Supporting Information

Wang *et al.* 10.1073/pnas.0804318105

SI Experimental Procedures

Multiple Whole Cell Recordings in Brain Slice Preparation. The main results reported in this paper were obtained from experiments using adult (postnatal days 85–110) rats. For comparison, some experiments were done with young (postnatal days 13–23) rats and adult rats (6–8 months). Rats were deeply anesthetized with sodium pentobarbital (150 mg/kg, intraperotoneal injection, *i.p.*) and euthanized through decapitation. The brains were rapidly removed and placed in ice-cold (< 4° C) sucrose solution containing (mM): KCl 2.5, NaH_2PO_4 1.25, $NaHCO_3$ 26, $CaCl_2$ 0.5, $MgSO_4$ 7.0, sucrose 213 and aerated with 95% O_2 and 5% CO_2. A block of neocortex containing medial PFC (PrL; Paxinos and Watson) was trimmed and glued to the ice-cold stage (< 4° C) of a Vibratome (Vibratome Co.). Horizontal brain slices at thickness of 300 μm were cut and incubated in oxygenated Ringer solution containing (in mM): NaCl 128, KCl 2.5, NaH_2PO_4 1.25, $CaCl_2$ 2, $MgSO_4$ 1, $NaHCO_3$ 26 and dextrose 10, pH 7.4 at 35° C for 1 h and then kept at room temperature until being transferred to a submerged recording chamber (1).

Whole cell recordings were obtained from multiple (up to four) pyramidal neurons simultaneously in cortical slices maintained in a submerged chamber on upright microscopes (Axioscope ; Zeiss or BX61; Olympus) equipped with IR-DIC optics. The recordings were conducted at approximately 36° C. The resistance of the recording pipette (1.2 mm borosilicate glass) was 5–9 MΩ. Monosynaptic connections between layer 5 pyramidal neurons were recorded with one cell (presynaptic neuron) filled with K^+-gluconate based intracellular solution (in mM): K^+-gluconate 120, KCl 6, ATP-Mg 4, Na_2GTP 0.3, EGTA 0.1, Hepes 10, and 0.3% biocytin, pH 7.3, 310 mOsm, and the remaining neurons (postsynaptic neurons) filled with pipette solution containing Cs^+ and QX-314 to block K^+ and Na^+ channels, respectively. The solution contained (in mM): Cs-gluconate 120, lidocaine 5 (QX-314), $CsCl_2$ 6, ATP-Mg 1, Na_2GTP 0.2, Hepes 10, and 0.3% biocytin at pH 7.3 (adjusted with CsOH). All currents were recorded with addition of $GABA_A$ antagonist picrotoxin (50–100 μM; Sigma-Aldrich) in the bath solution to block inhibitory transmission while the NMDA receptor-mediated currents were recorded with membrane potentials of the postsynaptic neurons held at +60 mV and bath-applied both picrotoxin and AMPA receptor antagonist CNQX (20 μM; Sigma-Aldrich). The electric signals were amplified and filtered at 2 kHz in voltage clamp mode with MultiClamp 700A or 700B (Molecular Devices), and acquired at sampling intervals of 20–50 μs through a DigiData 1322A (data acquisition system) and pCLAMP 9.2 (Molecular Devices) software. Access resistances were continuously monitored during recording and neurons with more than 20% change of series resistance were excluded from data analysis. The liquid junction potential in whole cell recordings (typically approximately 9 mV in K-gluconate solutions) was not adjusted. Only cells exhibiting a stable membrane potential without the injection of holding current were used for analysis.

Morphological Analysis of Recorded Neurons. The morphologies of the recorded cells were examined as previous reported (1). Briefly, slices were immediately fixed in cold 4% paraformaldehyde for at least three days and then directly reacted in 3% H_2O_2 for 30 min. After thorough rinsing, ABC reactions were conducted overnight, followed by the Ni-DAB reaction. The slices were directly mounted from 0.1 mM PB (pH 7.4) and covered with water-soluble mounting media. Selected slices were re-sectioned into 150 μm and mounted with water-soluble Permount. The labeled cells were fully reconstructed with Neurolucida (Microbrightfield) and edited in PhotoShop.

