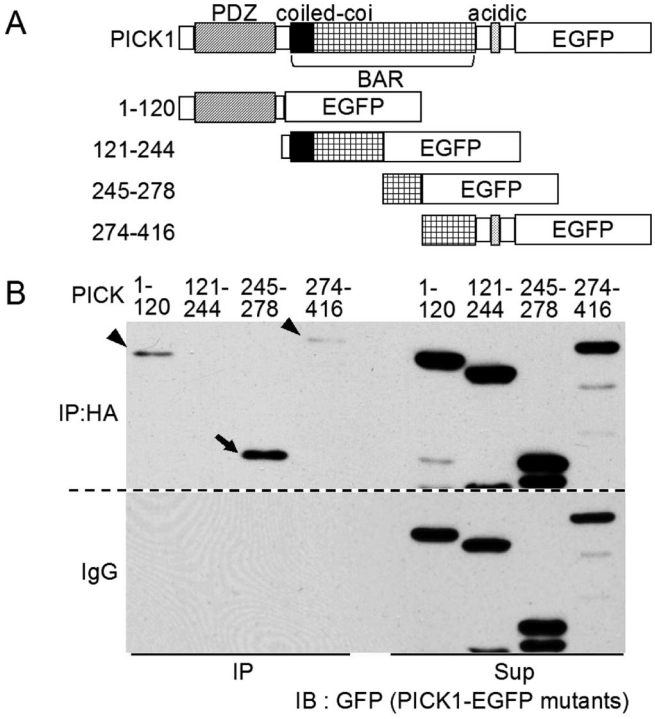

Figure 6. The region of PICK1 involved in binding to GluRδ2. ***A***, The PICK1 mutants. ***B***, PICK1 deletion mutants fused to EGFP were immunoprecipitated with the anti-HA antibody recognizing HA-GluRδ2. PICK1(245–278) was clearly immunoprecipitated (arrow), whereas PICK1(1–120) and PICK1(274–416) were only slightly immunoprecipitated (arrowheads). Degradation products were also stained in Sup. IP, Immunoprecipitated; IB, immunoblot; Sup, supernatant.

al., 2002; Yue et al., 2002; Yap et al., 2003a). Here, we showed that all GluRδ2 mutants including C5 with a very short C-terminal sequence was localized in dendritic spines, suggesting that trafficking of GluRδ2 to dendritic spines depends on neither its C terminus nor binding partners. Thus, the intracellular loop region, extracellular domains or transmembrane regions might play roles in the transport of GluRδ2 to the cell membrane, although regulatory involvement of C terminus cannot be excluded. Unlike other GluRs (Hafidi and Hillman, 1997), limited GluRδ2 protein was detected in the cytoplasm (Takayama et al., 1996), which might be attributable to the distinct trafficking regulation mechanism for GluRδ2. The present results that C21 but not C13 rescued LTD and that SKEDDKE [C(14–20)]-EGFP inhibited LTD in wild-type PNs indicate that C(14–20) are essential for LTD. Thus, the PDZ binding motif is not only dispensable for trafficking to dendritic spines but also for LTD. The C(4–30) (855–881 aa) of GluRδ2 is demonstrated to be essential for stable localization of GluRδ2 on the plasma membrane in MDCK cells (Matsuda and Mishina, 2000). It is also reported that the mutant GluRδ2 protein lacking C(1–13) is retained in the endoplasmic reticulum and not transported to the cell surface (Matsuda et al., 2004). C(14–20) (SKEDDKE) of GluRδ2, which is enriched with charged amino acids, does not show clear homology with any functional motifs identified to date.

