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Rescue of abnormal phenotypes of the δ2 glutamate receptor-null mice by mutant δ2 transgenes

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The δ2 glutamate receptor (GluRδ2) has a crucial role in cerebellar functions; disruption of *GluRδ2* alleles in mice (*δ2*$^{-/-}$) impairs synapse formation and long-term depression, which is thought to underlie motor learning in the cerebellum, and consequently leads to motor discoordination. However, it has been unclear whether GluRδ2 is activated by glutamate analogues. Here we introduced a *GluRδ2* transgene, which had a mutation (Arg514Lys) in the putative ligand-binding motif conserved in all mammalian ionotropic glutamate receptors (iGluRs) and their ancestral bacterial periplasmic amino-acid-binding proteins, into *δ2*$^{-/-}$ mice. Surprisingly, a mutant *GluRδ2* transgene, as well as a wild-type *GluRδ2* transgene, rescued all abnormal phenotypes of *δ2*$^{-/-}$ mice. Therefore, these results indicate that the conserved arginine residue, which is crucial for the binding of iGluRs to glutamate analogues, is not essential for the restoration of GluRδ2 functions in *δ2*$^{-/-}$ mice.

Keywords: glutamate receptor; Purkinje cell; cerebellum; LTD; mouse

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INTRODUCTION

Fast excitatory neurotransmission in the mammalian central nervous system is mainly mediated by L-glutamate, which activates postsynaptic ionotropic glutamate receptors (iGluRs): α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate and *N*-methyl-D-aspartate (NMDA) receptors. On the basis of amino-acid sequence, the δ2 glutamate receptor (GluRδ2) family is positioned at equal distances in a phylogenetic tree from these three receptor families (Araki *et al*, 1993; Lomeli *et al*, 1993). Although GluRδ2 is predominantly expressed at parallel fibre (PF)–Purkinje cell synapses in the cerebellum, it does not seem to mediate normal synaptic transmission, which is completely blocked by the AMPA receptor-specific antagonist (Kano & Kato, 1987). Instead, GluRδ2 has a crucial role in forming PF–Purkinje cell and climbing fibre (CF)–Purkinje cell synapses during development, and impaired synaptogenesis is associated with motor discoordination in *δ2*$^{-/-}$ mice (Kurihara *et al*, 1997; Morando *et al*, 2001; Ichikawa *et al*, 2002). In addition to its role during development, GluRδ2 signalling has a unique role in modulating existing synapses in adult mice (Hirai *et al*, 2003).

Despite their importance, the mechanisms by which GluRδ2 participates in cerebellar functions have been elusive: GluRδ2 does not form functional glutamate-gated ion channels when they are expressed, either alone or with other iGluRs, in heterologous cells (Araki *et al*, 1993; Lomeli *et al*, 1993), and radio-ligand-binding assays failed to detect binding of GluRδ2 to glutamate analogues (Lomeli *et al*, 1993). Interestingly, the extracellular amino-terminal region of GluRδ2 contains a putative ligand-binding motif conserved in all mammalian iGluRs but not in metabotropic glutamate receptors (Fig 1). An arginine residue in this domain is especially conserved from the ancestral bacterial periplasmic amino-acid-binding proteins to mammalian iGluRs; X-ray crystallographic analyses demonstrated its essential interaction with the α-carboxyl moieties of amino-acid ligands (Oh *et al*, 1993; Hsiao *et al*, 1996; Armstrong *et al*, 1998; Mayer *et al*, 2001; Furukawa & Gouaux, 2003). Indeed, a substitution of this arginine residue with lysine completely abolishes the ligand-binding or channel activities of iGluRs (Hirai *et al*, 1996; Kawamoto *et al*, 1997; Laube *et al*, 1997; Jouppila *et al*, 2002). Therefore, to address the question whether GluRδ2 is activated by glutamate

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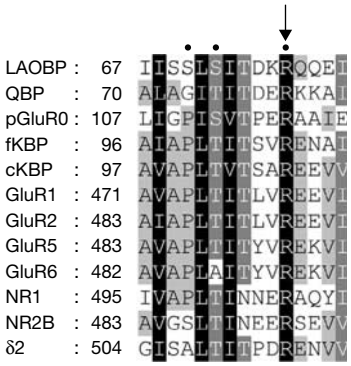

