Supporting Information

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SI Text

Electrophysiology. Transverse cerebellar acute slices (300 μm) of Wistar rats (17- to 24-day-old) were prepared following the method described by Llano et al. (1) with 50 μM D-APV added to the slicing solution to protect the tissue during slicing. Slices were visualized using a 40× water-immersion objective (0.75 NA, Axioskop, Carl Zeiss) and infrared optics (illumination filter 750 ± 50 nm; Cool Snap Photometrics, Roper Scientific). The recording chamber was continuously perfused at a rate of 2 mL/min with a solution containing (mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 25 glucose, and 10 tricine (a Zn^{2+} ion buffer, ref.2) bubbled with 95% O_2 and 5% CO_2 (pH 7.4)Patch pipettes had resistances of 2.0–4.0 MΩ with the internal solution given below. Cells were voltage-clamped at −70 mV in the whole-cell configuration. Series resistance was held between 4 and 10 MΩ and compensated with settings of 95%. pCLAMP8 software (Molecular Dynamics) was used for data acquisition and analysis. Whole-cell recordings were filtered at 1 kHz and digitized at 10 kHz.

Experiments were performed at 32 °C (Single Channel Heater Controller, Warner Instruments). Ten micromolar bicuculline methochloride, 1 μM CGP55845, and 200 nM DPCPX were added to the bath solution to block GABA-A-mediated fast inhibitory transmission, GABA-B, and adenosine A1 receptors, respectively. The internal solution contained (mM): 140 K-gluconate, 10 Hepes, 0.5 EGTA, 4 KCl, and 5 Mg-ATP (pH adjusted to 7.3 with KOH).

EPSCs were evoked by stimulating parallel fibers (PF) extracellularly by means of a glass pipette (tip diameter 8–12 μm) filled with Hepes-buffered saline. The stimulation electrode was placed at the surface of the molecular layer at a distance of 100–500 μm from the recorded PC, or in the granule cell layer (Fig. 1*E*). Stimulation intensity was fixed at the beginning of the experiment (between 3 and 15V; 30 to 150 μs) and remained unchanged during the experiment. Test stimulation was applied at 0.05 Hz. Stimulation consisted of two pulses separated by 100 ms, allowing the quantification of PPF.

Recordings were made in the vermis of lobules three to eight of the cerebellar cortex. LTD was induced by pairing (at 1 Hz for 2 min) the PC depolarization (to ≈−20 mV for 120 ms) with a doublet of PF stimulations (see *Results*). The PC depolarization resulted always in calcium spikes. In a set of experiments (Fig. S1), PC depolarization was replaced by a single climbing fiber (CF) stimulation coincident with the second PF stimulation. CF synaptic inputs were easily identified because of their large amplitude, all-or-none nature and paired-pulse depression. LTD was quantified as the ratio between EPSC amplitude after induction (mean of 15 sweeps between 30 and 35 min after pairing) and control EPSC amplitude (mean of 15 sweeps immediately before pairing). Means and SEM are given in text and figures. Unless otherwise stated, significance was tested using the nonparametric Sign test (two-tailed) in SPSS for Windows software.

HEK293 cells plated on glass coverslips in 35-mm Petri dishes were transfected with 2 μg cDNAs mixed at a ratio of 1 NR1:3 NR2:3 GFP (green fluorescent protein) using calcium phosphate precipitation. Positive cells were visualized by fluorescence. The NMDA subunit cDNAs were subcloned into a modified expression vector (pcDNA3; Invitrogen) (2). Recordings were performed in the whole-cell configuration. Experiments were performed at 32 °C. Patch pipettes of 6–8 MΩ were filled with a solution containing (mM): 125 CsGluconate, 10 CsCl, 10 EGTA, and 10 Hepes (pH adjusted to 7.2 with CsOH). The osmolality was 295 mosm/kg. The standard external solution contained (mM): 130 NaCl, 2.8 KCl, 1 $CaCl_2$, 10 tricine, 0.1 glycine, and 10 Hepes. (pH adjusted to 7.3 with NaOH). Glutamate (1 mM) was applied to the cell by means of a multibarrel fast-perfusion system. Solutions flowed continuously by gravity from all barrels. Currents were recorded with a Multiclamp 700B amplifier (Molecular Dynamics). The voltage-clamp current was filtered (8-pole Bessel) with a corner frequency of 4 kHz. The sampling frequency was 20 kHz. Data were acquired and analyzed with pCLAMP 9 (Molecular Dynamics).