We demonstrated that both the interaction between GluRδ2 and PICK1 and the induction of LTD were blocked by SKEDDKE-GFP, suggesting implication of GluRδ2-PICK1 interaction in LTD. Involvement of PICK1 in LTD has been reported. It is suggested that phosphorylation of GluR2 Ser880 residue by PKC releases the GluR2 containing AMPA receptor from GRIP, which might be anchoring GluR2 to the postsynaptic membrane. Then, the GluR2 containing AMPA receptor released from GRIP binds to PICK1 and is internalized (Matsuda et al., 2000; Xia et al., 2000; Chung et al., 2003). At present, the precise role of GluRδ2 and PICK1 interaction in LTD is unclear. It might somehow help GluR2 and PICK1 interaction (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). PICK1 interacts with GluR2 mainly through its PDZ domain (Xia et al., 1999), although it is also reported that PICK1 180–378 aa interact with the NSF (*N*-ethylmaleimide-sensitive factor)-binding region of GluR2 located in the juxtamembrane C terminus (Hanley et al., 2002). As shown here, PICK1 is likely to interact with GluRδ2 through 245–278 aa distinct from the PDZ domain. Both the PDZ domain (20–110 aa) and the coiled-coil region (139–166 aa) in the BAR domain of PICK1 are essential for LTD (Xia et al., 2000). The 245–278 aa of PICK1, the main interacting region with GluRδ2, is located within the BAR domain (140–355 aa). BAR domains are found in numbers of proteins such as amphiphysins, endophilins, and arfaptins. The amphiphysin BAR domain is reported to form a crescent-shaped dimer that interacts with curved and negatively charged membrane (Peter et al., 2004). The net negative charge of SKEDDKE sequence of GluRδ2 might provide the interacting site for the PICK1 BAR domain. Lu and Ziff (2005) showed that the PICK1 BAR domain interacts with the PICK1 PDZ domain intramolecularly and with GRIP intermolecularly, and suggested that the latter interaction helps

efficient phosphorylation of GluR2 Ser880 residue by PKC and facilitating the GluR2 and PICK1 interaction.

By the way, GluRδ2 also plays a role in stabilization of parallel fiber–PN synapse structure possibly through interaction with a molecule in the presynaptic active zone (Kurihara et al., 1997; Takeuchi et al., 2005; Hirano 2006). GluRδ2 is efficiently transported to plasma membrane and relatively evenly distributed in the postsynaptic density of parallel fiber–PN synapse. Taking all of the above information together, we speculate that GluRδ2 might provide a slot or a place, which helps the efficient interaction between PICK1, PKC, and GluR2 that is essential for the induction of LTD in the postsynaptic density of parallel fiber–PN synapse (supplemental Fig. 1, available at www.jneurosci.org as supplemental material).

To summarize, we have identified the membrane-proximal C-terminal residues C(14–20) of GluRδ2 as a critical region for LTD and showed that it also mediates interaction with PICK1, an essential molecule for LTD. Thus, it is suggested that GluRδ2 might participate in the induction of LTD through its interaction with PICK1 in PNs.

References

Araki K, Meguro H, Kushiya E, Takayama C, Inoue Y, Mishina M (1993) Selective expression of the glutamate receptor channel δ2 subunit in cerebellar Purkinje cells. Biochem Biophys Res Commun 197:1267–1276.

Boudin H, Craig AM (2001) Molecular determinants for PICK1 synaptic aggregation and mGluR7a receptor coclustering: role of the PDZ, coiled-coil, and acidic domains. J Biol Chem 276:30270–30276.

Chung HJ, Xia J, Scannevin RH, Zhang X, Huganir RL (2000) Phosphorylation of the AMPA receptor subunit GluR2 differentially regulates its interaction with PDZ domain-containing proteins. J Neurosci 20:7258–7267.

Chung HJ, Steinberg JP, Huganir RL, Linden DJ (2003) Requirement of AMPA receptor GluR2 phosphorylation for cerebellar long-term depression. Science 300:1751–1755.

Crepel F, Krupa M (1988) Activation of protein kinase C induces a long-term depression of glutamate sensitivity of cerebellar Purkinje cells. An in vitro study. Brain Res 458:397–401.

De Zeeuw CI, Hansel C, Bian F, Koekkoek SK, van Alphen AM, Linden DJ, Oberdick J (1998) Expression of a protein kinase C inhibitor in Purkinje cells blocks cerebellar LTD and adaptation of the vestibulo-ocular reflex. Neuron 20:495–508.

Hafidi A, Hillman DE (1997) Distribution of glutamate receptors GluR 2/3 and NR1 in the developing rat cerebellum. Neuroscience 81:427–436.

Hanley JG, Khatri L, Hanson PI, Ziff EB (2002) NSF ATPase and α-/β-SNAPs disassemble the AMPA receptor-PICK1 complex. Neuron 34:53–67.

Hirai H, Matsuda S (1999) Interaction of the C-terminal domain of δ glutamate receptor with spectrin in the dendritic spines of cultured Purkinje cells. Neurosci Res 34:281–287.

Hirai H, Miyazaki T, Kakegawa W, Matsuda S, Mishina M, Watanabe M, Yuzaki M (2005) Rescue of abnormal phenotypes of the δ2 glutamate receptor-null mice by mutant δ2 transgenes. EMBO Rep 6:90–95.