Fig 1 | Amino-acid alignment involving the conserved arginine of iGluRs and bacterial periplasmic binding proteins. The conserved arginine residue of GluRδ2 aligns with that of the following: bacterial periplasmic binding proteins leucine-arginine-ornithine-binding protein (LAOBP) and glutamine-binding protein (QBP), prokaryotic glutamate receptor (pGluR0), chick and frog kainate-binding proteins (cKBP and fKBP, respectively), AMPA receptor subunits GluR1 and GluR2, kainate receptor subunits GluR5 and GluR6 and NMDA receptor subunits NR1 and NR2B. Letters are shaded according to the percentage of conserved similar amino acids at each position (100%, black shading; 80%, mid-grey; 60%, light grey). The amino-acid position of the first residue of each sequence is indicated. Critical residues in LAOBP, QBP, pGluR0, GluR2 and NR1 that interact directly with ligands are indicated by dots above the sequences (Oh *et al*, 1993; Hsiao *et al*, 1996; Armstrong *et al*, 1998; Mayer *et al*, 2001; Furukawa & Gouaux, 2003). The arrow indicates the position of the highly conserved arginine residue.

analogues, we did not rely on *in vitro* binding or functional assays, but instead we generated mice that express a mutant *GluRδ2* transgene in which lysine replaced the conserved arginine at position 514 ($Tg_{R/K}$) onto a $\delta2^{-/-}$ background. We hypothesized that the function of GluRδ2 is abrogated by the mutation if GluRδ2 is activated by glutamate or related amino acids in a conventional manner similar to that of other iGluRs.

RESULTS

We used the Purkinje-cell-specific L7 promoter (Tomomura *et al*, 2001; Fig 2A) to drive the expression of a wild-type *GluRδ2* transgene (Tg_{wt}) and $Tg_{R/K}$. By breeding transgenic mice onto a $\delta2^{-/-}$ background, we obtained transgenic 'rescue' lines called $\delta2^{-/-}/Tg_{wt}$ and $\delta2^{-/-}/Tg_{R/K}$. Proper insertion of transgenes into the genome was confirmed by sequencing the genomic DNA from each transgenic line (supplementary Fig S1 online). We further analysed lines that expressed equivalent levels of construct-encoded proteins (Fig 2B). Immunohistochemical staining showed cerebellum-specific expression of $Tg_{R/K}$ (Fig 2C) and Tg_{wt} (data not shown). Although the levels of expression of transgenic GluRδ2 protein were about 20% of those of endogenous GluRδ2 protein in wild-type cerebellum (Fig 2B), $\delta2^{-/-}/Tg_{wt}$ mice showed no ataxic gait and could walk along a straight line (Fig 2D). Surprisingly, the ataxic gait was not observed in $\delta2^{-/-}/Tg_{R/K}$ mice (Fig 2D). Results of the rotarod test confirmed the complete restoration of the motor performance of $\delta2^{-/-}/Tg_{R/K}$ mice (Fig 2E). These results indicated that $Tg_{R/K}$ as well as Tg_{wt} could rescue the motor discoordination of $\delta2^{-/-}$ mice.

Immature Purkinje cells are innervated by multiple CFs that originate from the inferior olive of the medulla (Crepel *et al*, 1976). As animals grow, redundant CFs are gradually eliminated, and a relationship of one CF to one Purkinje cell is established by the end of the third postnatal week. Previous studies showed that GluRδ2 has an essential role in this elimination process (Kashiwabuchi *et al*, 1995; Hashimoto *et al*, 2001). To examine whether each Purkinje cell was innervated by a single CF in the $\delta2^{-/-}/Tg_{R/K}$ cerebellum, we recorded the CF-evoked excitatory postsynaptic currents (EPSCs) from postnatal day 21 (P21) to P30 Purkinje cells in slice preparations. Because a single CF has a single threshold for excitation, increasing the stimulus intensity normally elicits CF-evoked EPSCs in an all-or-none manner. Single EPSCs were elicited in about 90% of wild-type P30 Purkinje cells (Fig 3A), whereas only 40% of $\delta2^{-/-}$ Purkinje cells attained a one-to-one relationship with CFs (Hashimoto *et al*, 2001; Fig 3B). In contrast, $\delta2^{-/-}/Tg_{R/K}$ mice had almost the same percentage (86%; Fig 3C) of single Purkinje cell–single CF innervation as the wild-type mice, which indicates that $Tg_{R/K}$ restored the normal process of CF synapse elimination.

