ONLINE SUPPLEMENTAL MATERIAL

Supplemental Methods

Genotyping of mice. Genomic DNA from the mouse tail was prepared by the HotSHOT method (Truett *et al.*, 2000). To amplify GluRδ2 transgenes by PCR, we used the sense primer 5'-CGA GTG GTG ACT GTA TTG-3', which corresponds to exon 9 of the GluRδ2 genome, and the antisense primer 5'-GTT TAA GAG GTA GAC CAA GAG-3', which corresponds to exon 11. Because the estimated distance between these exons is more than 69 kb (Wang *et al.*, 2003), only GluRδ2 transgenes, but not the GluRδ2 genome, were amplified. PCR was performed in a RoboCycler (Stratagene, La Jolla, CA) with *Taq* DNA polymerase (Qiagen, Valencia, CA) according to the following schedule: preincubation at 94 °C for 5 min followed by 35 cycles in which each cycle consisted of incubation at 94°C for 45 s, at 54°C for 45 s, and at 72°C for 45 s. The last cycle was followed by a 5-min incubation at 72°C. The amplified DNA fragments were purified by using the MinElute PCR purification kit (Qiagen) and bidirectionally sequenced by using the primers designed for PCR.

Electrophysiological analysis of heteromeric iGluR channels. AMPA receptors GluR1 and GluR2 were the 'flop' version. To examine the contribution of glutamate binding to GluR2 in heteromeric channels composed of GluR1 and GluR2, an

arginine residue at position 493 of GluR2, which constitutes the essential glutamate-binding site (Fig. 1), was replaced with lysine ($GluR2^{R/K}$), as previously described (Kohda *et al.*, 2000). For coexpression studies, GluR1 was subcloned into pTracer-nucYFP, which expresses yellow fluorescent protein (YFP) fused with a nuclear localization signal by a promoter separate from that of GluR1, and GluR2 was subcloned into pTracer-mitCFP, which expresses cyan fluorescent protein (CFP) fused with the mitochondrial localization signal (Kohda *et al.*, 2003). GluR1 and GluR2 clones were co-transfected into HEK293 cells (American Type Culture Collection, Rockville, MD, USA) by a calcium-phosphate precipitation method (ratio of GluR1 to GluR2 plasmids, 1:4). When single transfection was done as a control, the total amount of DNA was kept constant by replacing pTracer-mitCFP-GluR2 with pTracer-mitCFP (mock). Expression of the clone of interest was confirmed by immunoblot analysis using anti-GluR1 and anti-GluR2 antibodies as described (Hirai *et al.*, 2003). Twenty-four hours after transfection, cells that expressed both CFP and YFP were subjected to whole-cell patch-clamp recordings in which an Axopatch 200B (Axon Instruments, Foster City, CA, USA) amplifier was used. The recording pipettes had resistances of 4 to 6 MΩ when filled with the intracellular solution (150 mM CsCl, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM EGTA [pH 7.3]), and series resistances were 8

to 15 MΩ. The extracellular solution was composed of 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 20 mM HEPES, and 20 mM D-glucose (pH 7.3). To apply glutamate (10 mM), cells were lifted to a flow pipe constructed from theta glass tubing (tip diameter, 250 μm; Sutter Instruments, Novato, CA, USA) and placed in the control-solution stream close to the interface between the continuously flowing control and drug solutions. Solution exchange was made by rapidly moving the theta glass with a Piezo translator (LSS-3100, Burleigh Instruments, Fishers, NY, USA) (Kohda et al., 2000). During recordings, 100 μM cyclothiazide was added to both the control and ligand solutions to block AMPA receptor desensitization.