Hirano T (2006) Cerebellar regulation mechanisms learned from studies on GluRδ2, a unique glutamate-receptor-related molecule specifically expressed at parallel fiber-Purkinje cell synapses. Mol Neurobiol 33:1–16.

Hirano T, Kasono K (1993) Spatial distribution of excitatory and inhibitory synapses on a Purkinje cell in a rat cerebellar culture. J Neurophysiol 70:1316–1325.

Hirano T, Kasono K, Araki K, Shinozuka K, Mishina M (1994) Involvement of the glutamate receptor δ2 subunit in the long-term depression of glutamate responsiveness in cultured rat Purkinje cells. Neurosci Lett 182:172–176.

Hirano T, Kasono K, Araki K, Mishina M (1995) Suppression of LTD in cultured Purkinje cells deficient in the glutamate receptor δ2 subunit. NeuroReport 6:524–526.

Hironaka K, Umemori H, Tezuka T, Mishina M, Yamamoto T (2000) The protein-tyrosine phosphatase PTPMEG interacts with glutamate receptor δ2 and ε subunits. J Biol Chem 275:16167–16173.

Hollmann M, Heinemann S (1994) Cloned glutamate receptors. Annu Rev Neurosci 17:31–108.

Ito M (2001) Cerebellar long-term depression: characterization, signal transduction, and functional roles. Physiol Rev 81:1143–1195.

Jeromin A, Huganir RL, Linden DJ (1996) Suppression of the glutamate receptor δ2 subunit produces a specific impairment in cerebellar long-term depression. J Neurophysiol 76:3578–3583.

Kashiwabuchi N, Ikeda K, Araki K, Hirano T, Shibuki K, Takayama C, Inoue Y, Kutsuwada T, Yagi T, Kang Y, Aizawa S, Mishina M (1995) Impairment of motor coordination, Purkinje cell synapse formation, and cerebellar long-term depression in GluR δ2 mutant mice. Cell 81:245–252.

Kawaguchi SY, Hirano T (2006) Integrin α3β1 suppresses long-term potentiation at inhibitory synapses on the cerebellar Purkinje neuron. Mol Cell Neurosci, in press.

Kim E, Sheng M (2004) PDZ domain proteins of synapses. Nat Rev Neurosci 5:771–781.

Kohda K, Kamiya Y, Matsuda S, Kato K, Umemori H, Yuzaki M (2003) Heteromer formation of δ2 glutamate receptors with AMPA or kainate receptors. Brain Res Mol Brain Res 110:27–37.

Kurihara H, Hashimoto K, Kano M, Takayama C, Sakimura K, Mishina M, Inoue Y, Watanabe M (1997) Impaired parallel fiber→Purkinje cell synapse stabilization during cerebellar development of mutant mice lacking the glutamate receptor δ2 subunit. J Neurosci 17:9613–9623.

Landsend AS, Amiry-Moghaddam M, Matsubara A, Bergersen L, Usami S, Wenthold RJ, Ottersen OP (1997) Differential localization of δ glutamate receptors in the rat cerebellum: coexpression with AMPA receptors in parallel fiber-spine synapses and absence from climbing fiber-spine synapses. J Neurosci 17:834–842.

Linden DJ, Connor JA (1991) Participation of postsynaptic PKC in cerebellar long-term depression in culture. Science 254:1656–1659.

Linden DJ, Dickinson MH, Smeyne M, Connor JA (1991) A long-term depression of AMPA currents in cultured cerebellar Purkinje neurons. Neuron 7:81–89.

Lomeli H, Sprengel R, Laurie DJ, Kohr G, Herb A, Seeburg PH, Wisden W (1993) The rat delta-1 and delta-2 subunits extend the excitatory amino acid receptor family. FEBS Lett 315:318–322.

Lu W, Ziff EB (2005) PICK1 interacts with ABP/GRIP to regulate AMPA receptor trafficking. Neuron 47:407–421.

Ly CD, Roche KW, Lee HK, Wenthold RJ (2002) Identification of rat EMAP, a δ glutamate receptor binding protein. Biochem Biophys Res Commun 291:85–90.

Matsuda I, Mishina M (2000) Identification of a juxtamembrane segment of the glutamate receptor δ2 subunit required for the plasma membrane localization. Biochem Biophys Res Commun 275:565–571.

Matsuda S, Mikawa S, Hirai H (1999) Phosphorylation of serine-880 in GluR2 by protein kinase C prevents its C terminus from binding with glutamate receptor-interacting protein. J Neurochem 73:1765–1768.