Purkinje cell dendrites have two separate domains (Bravin *et al*, 1999): proximal regions innervated by CFs and distal regions innervated by PFs. CFs abnormally invade the 'PF domain' of distal dendrites of $\delta2^{-/-}$ Purkinje cells (Ichikawa *et al*, 2002). To examine the pattern of CF innervation of Purkinje cells, sections were stained with antibody to vesicular glutamate transporter 2 (VGluT2), which is predominantly expressed in CF terminals. The most distal terminal of CFs penetrated 95.1 ± 0.4% of the molecular layer thickness in $\delta2^{-/-}$ cerebella (Ichikawa *et al*, 2002), 81 ± 1% in $\delta2^{-/-}/Tg_{wt}$ cerebella (Fig 3D,E) and 80 ± 2% in $\delta2^{-/-}/Tg_{R/K}$ cerebella (Fig 3F,G; mean ± s.e.m. from three mice). In high-power micrographs, CF terminals were mainly associated with shaft dendrites in $\delta2^{-/-}/Tg_{R/K}$ cerebella (Fig 3H,I), whereas they terminate at distal spiny branchlets in $\delta2^{-/-}$ cerebella (Ichikawa *et al*, 2002). Similarly, although CFs innervating the distal dendrites of $\delta2^{-/-}$ Purkinje cells are associated with EPSCs with a slow rise time (Hashimoto *et al*, 2001), CF-evoked EPSCs in $\delta2^{-/-}/Tg_{R/K}$ Purkinje cells had a fast rise time (0.6 ± 0.1 ms, $n = 12$) similar to those in wild-type cells (0.6 ± 0.1 ms, $n = 13$). Therefore, we concluded that the abnormal distal CF innervation of $\delta2^{-/-}$ Purkinje cells was also rescued by $Tg_{R/K}$.

Previous studies revealed that about 40% of spines on $\delta2^{-/-}$ Purkinje cells are 'naked' ones that lack presynaptic contact but have postsynaptic density (PSD)-like condensations (Kurihara *et al*, 1997; Lalouette *et al*, 2001). Furthermore, the remaining PF–Purkinje cell synapses in $\delta2^{-/-}$ mice frequently show another specific abnormality—the length of the PSD does not equal that of the opposing presynaptic active zone (Lalouette *et al*, 2001). However, serial electron microscopic analysis (Fig 3J,K) revealed few free spines in both strains of transgenic mice (0.7 ± 0.7% in $\delta2^{-/-}/Tg_{wt}$ cerebella and 0.3 ± 0.3% in $\delta2^{-/-}/Tg_{R/K}$ cerebella; 300 total spines counted in three representative mice of each line). Similarly, mismatching between the PSD and the active zone at PF synapses was rare (0.3 ± 0.3% in $\delta2^{-/-}/Tg_{wt}$ cerebella and 0.3 ± 0.3% in $\delta2^{-/-}/Tg_{R/K}$ cerebella; 300 spines counted in three representative mice of each line). These results indicate that abnormal PF–Purkinje cell synaptogenesis in $\delta2^{-/-}$ cerebella was rescued by $Tg_{R/K}$ as well as by Tg_{wt}.