Immunoprecipitation and silver staining. Whole cerebella of $\delta2^{-/-}/Tg_{wt}$ and $\delta2^{-/-}/Tg_{R/K}$ mice (3 month old) were homogenized and solubilized in 500μl TNE buffer (50mM NaCl, 10% NP-40, 20mM EDTA, 0.1% SDS, 50mM Tris-HCL, pH 8.0) containing protease inhibitor cocktail (Calbiochem, San Diego, CA, USA) as described previously (Matsuda and Yuzaki, 2002). Two micrograms of anti-GluRδ2 antibody (Chemicon, Temecula, CA, USA) were added to each lysate, and the mixture was incubated for 1 h at 4°C. Then 50 μl of protein G–conjugated agarose (Roche, Indianapolis, IN, USA) was added and incubated for another 1 h at 4°C. After the precipitates were washed four times with TNE buffer, 50 μl of SDS-PAGE sample

buffer was added and the samples were incubated for 5 min at 95°C. After centrifugation, 20 μL of the supernatant was subjected to SDS-PAGE (5-20%) and analyzed by the silver staining kit (Amersham, Piscataway, NJ, USA).

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SUPPLEMENTAL FIGURE LEGENDS

Fig. S1. Examples of sequence traces of genomic PCR products from $\delta 2^{-/-}/Tg_{wt}$ and $\delta 2^{-/-}/Tg_{R/K}$ mice. Boxes indicate the codons for the amino acids at position 514 in Tg_{wt} and $Tg_{R/K}$.

Fig. S2. Representative traces of PF–evoked (upper) and CF–evoked (lower) EPSCs elicited in wild-type (left, 1) or $\delta 2^{-/-}/Tg_{R/K}$ (middle, 2) Purkinje cells. For the right trace (1+2), the peak amplitude of each trace was adjusted and superimposed. There were no fundamental differences in the time course of both PF– and CF–EPSCs between wild-type and $\delta 2^{-/-}/Tg_{R/K}$ Purkinje cells (see Supplemental Table S1).

Fig. S3. No activation by glutamate of heteromeric AMPA receptor channels formed by $GluR2^{R/K}$ and $GluR1^{wt}$. (**A–C**) Representative glutamate-induced currents (left) and the corresponding current-voltage (I-V) curves (right) recorded from HEK293 cells that expressed wild-type GluR1 ($GluR1^{wt}$) alone (**A**), $GluR1^{wt}$ plus wild-type GluR2 ($GluR2^{wt}$) (**B**), or $GluR1^{wt}$ plus GluR2 with a mutation at the putative ligand-binding site ($GluR2^{R/K}$) (**C**). Currents induced by glutamate application were recorded from the cells clamped at various membrane potentials between –100 mV and +100 mV in the presence of cyclothiazide (100 μM). (**D**) Rectification indices of I-V curves from

HEK293 cells expressing each combination of clones. The rectification index was calculated as the ratio of the slope conductance at -60 mV to the slope conductance at 40 mV. (**E**) Amplitudes of glutamate-induced currents recorded from cells clamped at –80 mV. *** $P < 0.001$, ** $P < 0.01$ as determined by the Mann-Whitney U test. Error bars indicate SEM. As reported previously (Verdoorn *et al.*, 1991), properties of homomeric and heteromeric glutamate receptors are determined by the presence of glutamine (Q) or arginine (R) at the Q/R site in the channel pore; the presence of R dominantly determines the linear I-V relationship. Indeed, cells expressing homomeric AMPA receptors formed by $GluR1^{wt}$, which had Q in the channel pore, displayed strong inward rectification (**A, D**), whereas heteromeric receptors containing GluR1 and $GluR2^{wt}$, which had R, exhibited a linear I-V relationship (**B, D**). In contrast, cells expressing $GluR1^{wt}$ and $GluR2^{R/K}$ displayed inwardly rectifying glutamate-induced currents (**C, D**) whose amplitudes were significantly smaller than those from cells expressing $GluR1^{wt}$ and $GluR2^{wt}$ (**E**). Because similar quantities of $GluR2^{R/K}$ and $GluR2^{wt}$ proteins were coimmunoprecipitated by anti-GluR1 antibody (data not shown), these findings suggested that heteromeric channels composed of GluR1 and $GluR2^{R/K}$ are not effectively gated by glutamate and that glutamate-induced currents (**C**) are those that originate from a small population of homomeric GluR1 channels.