Data Analysis of Electrophysiology. The EPSC amplitudes were measured by averaging 30–50 traces from the onset to peak of EPSCs using Clampfit 9.2 software (Molecular Devices). Only the connections giving stable EPSCs without rundown for at least 5 min were considered for further analysis of drug and stimulus effects. The amplitudes of 2nd and following EPSCs were measured from onset to peak. The normalized ratios of the 2nd divided by the 1st EPSC, as well as the ratios of nth/1st were calculated to show the temporal summation of synaptic transmission at 20 Hz train. The decay time course, measured from the repolarization curve of a unitary EPSC, was fitted with either a single exponential or a sum of two exponentials using standard exponential formulas in Clampfit 9.2. When an EPSC decay time course was fitted with double exponentials, $EPSC(t) = w_1 \exp(-t/\tau_1)+w_2 \exp(-t/\tau_2)$, the weighted time constant in Table S1 was defined as $\tau_w = (w_1\tau_1+w_2\tau_2)/(w_1+w_2)$. The integrated EPSC areas (charge transfer) of NMDAR- and AMPAR-mediated currents were measured with pClamp 9.2 and expressed in the unit of picocoulomb (pC). The contribution of NMDA receptor subunit NR2B to the overall NMDAR-mediated current was estimated from measurements in the control condition and with the application of the NR2B antagonist ifenprodil, i.e., (control trace − ifenprodil trace)/control × 100. All data were presented as group measures with mean ± standard error (SE) along with Student's *t* test or ANOVA to examine statistical significance.

Immunocytochemistry. Adult animals (4 months) were euthanized with an overdose of sodium pentobarbital (100 mg/kg, i.p.), and perfused through the heart with 0.1 mM PBS (PBS; pH 7.4) followed by 4% paraformaldehyde in 0.1 mM phosphate buffer (PB; pH 7.4). Brains were extracted from the skull, postfixed overnight with 4% paraformaldehyde and then transferred into 30% sucrose in PB at 4° C. Coronal sections were cut frozen at thicknesses of 30 μm and collected in 0.1 mM PB. Sections containing brain regions of PFC or V1 were simultaneously processed for immunofluorescence staining. All steps were performed under constant agitation. Sections were first rinsed in 0.1 mM PBS with 0.3% Triton X-100 and nonspecific binding was suppressed by preincubation with 3% normal goat serum (NGS) in 0.1 mM PBS with Triton for 1 h at room temperature. Sections were then transferred into primary antibodies (goat anti-NR2B and anti-NR1, 1:800; Santa Cruz Biotechnology, Inc.) in PBS with Triton and 3% NGS, and incubated at 4°C for 48–72 h. After thorough rinsing, bovine anti-goat IgG-FITC at a dilution of 1:800 was used as the secondary antibody (incubation for 2 h at room temperature). The primary antibodies have been well characterized and have been used in numerous studies in several different species (Santa Cruz Biotechnology). We demonstrated the specificity of the secondary antibody by preblocking with serum and by including primary antibody-free controls in each experiment. We never observed any immunoreactivity in these control sections. The specificity of the staining was further evidenced by the fact that only certain cell types showed immunolabeling with this antibody. To quantify the staining, sections from three animals were used for quantitative analysis at both PFC and V1. We manually counted the puncta numbers localized in labeled neurons and more than one section per brain

region was analyzed quantitatively. Data from a previous study of Nissl-stained neurons was used to delineate the cortical layers. The data were analyzed with Student's t test for statistical significance and the comparisons are expressed as means ± SE. Photomicrographs were prepared in Adobe Photoshop and Canvas.

Western Blot. Tissues containing PFC or V1 were dissected and homogenized in lysis buffer. Equal amounts of protein samples were separated and electrotransferred onto nitrocellulose membranes (Invitrogen Inc.), which are probed with anti-NR2A and anti-NR2B (Millipore Corp.), with β-actin as a loading control (Sigma-Aldrich). The secondary antibody is an HRP-conjugated goat anti-rabbit (or mouse) IgG (ECL plus) diluted at 1:2,000. Signals are detected with ECL Western Blotting System (Amersham Bioscience). Band densities are measured with Image J (NIH) and are normalized to those of β-actin, with background subtraction. This normalization allows comparisons of band densities from different immunoblot probed with the same antibody.

1. Gao WJ, Krimer LS, Goldman-Rakic PS (2001) Presynaptic regulation of recurrent excitation by D1 receptors in prefrontal circuits. *Proc Natl Acad Sci USA* 98:295–300.