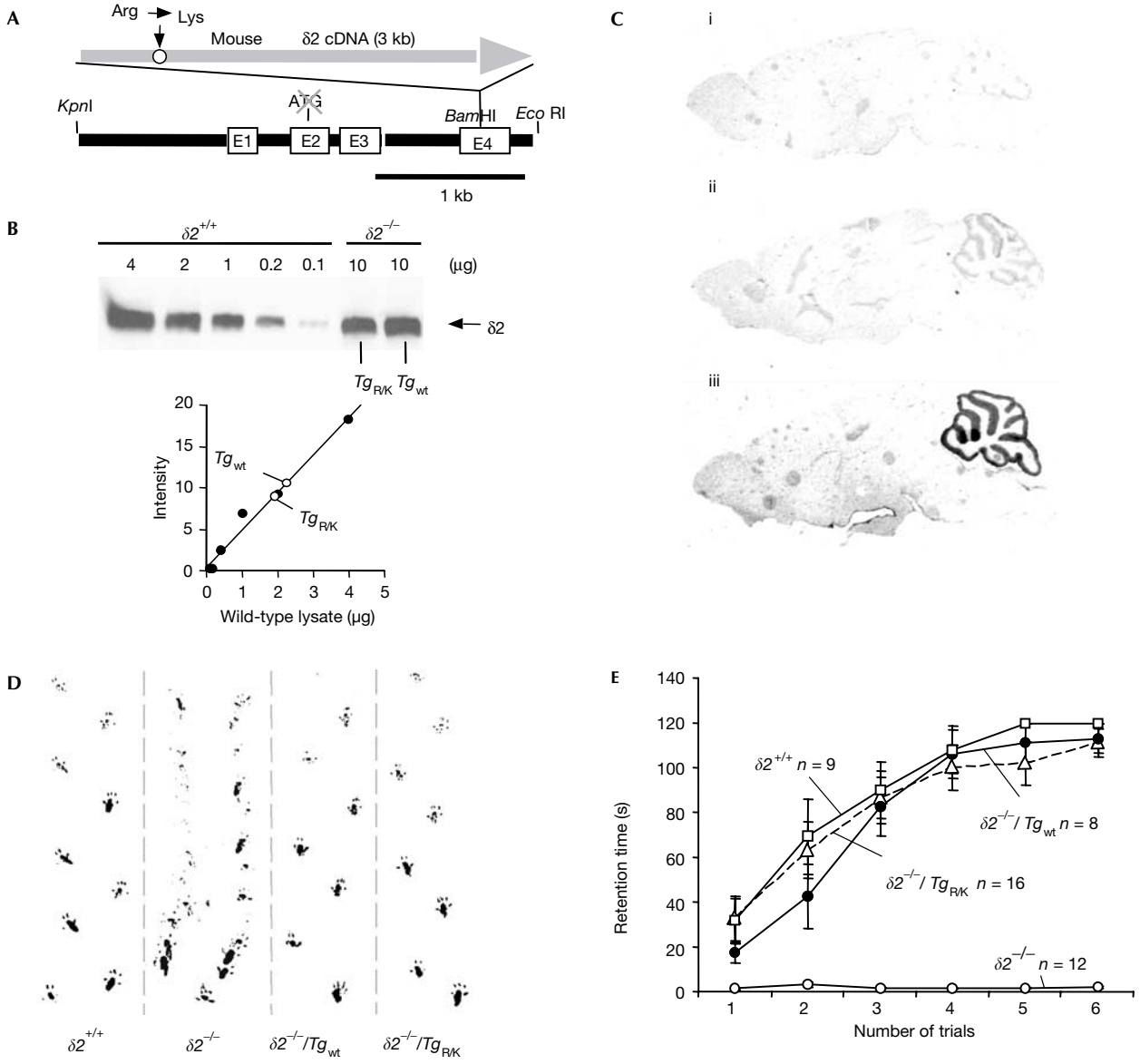

Fig 2 | Rescue of the ataxic phenotype of $\delta2^{-/-}$ mice by *GluRδ2* transgene expression. (**A**) Structure of the transgene construct. Mouse wild-type or mutant GluRδ2 cDNA was inserted into the *Bam*HI site of the L7 gene in which the translational start codon was disrupted (Tomomura *et al*, 2001). E1–E4, exons. (**B**) Western blot of $\delta2^{+/+}$, $\delta2^{-/-}/Tg_{wt}$ and $\delta2^{-/-}/Tg_{R/K}$ cerebellar cells. Total lysates were blotted, and the blots were incubated with a polyclonal antibody against GluRδ2. The total amount of protein is indicated above each lane. To quantify the expression level of transgenes (lower graph), band intensities of the GluRδ2 protein in 10 μg of $\delta2^{-/-}/Tg_{wt}$ or $\delta2^{-/-}/Tg_{R/K}$ cerebellar cell lysates were compared with those in various amounts of wild-type cerebellar cell lysates. (**C**) Immunohistochemical analysis of parasagittal sections of brains from (i) $\delta2^{-/-}$, (ii) $\delta2^{-/-}/Tg_{R/K}$ and (iii) $\delta2^{+/+}$ (wild-type) mice. These sections were stained with anti-GluRδ2 antibody. (**D**) Footprint patterns. Tottering