Immunohistochemistry. Western blot analysis showed that NR1, NR2A, and NR2B proteins were present in the cerebellar cortex of juvenile rats (P19). Although in situ hybridization experiments have shown that NR2B mRNA expression fades away after P14 in the cerebellar cortex of rats (3–5), our results indicate the presence of NR2B proteins in addition to NR2A and NR1 proteins.

For fluorescence immunohistochemistry, Purkinje cells in 400-μm slices were filled with neurobiotin by the means of a patch pipette. Slices were then fixed in 4% paraformaldehyde (PFA) for 20 min, coated with gelatin (G2500; Sigma) and resliced in 70-μm sections that were air-dried overnight on gelatined coated slides. Tissue pretreatment consisted in a microwave heating in sodium citrate buffer and incubation in 1% H_2O_2 solution. Samples were then incubated in 0.5% blocking reagent solution (FP1020; Perkin-Elmer) with 0.2% Triton X-100. NR2A labeling was performed by incubating sections with A12W rabbit monoclonal antibody (1:100; ref 05901, Upstate Biotechnology); then with anti-rabbit IgG horseradish peroxidase whole antibody (0.5 μg/mL; ref NA934, Amersham-GE), and finally incubated 10 min in Cy3-tyramide (1/50; FP1170; Perkin-Elmer) diluted in FP1050 (Perkin-Elmer) containing 12% dextran sulfate sodium salt (MW 500,000). After peroxidase inactivation by HCl treatment, Purkinje cell labeling was revealed with streptavidin Alexa Fluor 488 (4 μg/mL; Molecular Probes).

For preembedding immuno electron microscopy, a 24-day-old Wistar rat was anesthetized with pentobarbital (60 mg/kg) and intracardially perfused with 4% PFA and 0.1% glutaraldehyde in PBS. After dissection, the cerebellum was kept overnight at 4 °C in 4% PFA. Vibratome saggital sections (100 μm) were collected in ice-cold PBS, cryoprotected for 3 h in 20% sucrose and 20% glycerol at room temperature and permeabilized by freezing and thawing, and then incubated overnight at 4 °C in anti-NR1 mouse monoclonal antibody (1:50; ref MAB363, Chemicon), anti-NR2A rabbit monoclonal antibody (1:50; ref 05901, Upstate Biotechnology), anti-NR2B mouse monoclonal antibody (1:50; ref 610416, BD Transduction Laboratories) or no primary antibody for control sections. All primary antibodies were diluted in PBS containing 0.1% gelatin (PBSg). After extensive washes, NMDA receptor subunits were detected by using the avidin-biotin complex method (Elite Vectastain, Vector Laboratories). For this purpose, the sections were incubated for 4 h at room temperature with horse anti-mouse or horse anti-rabbit biotinylated antibody (1:200 in PBSg, Vector Laboratories). After a preincubation for 20 min in 0.6% diaminobenzidine (DAB) in Tris-buffered saline (0.06 M, pH 7.4), the sections were incubated with DAB and hydrogen peroxide (Sigma Fast DAB) in the same buffer and revealed under visual control. They were then postfixed for 1 h at 4 °C in 2% osmium tetroxide in

PBS with 0.07% glucose, dehydrated in ethanol, and finally flat-embedded in araldite resin (Polysciences). After blocks were trimmed, ultrathin sections (≈70 nm) were collected on copper grids and counterstained for 10 min with 2% uranyl acetate in H_2O and then with 0.2% Reynolds lead citrate. Observations of ultrathin sections were performed with a Philips Tecnaï 12 electron microscope (FEI). Micrographs were analyzed using Image J software.

Excitatory synapses were recognized for their postsynaptic densities. PF-PC synapses constitute 85% of excitatory synapses in the molecular layer of the cerebellar cortex (6). The precise identification of PF-PC synapses was done following the morphological criteria described by Palay and Chan-Palay (7): PFs make synapses on PC spines, which present characteristic submembrane cisternae. Little confusion was possible with PF synapses on interneurons/Golgi/Lugaro cells because they are mostly formed on shafts. Rare climbing fiber to PC synapses were recognized by their numerous densely packed synaptic vesicles and thus discarded from analysis. The quantification of the peroxidase labeling was done on ≈2,500 μm^2 for each section, in lobules 5 to 7 of the cerebellar cortex. Control sections (i.e., without primary antibody) for NR1 and NR2B (count on 342 synapses) or for NR2A (count on 379 synapses) showed no labeling.