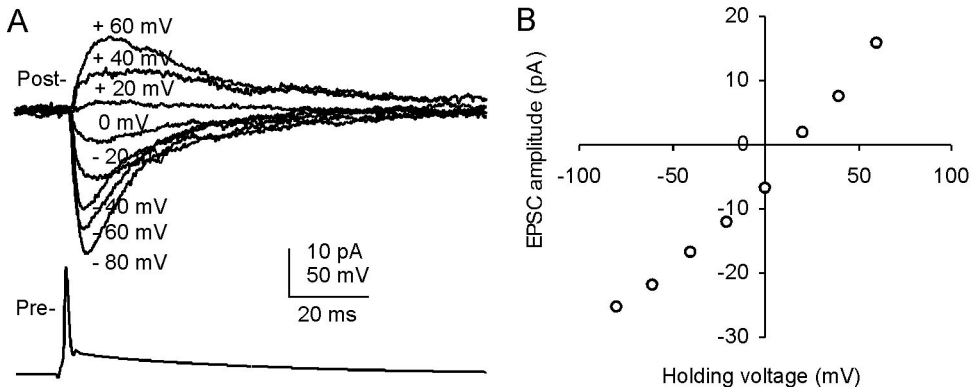

Fig. S1. (*A*) EPSC measured with the membrane potential of the postsynaptic neurons held at different levels. (*B*) Peak EPSC as a function of the holding voltage. The reversal potential was about +10 mV under our recording conditions.

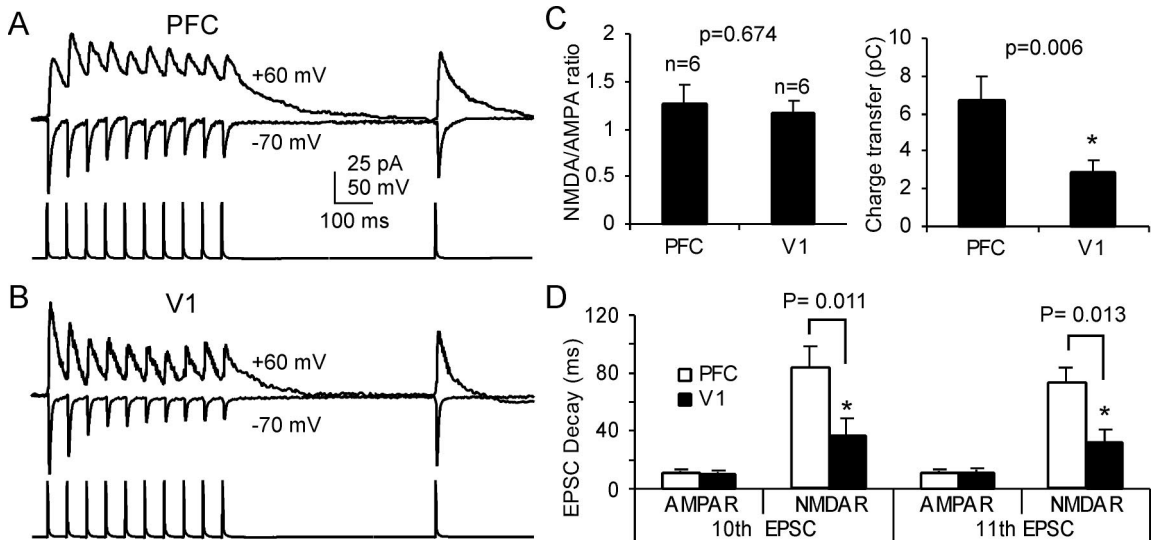

Fig. S2. Examples and summary graphs showing the layer 5 pyramidal-pyramidal (P-P) connections recorded from young adult (3–4.5 months) ferret mPFC ($n = 6$) and V1 ($n = 6$). (*A* and *B*) a sample P-P connection of PFC (*A*) and a P-P pair from V1 (*B*) showing the NMDAR-mediated EPSCs recorded at +60 mV in the presence of CNQX (20 μM) and picrotoxin (50 μM). The NMDAR-mediated EPSCs in the PFC synapses exhibited more summation than that in V1 synapses because of the significant slower time constant decay. (*C*) PFC neurons exhibited a similar NMDA/AMPA peak current ratio ($P = 0.674$) but significantly larger charge ($P < 0.01$) compared to V1. (*D*) The decay time constant of the NMDAR-mediated EPSC is longer in PFC vs. V1, both at the end of a train of ten stimuli at 20 Hz and for a single recovery stimulus ($P < 0.05$).