steps were made by the $\delta2^{-/-}$ mice, and their feet tended to sweep along as they moved. In contrast, $\delta2^{-/-}/Tg_{R/K}$ and $\delta2^{-/-}/Tg_{wt}$ mice walked along a straight line as did $\delta2^{+/+}$ mice. (**E**) Results of the rotarod task. Mice were allowed a maximum retention time of 120 s per trial. The number of mice in each group is indicated in the graph. Error bars indicate s.e.m.

Simultaneous activation of PFs and CFs induces long-term depression (LTD) of PF–Purkinje cell transmission (Ito, 1989). CF stimulation can be replaced by direct depolarization of Purkinje cells to rule out any effect due to the CF innervation pattern. This protocol robustly induced LTD of PF-EPSCs in wild-type (Fig 4A,D) but not in $\delta2^{-/-}$ (Fig 4B,E) Purkinje cells. The amplitudes of PF-EPSCs 40 min after conjunctive stimulation (eight Purkinje cells isolated from seven wild-type mice) were 68±2% of those before the start of conjunctive stimulation. Similarly, the same protocol induced LTD of PF-EPSCs in $\delta2^{-/-}/Tg_{R/K}$ Purkinje cells (Fig 4C,F). The amplitudes of such EPSCs 40 min after conjunctive stimulation were 71±5% (seven