Western Blot Analysis. Recombinant NMDA receptors were expressed in *Xenopus laevis* oocytes after coinjection of cDNAs (10 ng/μL) coding for NR1–1a and NR2A or NR2B subunits. Some oocytes were not injected to perform control experiments. Three days after injection, oocytes were screened to check the amount of expression of NMDAR. Oocytes were then lysed in a lysis buffer (100 mM Tris pH 8.0, 100 mM NaCl, and 0.5% Triton X-100 in the presence of the protease inhibitor mixture indicated above). Nuclei were removed by 10,000 × *g* centrifugation. The supernatant was used for Western blot analysis.

Cerebellar cortex from a 19-day-old Wistar rat was homogenized in homogenization buffer (5 mM Hepes, pH 7.4, 320 mM sucrose) containing a protease inhibitor mixture (pepstatin A 1:200, leupeptin 1:100, PMSF 1:1,000, Sigma). Nuclei were removed by 1000 × *g* centrifugation. The supernatant was spun at 100,000 × *g* for 30 min at 4 °C. The pellet (i.e., the crude membrane fraction) was resuspended and solubilized in 5 mM Tris·HCl, pH 8.1, 0.5% Triton X-100, and the protease inhibitor mixture indicated above.

Equivalent amounts of preparation (50 μg of cerebellar proteins or 1 oocyte equivalent) were loaded onto 8% SDS/PAGE and transferred onto polyvinylidene difluoride membrane. Antibodies against the following antigens were used: NR1 (1:1000; ref MAB363, Chemicon), NR2A (1:2000; ref 05901, Upstate Biotechnology), NR2B (1:250; ref 610416, BD Transduction Laboratories). Horseradish peroxidase-labeled anti-rabbit or anti-mouse antibodies (1:10,000, Jackson ImmunoResearch) were used as secondary antibodies. Immunoblots were processed with the SuperSignal West Pico Trial kit Chemiluminescent system (Pierce). Western blots were analyzed using Image J software; background noise was subtracted and contrast adjusted.

Chemicals. Tricine (10 mM) was used for Zn^{2+} buffering. To obtain 300 nM free Zn^{2+}, 60 μM total $ZnCl_2$ was added to 10 mM tricine as described by Paoletti et al. (2). All drugs, except D-APV and CPCCOEt were continuously perfused. CPCCOEt, bicuculline methochloride, CGP55845, DPCPX, and NBQX were purchased from Tocris Cookson. D-APV was from Ascent Scientific. Ro25–6981 was from Roche. LY303070 was a kind gift of Dr. C. Mulle (Bordeaux Neuroscience Institute, France). All of the other chemicals were from Sigma.

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Fig. S1. LTD induced by pairing PF and CF activity is frequency and NMDAR dependent. (*A*) The LTD induction protocol consisted of pairing (at 1 Hz for 2 min) a climbing fiber stimulation with a double PF stimulation. Single CF stimulation was applied simultaneously to the second PF stimulation. (*B1*) Time course of the normalized EPSC amplitude in an experiment where pairing was done with a double PF stimulus at a 5-ms interval. (*B2*) Traces from the experiment shown in (*B1*) are averages of 10 successive sweeps, before and 30 min after pairing. (*C*) LTD induction depends on the interval between the 2 PF stimuli and on the activation of NMDA receptors. Depression induced by pairing CF and doublets of PF stimuli at 5-ms and at 60-ms intervals, in control conditions (white; $n = 8$, $n = 4$ respectively), or in the presence of 100 μM D-APV during the whole experiment (black; $n = 6$). *, $P < 0.02$; *t* test.

Fig. S2. NMDA receptors are not present at CF-PC synapses of young rats. Climbing fiber-evoked EPSC in PCs of 17–21 days old (young) (*A*) and 2–3 months old (adult) (*B*) rats. Superimposed representative traces in control conditions (black), after addition of 10 μM NBQX plus 25 μM LY303070 (green) and after subsequent addition of 100 μM D-APV (red). In gray, the difference between currents in the absence and in the presence of D-APV. Addition of NBQX and LY303070 (the active enantiomer of GYKI53655) (8) blocks the large AMPA-mediated currents to unveil the small but measurable NMDA component in PCs from adult rats. In the same conditions, no NMDA component was detectable in the younger rats used in the rest of this study. (*C*) Summary histogram of the NMDA component amplitude of the CF evoked EPSC. Data are means ± SEM of three experiments performed at room temperature. *, $P < 0.005$; *t* test).