Fig. S4. Proteins co-immunoprecipitated by anti-GluRδ2 antibodies from $\delta2^{-/-}/Tg_{wt}$ and $\delta2^{-/-}/Tg_{R/K}$ cerebella. Cerebella of $\delta2^{-/-}/Tg_{wt}$ and $\delta2^{-/-}/Tg_{R/K}$ mice were solubilized and immunoprecipitated by anti-GluRδ2 antibody. Co-immunoprecipitated proteins were analyzed by SDS-PAGE and silver staining. There were no differences in the intensity or migration of the protein bands, a result suggesting that $Tg_{R/K}$ and Tg_{wt} associated with similar proteins. Arrows indicate GluRδ2 derived from transgene (Tg), heavy chain of IgG (IgG_{HC}), and light chain of IgG (IgG_{LC}), respectively.

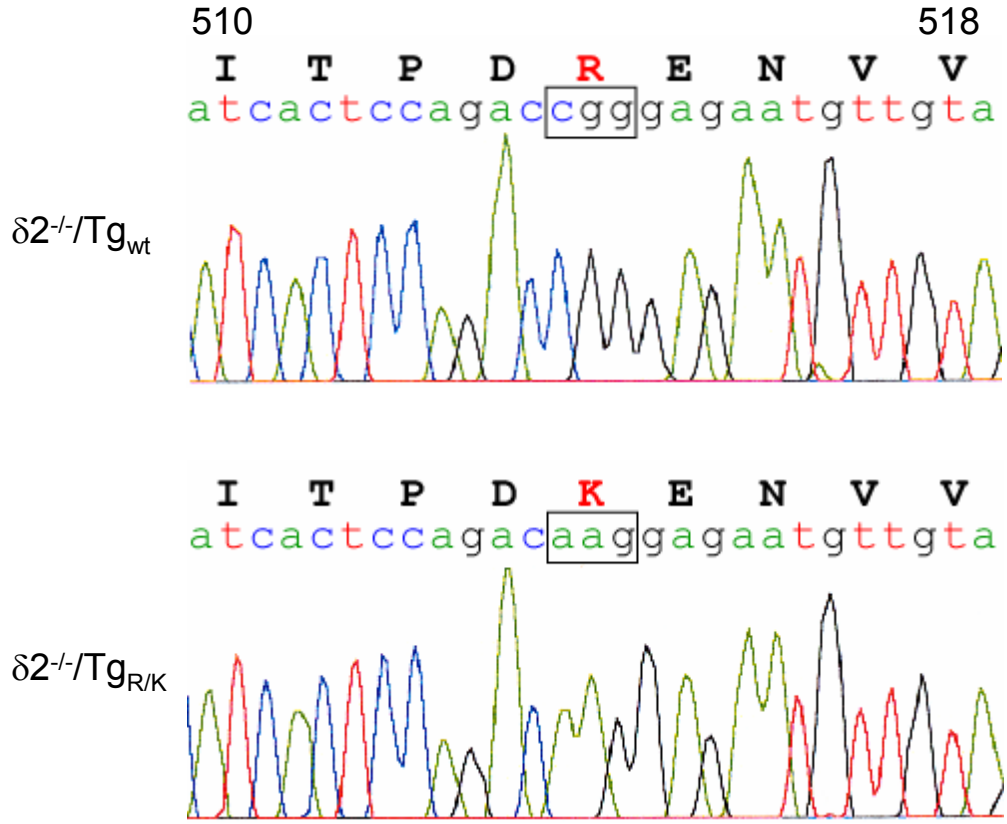
Table S1. Kinetic properties of CF-EPSCs and PF-EPSCs in wild-type and $Tg_{R/K}$ Purkinje cells [a]

		10%-90% Rise time [b] (ms)	Decay time constant [c] (ms)	No. of cells	No. of animals
CF-EPSC	Wild-type	0.6 ± 0.1	13.3 ± 1.1	16	7
	$Tg_{R/K}$	0.6 ± 0.1	13.8 ± 1.0	21	6
PF-EPSC	Wild-type	3.6 ± 0.3	21.6 ± 1.4	15	7
	$Tg_{R/K}$	3.4 ± 0.3	22.8 ± 1.9	12	5