Matsuda S, Launey T, Mikawa S, Hirai H (2000) Disruption of AMPA receptor GluR2 clusters following long-term depression induction in cerebellar Purkinje neurons. EMBO J 19:2765–2774.

Matsuda S, Hannen R, Matsuda K, Yamada N, Tubbs T, Yuzaki M (2004) The C-terminal juxtamembrane region of the δ2 glutamate receptor controls its export from the endoplasmic reticulum. Eur J Neurosci 19:1683–1690.

Miyagi Y, Yamashita T, Fukaya M, Sonoda T, Okuno T, Yamada K, Watanabe M, Nagashima Y, Aoki I, Okuda K, Mishina M, Kawamoto S (2002) Delphilin: a novel PDZ and formin homology domain-containing protein that synaptically colocalizes and interacts with glutamate receptor δ2 subunit. J Neurosci 22:803–814.

Niwa H, Yamamura K, Miyazaki J (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108:193–199.

Peter BJ, Kent HM, Mills IG, Vallis Y, Butler PJ, Evans PR, McMahon HT (2004) BAR domains as sensors of membrane curvature: the amphiphysin BAR structure. Science 303:495–499.

Roche KW, Ly CD, Petralia RS, Wang YX, McGee AW, Bredt DS, Wenthold RJ (1999) Postsynaptic density-93 interacts with the δ2 glutamate receptor subunit at parallel fiber synapses. J Neurosci 19:3926–3934.

Shigemoto R, Abe T, Nomura S, Nakanishi S, Hirano T (1994) Antibodies inactivating mGluR1 metabotropic glutamate receptor block long-term depression in cultured Purkinje cells. Neuron 12:1245–1255.

Staudinger J, Zhou J, Burgess R, Elledge SJ, Olson EN (1995) PICK1: a perinuclear binding protein and substrate for protein kinase C isolated by the yeast two-hybrid system. J Cell Biol 128:263–271.

Staudinger J, Lu J, Olson EN (1997) Specific interaction of the PDZ domain protein PICK1 with the COOH terminus of protein kinase C-α. J Biol Chem 272:32019–32024.

Takayama C, Nakagawa S, Watanabe M, Mishina M, Inoue Y (1996) Developmental changes in expression and distribution of the glutamate receptor channel δ2 subunit according to the Purkinje cell maturation. Brain Res Dev Brain Res 92:147–155.

Takeuchi T, Miyazaki T, Watanabe M, Mori H, Sakimura K, Mishina M (2005) Control of synaptic connection by glutamate receptor δ2 in the adult cerebellum. J Neurosci 25:2146–2156.

Uemura T, Mori H, Mishina M (2004) Direct interaction of GluRδ2 with Shank scaffold proteins in cerebellar Purkinje cells. Mol Cell Neurosci 26:330–341.

Xia J, Zhang X, Staudinger J, Huganir RL (1999) Clustering of AMPA receptors by the synaptic PDZ domain-containing protein PICK1. Neuron 22:179–187.

Xia J, Chung HJ, Wihler C, Huganir RL, Linden DJ (2000) Cerebellar long-term depression requires PKC-regulated interactions between GluR2/3 and PDZ domain-containing proteins. Neuron 28:499–510.

Yamazaki M, Araki K, Shibata A, Mishina M (1992) Molecular cloning of a cDNA encoding a novel member of the mouse glutamate receptor channel family. Biochem Biophys Res Commun 183:886–892.

Yap CC, Muto Y, Kishida H, Hashikawa T, Yano R (2003a) PKC regulates the δ2 glutamate receptor interaction with S-SCAM/MAGI-2 protein. Biochem Biophys Res Commun 301:1122–1128.

Yap CC, Murate M, Kishigami S, Muto Y, Kishida H, Hashikawa T, Yano R (2003b) Adaptor protein complex-4 (AP-4) is expressed in the central nervous system neurons and interacts with glutamate receptor δ2. Mol Cell Neurosci 24:283–295.

Yue Z, Horton A, Bravin M, DeJager PL, Selimi F, Heintz N (2002) A novel protein complex linking the δ2 glutamate receptor and autophagy: implications for neurodegeneration in *lurcher* mice. Neuron 35:921–933.

Zuo J, De Jager PL, Takahashi KA, Jiang W, Linden DJ, Heintz N (1997) Neurodegeneration in *Lurcher* mice caused by mutation in δ2 glutamate receptor gene. Nature 388:769–773.