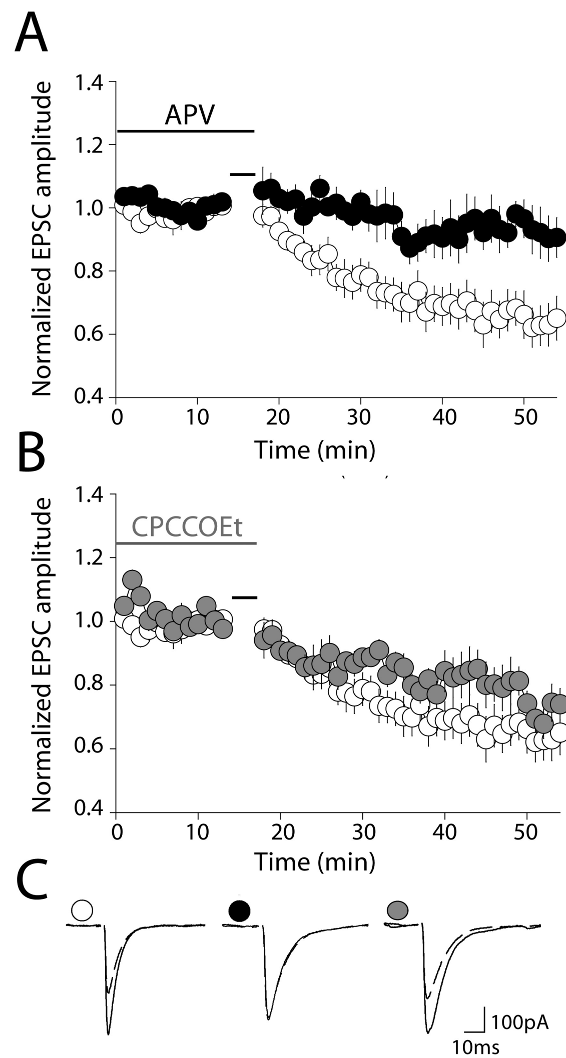

Fig. S3. NMDARs but not mGluR1s are required for LTD induction. (*A*) D-APV prevented LTD induction. Time course of the EPSC amplitude in control conditions (white; $n = 9$; same data as in Fig. 1) or when the pairing was done in the presence of 200 μM D-APV (black; $n = 5$). In each case, pairing was done with double PF stimuli at a 5-ms interval. (*B*) LTD could be induced when mGluR1s were blocked by CPCCOEt. Time course of the EPSC amplitude in control conditions (white; $n = 9$) or when the pairing was done in the presence of 50 μM CPCCOEt (gray; $n = 6$). Double PF stimuli at a 5-ms interval were used. (*C*) Records from representative experiments in control conditions (*Left*), in the presence of 200 μM D-APV (*Center*) or 50 μM CPCCOEt (*Right*). Means of 10 successive sweeps, before pairing (solid traces) and 30 min after pairing (dashed traces).