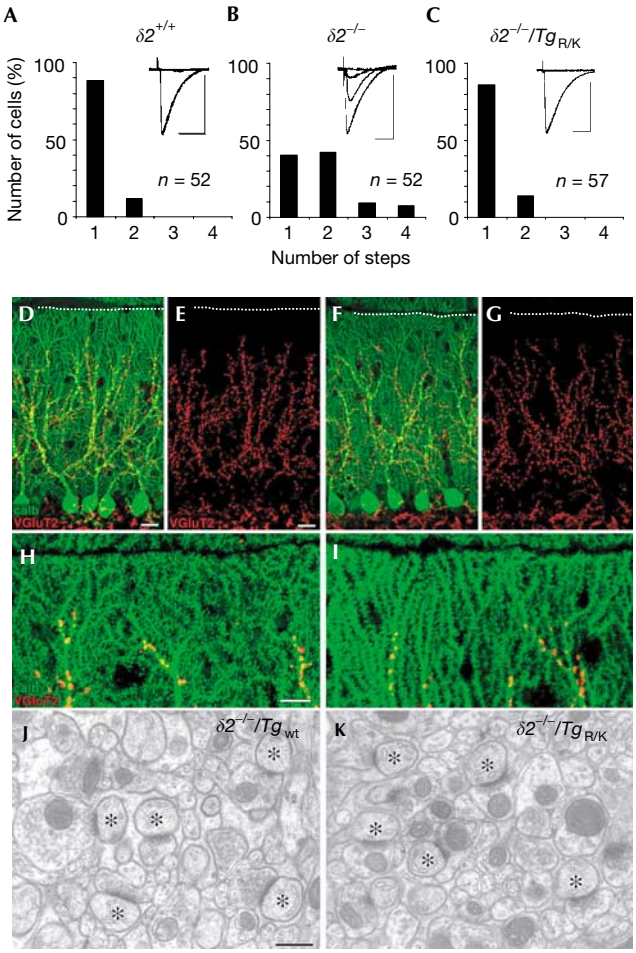

Fig 3 | Restoration of impaired synapse formation of $\delta2^{-/-}$ Purkinje cells by $Tg_{R/K}$ expression. (**A–C**) Electrophysiological estimation of the number of CFs innervating single Purkinje cells in $\delta2^{+/+}$ (**A**), $\delta2^{-/-}$ (**B**) and $\delta2^{-/-}/Tg_{R/K}$ (**C**) cerebella. EPSCs were elicited by stimulation of CFs in the granule cell layer. The number of EPSCs induced by different stimulus thresholds was counted (inset traces). The number of Purkinje cells tested is indicated. Horizontal scale bars, 20 ms; vertical scale bars, 500 pA. (**D–I**) Normal distribution of CF terminals in $\delta2^{-/-}/Tg_{wt}$ (**D,E,H**) and $\delta2^{-/-}/Tg_{R/K}$ (**F,G,I**) cerebella. Cerebellar sections were stained with antibodies to VGluT2 (red; a marker of CF terminals) and calbindin (green; a marker of Purkinje cells). The pial surface is indicated by the dotted line. (**J,K**) Electron micrographs of PF–Purkinje cell synapses in the molecular layer. The asterisks indicate Purkinje cell spines in contact with PF terminals. Scale bars: (**D,E**) 20 μm; (**H**) 10 μm; (**J**) 500 nm.

Purkinje cells isolated from six mice) and were not significantly different from those in wild-type Purkinje cells ($P > 0.5$; as determined by Student's *t*-test). Cerebellar LTD is believed to occur solely in postsynaptic Purkinje cells, probably by increased endocytosis of postsynaptic iGluRs (Matsuda *et al*, 2000; Wang & Linden, 2000). Therefore, $Tg_{R/K}$ probably has a role similar to that of wild-type GluRδ2 in postsynaptic signalling pathways involved in synaptic plasticity at PF–Purkinje cell synapses.

DISCUSSION

Here we demonstrated that $Tg_{R/K}$ expression effectively rescued all principal abnormal phenotypes of $\delta2^{-/-}$ mice: motor discoordination, multiple CF innervation, impaired PF synaptogenesis and abrogated LTD. In contrast, a substitution of the corresponding arginine residue with lysine completely abolishes the ligand-binding or channel activities of other iGluRs (Hirai *et al*, 1996; Kawamoto *et al*, 1997; Laube *et al*, 1997; Jouppila *et al*, 2002). In addition, the arginine residue is essential for the binding of bacterial periplasmic proteins to amino-acid ligands, including glutamate, aspartate, glutamine, glycine, lysine, serine, arginine, ornithine and histidine (Oh *et al*, 1993; Hsiao *et al*, 1996; Armstrong *et al*, 1998; Mayer *et al*, 2001; Furukawa & Gouaux, 2003). Therefore, we speculate that GluRδ2 does not require glutamate-like amino acids to function in Purkinje cells.

However, we do not have direct evidence that $Tg_{R/K}$ does not bind any glutamate analogues; it may still bind them in an unconventional way. It is also theoretically possible that, although $Tg_{R/K}$ does not bind glutamate analogues, wild-type GluRδ2 does. For example, if $Tg_{R/K}$ makes an abnormal heteromeric channel with other iGluRs, glutamate binding to these subunits may substitute for glutamate binding to wild-type GluRδ2 and restore the altered phenotypes of $\delta2^{-/-}$ mice. However, the binding of three or more glutamate molecules to each receptor-channel complex is required for full activation of iGluRs (Rosenmund *et al*, 1998; supplementary Fig S3 online). Thus, it seems unlikely that a heteromeric channel composed of $Tg_{R/K}$ and iGluR subunits could substitute for a channel formed by wild-type GluRδ2. In any case, GluRδ2 is unique in that its conserved arginine is not essential for the rescue of the altered phenotypes of $\delta2^{-/-}$ mice.