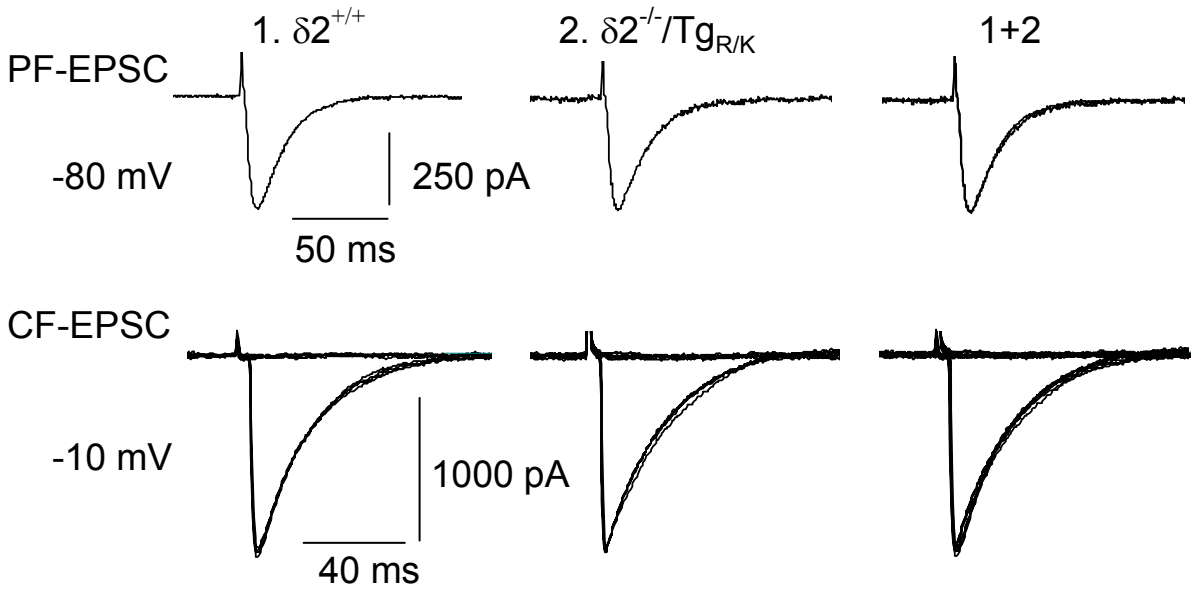
a Data are expressed as the mean ± SEM.

b The rise time was defined as the time required for the glutamate-induced current to reach 10% to 90% of its maximum level.