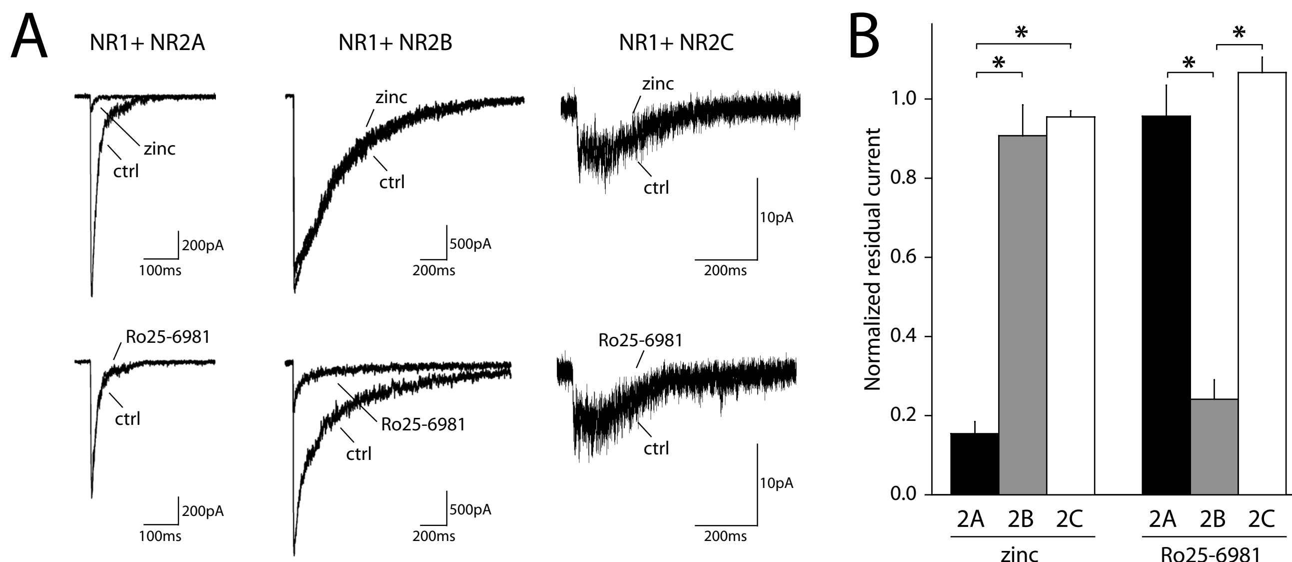

Fig. S4. Zinc (300 nM) and Ro25–6981 (300 nM) are selective antagonists of NR2A- and NR2B-containing NMDARs, respectively. (*A*) Currents were elicited by 100 ms-long applications of L-glutamate (1 mM) in the presence of 100 μM glycine to small lifted HEK-293 cells transfected with different NMDA receptor subunit DNAs (NR1–1a combined with different NR2s). (*B Left*) Zinc inhibits NR2A-containing NMDARs without affecting NR2B- or NR2C-containing receptors. In the presence of 300 nM free zinc NR1+NR2A currents were 15.4 ± 3.0% of the control value (n = 4, black), NR1+NR2B currents 90.7 ± 7.7% (n = 4, gray) ,and NR1+NR2C currents 95.5 ± 1.5% (n = 3, white). (*Right*) Ro25–6981 inhibits NR2B-containing NMDARs without affecting NR2A- or NR2C-containing receptors. In the presence of 300 nM Ro25–6981, NR1+NR2A currents were 95.7 ± 7.8% of the control value (n = 3, black), NR1+NR2B currents 24.1 ± 4.9% (n = 4, gray) and NR1+NR2C currents 106.7 ± 3.9% (n = 3, white). *, $P < 0.0005$; t test).

A1

A2

B

Fig. S5. CPCCOEt, D-APV, Zinc, and Ro25–6981 have no effect on basal synaptic transmission. (*A*) Summary histogram of the effects of drugs on PF-PC synaptic transmission. (*A1*) The effect of washing out CPCCOEt or D-APV was measured (for LTD experiments, CPCCOEt and D-APV were applied before and during LTD induction, and then washed out). Residual EPSC amplitude after washing out 50 μM CPCCOEt was 95.0 $\pm$ 1.7% ($n = 4$) and 100.8 $\pm$ 6.0% after washing-out 200 μM D-APV ($n = 4$). (*A2*) The effect of washing in zinc or Ro25–6981 was measured. Residual EPSC amplitude was 89.9 $\pm$ 5.2% in the presence of 300 nM free zinc ($n = 4$) and 94.4 $\pm$ 6.5% in the presence of 300 nM Ro25–6981 ($n = 4$). (*B*) Recordings from representative experiments in control conditions (solid traces) and after washing out (dashed traces) 50 μM CPCCOEt or 200 μM D-APV, or in the presence (dashed traces) of 300 nM free zinc or 300 nM Ro25–6981. Traces are averages of 10 successive sweeps.

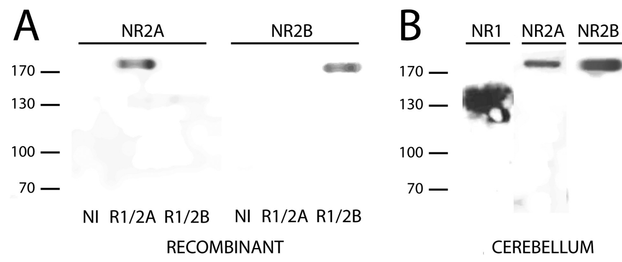

Fig. S6. Antibodies against NMDAR subunits are specific. (*A*) Western blot analysis of recombinant NR2A and NR2B proteins (∼180 kDa) from the lysate of noninjected oocytes (NI lanes) or oocytes injected with NR1–1a + NR2A (R1/2A lanes) or with NR1–1a + NR2B (R1/2B lanes) cDNAs. (*B*) Western blot analysis of native NR1, NR2A, and NR2B proteins in a cerebellar membrane preparation. Apparent molecular mass were ≈140 kDa for NR1 and ≈180 kDa for NR2A and NR2B subunits.