Recently, we found that the application of an antibody specific for GluRδ2's extracellular N-terminal region to Purkinje cells specifically abrogated LTD by disrupting the endocytosis of AMPA receptors and caused transient cerebellar ataxia (Hirai *et al*, 2003). This finding indicates that GluRδ2 signalling could be controlled by the binding of a ligand to the extracellular domain. In addition, the role in controlling endocytosis suggests that GluRδ2 acts as a metabotropic receptor (Yuzaki, 2004). Interestingly, the AMPA receptor GluR2 subunit also activates a metabotropic pathway to induce dendritic spine formation in cultured hippocampal neurons (Passafaro *et al*, 2003), and this activity is mediated by the extracellular N-terminal domain of GluR2, which is distinct from the conventional glutamate-binding domain. Therefore, further studies using $\delta2^{-/-}/Tg_{R/K}$ mice to explore such mechanisms exploited by GluRδ2 are warranted.

METHODS

Generation of transgenic mice. Mouse δ2 complementary DNA was inserted into the *Bam*HI site of pL7ΔAUG (Fig 2A). The resulting plasmid was digested with *Kpn*I and *Eco*RI, and the linearized L7-δ2 construct was injected into fertilized eggs (Tomomura *et al*, 2001). Seven Tg_{wt} and eight $Tg_{R/K}$ founders were bred onto a $\delta2^{-/-}$ background. Homozygous transgenic lines were established and confirmed by backcrossing with wild-type mice. All procedures relating to the care and treatment of animals were carried out according to NIH guidelines, and the experimental protocol was approved by the Animal Resource Committee of St Jude Children's Research Hospital.