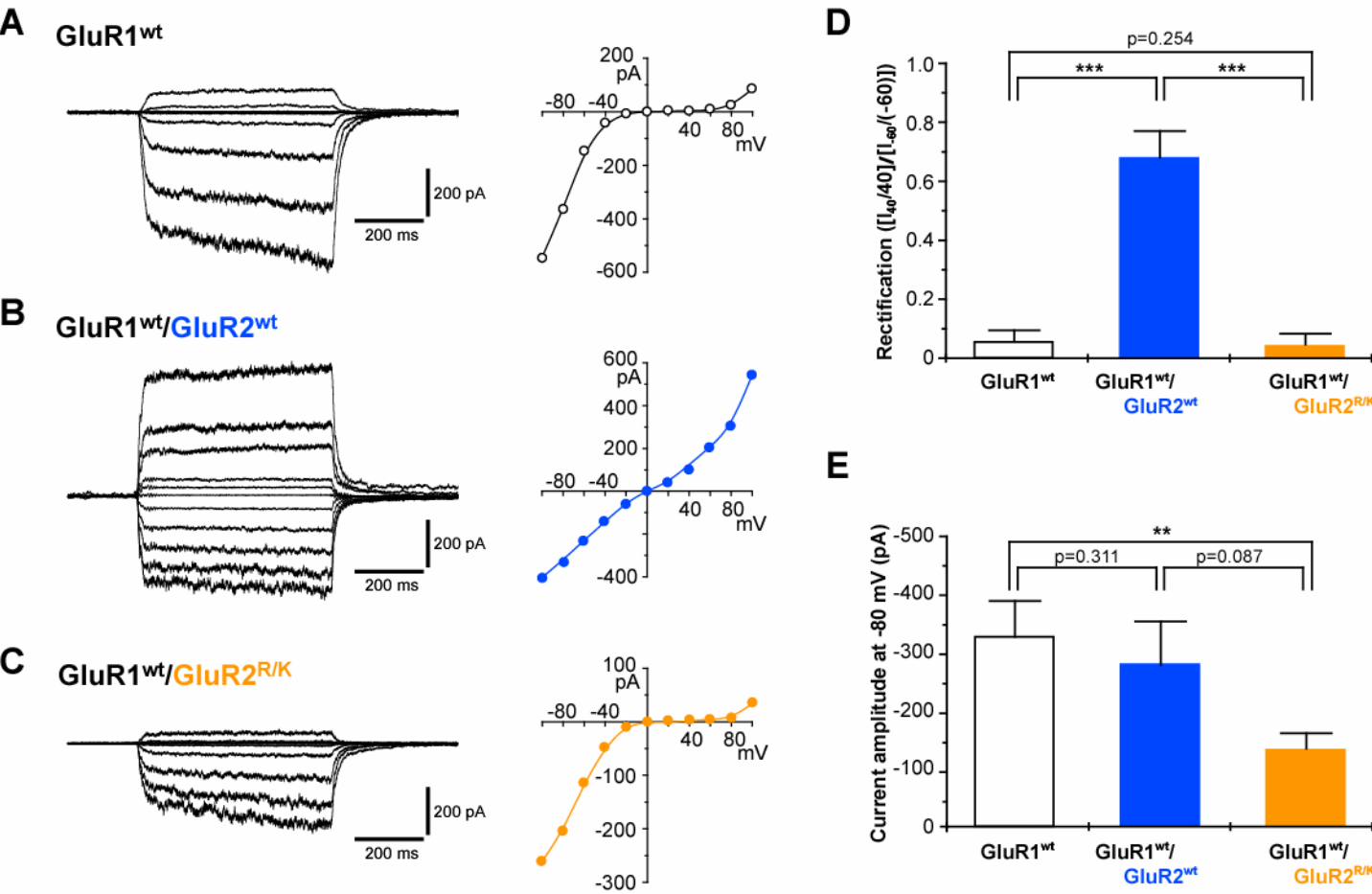
c Decay time constants were obtained by fitting the decay phase of EPSCs with single exponential curves.



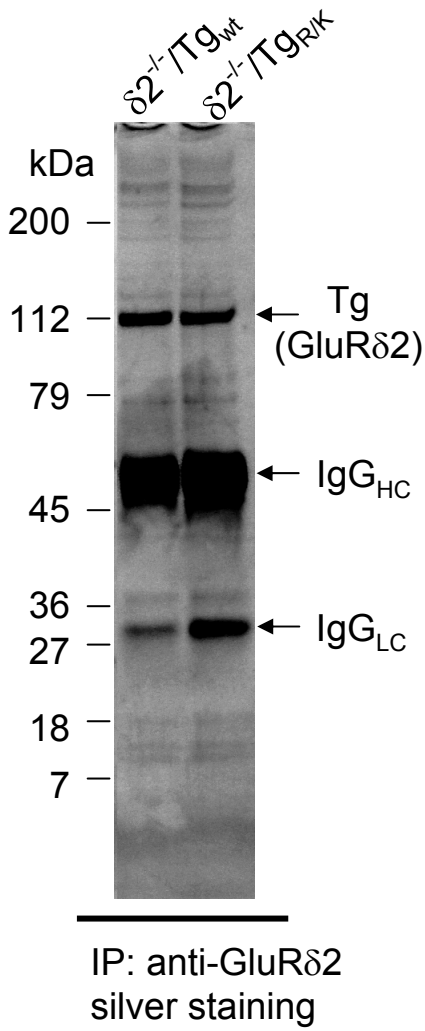
Supplemental Figure S1



Supplemental Figure S2



Supplemental Figure S3



Supplemental Figure S4