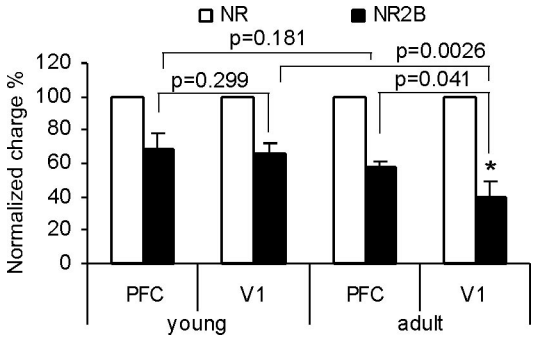

Fig. S3. In adult rats, the charge transfer of NR2B-containing NMDAR-mediated EPSCs is smaller at V1 recurrent synapses compared to PFC. There is also no difference between young and adult PFC synapses.

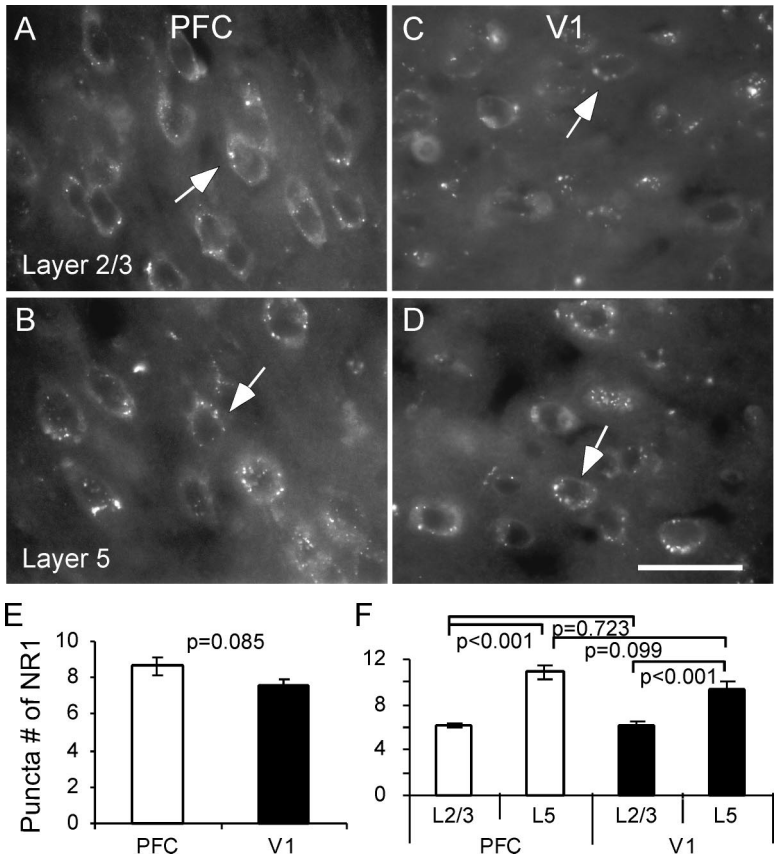

Fig. S4. Immunofluorescence labeling of NR1 subunits in the PFC and V1 in adult rats. (*A–D*) The NR1-labled neurons in the PFC exhibited similar density of puncta compared with those in V1. Arrows indicate sample cells selected for quantifying measurements. (Scale bar, 20 μm.) (*E* and *F*) Summary graphs showing the differences in puncta number (cell number = 100, $P = 0.085$) between PFC and V1. The immunofluorescence puncta were manually counted from individual labeled neurons and 50 neurons were selected for quantification in each cortical layer. In both PFC and V1, layer 5 neurons express significantly more puncta compared with those in layer 2/3.

Table S1. Weighted decay time constant of AMPA and NMDA receptor-mediated currents

		PFC		V1				
		τ_1	τ_2	τ_w	τ_1	τ_2	τ_w	*P*
10th EPSC	AMPA current	13.0 ± 1.84	5.7 ± 1.24	9.8 ± 1.16	14.5 ± 4.37	9.6 ± 2.23	11.2 ± 1.53	0.758
	NMDA current	131.0 ± 26.97	29.5 ± 7.24	92.7 ± 12.58	43.9 ± 9.56	14.3 ± 2.16	23.2 ± 5.81	0.0011*
11th EPSC (500 ms after 10th)	AMPA current	23.6 ± 5.65	9.1 ± 2.99	14.4 ± 3.58	17.4 ± 4.90	10.6 ± 2.09	10.9 ± 1.09	0.358
	NMDA current	91.9 ± 21.39	33.4 ± 9.08	81.8 ± 11.55	32.6 ± 3.92	20.6 ± 2.81	25.9 ± 5.11	0.0031*

*, $P < 0.01$ for comparison of τ_w between PFC and V1.