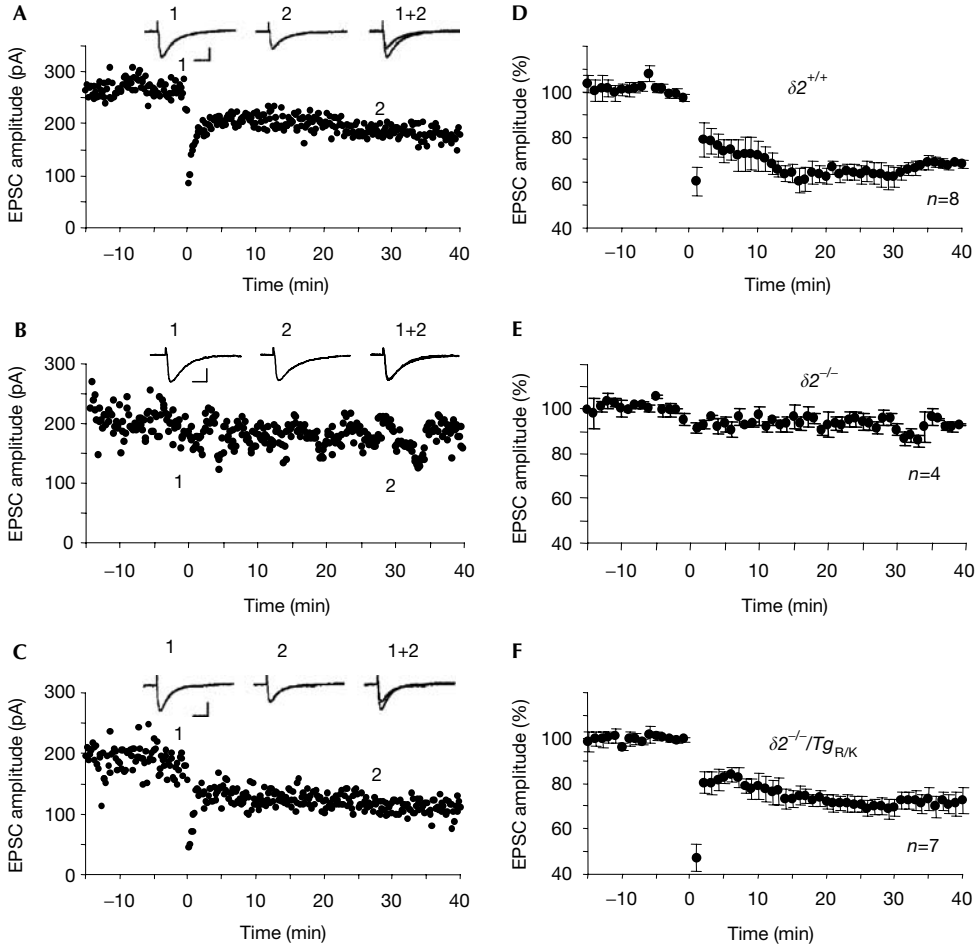

Fig 4 | Expression of long-term depression in $\delta2^{-/-}/Tg_{R/K}$ Purkinje cells. Representative EPSC amplitudes over time are shown. (**A**) Results for eight Purkinje cells isolated from seven wild-type mice. (**B**) Results for four Purkinje cells isolated from two $\delta2^{-/-}$ mice. (**C**) Results for seven Purkinje cells isolated from six $\delta2^{-/-}/Tg_{R/K}$ mice. The means (±s.e.m.) for each (wild type (**D**), $\delta2^{-/-}$ (**E**) and $\delta2^{-/-}/Tg_{R/K}$ (**F**)) are shown. The amplitude of the PF-EPSC was normalized to the baseline value, which was the average of 5-min responses that occurred just before conjunctive stimulation. Inset traces are EPSCs just before (1) and 30 min after (2) the conjunctive stimulation.

Immunoblotting and microscopic analysis. Whole cerebella of wild-type and transgenic mice were homogenized, and the homogenates were used for immunoblotting as described previously (Hirai *et al*, 2003). Adult mice were anaesthetized and perfused transcardially with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for light microscopy or with 2% paraformaldehyde plus 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for electron microscopy. Sagittal cryosections (20 μm in thickness) were immunostained with anti-GluRδ2 antibody as described previously (Hirai *et al*, 2003). To visualize CF terminals under a confocal laser scanning microscope (Fluoview FV1000, Olympus, Tokyo, Japan), microslicer sections (50 μm in thickness; VT1000S, Leica, Wien, Austria) were treated overnight with a mixture of calbindin antiserum (dilution, 1:10,000) and anti-VGluT2 antibody (0.5 μg/ml) as described previously (Ichikawa *et al*, 2002). For electron microscopy (H-7100, Hitachi, Tokyo, Japan), serial ultrathin sections (70 nm in thickness; UCT ultramicrotome, Leica) were prepared as described previously (Kurihara *et al*, 1997). When the edge of the PSD was more than 100 nm from the corresponding active zone in any serial sections, the spine was defined as a free spine.

Electrophysiology. Parasagittal cerebellar slices (200 μm) were prepared from wild-type, $\delta2^{-/-}$ and $\delta2^{-/-}/Tg_{R/K}$ mice. Whole-cell voltage-clamp recordings were made of Purkinje cells identified visually at 24 °C as described (Hirai *et al*, 2003). Patch pipettes were pulled from borosilicate glass capillaries to achieve a resistance of 4–5 MΩ when filled with a solution containing 140 mM cesium methanesulphonate, 2 mM Na_2ATP, 0.3 mM Na_2GTP, 10 mM HEPES and 0.4 mM EGTA (pH 7.3, 290 mOsm/kg). Square pulses were applied every 10 s using an isolation unit (Axon Instruments, Foster City, CA, USA) for focal stimulation.

After stable PF-EPSCs had been observed for at least 15 min, LTD was induced by conjunctive stimulation that consisted of 30 single PF stimuli together with a 50 ms depolarizing pulse from a holding potential of −70 to +20 mV. A hyperpolarizing pulse (−10 mV, 50 ms) was applied 420 ms before each PF stimulus to monitor the access resistance. If the resistance differed from the original value by more than 20%, the record was discarded.

PF-EPSCs were evoked every 10 s by a glass electrode placed in the molecular layer (pulse width, 10 μs; strength, 20–100 μA) about 100 μm away from the pial surface.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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