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Development of NMDA NR2 Subunits and Their Roles in Critical Period Maturation of Neocortical GABAergic Interneurons

Zhi Zhang and Qian-Quan Sun*

Department of Zoology and Physiology, University of Wyoming, Laramie, WY 82071

Abstract

The goals of this research are to 1) determine the changes in the composition of NMDA receptor (NMDAR) subunits in GABAergic interneurons during critical period (CP); and 2) test the effect of chronic blockage of specific NR2 subunits on the maturation of specific GABAergic interneurons. Our data demonstrate that: 1) The amplitude of NMDAR mediated EPSCs (EPSC_{NMDAR}) was significantly larger in the postCP group. 2) The coefficient of variation (CV), τ_{decay} and half-width of EPSC_{NMDAR} were significantly larger in the preCP group. 3) A leftward shift in the half-activation voltages in the postCP vs. preCP group. 4) Using subunit-specific antagonists, we found a postnatal shift in NR2 composition towards more NR2A mediated EPSC_{NMDAR}. These changes occurred within a 2-day narrow window of CP and were similar between fast-spiking (FS) and regular spiking (RSNP) interneurons. 5) Chronic blockage of NR2A, but not NR2B, decreased the expression of parvalbumin (PV), but not other calcium binding proteins in layer 2/3 and 4 of barrel cortex. 6) Chronic blockage of NR2A selectively affected the maturation of IPSCs mediated by FS cells. In summary, we have reported, for the first time, developmental changes in the molecular composition of NMDA NR2 subunits in interneurons during CP, and the effects of chronic blockage of NR2A but not NR2B on PV expression and inhibitory synaptic transmission from FS cells. These results support an important role of NR2A subunits in developmental plasticity of fast-spiking GABAergic circuits during CP.

Keywords

NR2A; NR2B; barrel cortex; synaptic plasticity; critical period; inhibitory network; fast-spiking interneuron

Introduction

Functional NMDARs are heteromers that contain multiple glycine-binding $\zeta 1$ (NR1) subunits and at least one type of $\epsilon 1-4$ (NR2 A–D) subunit that determines the receptor's distinct physiological properties and functions (Monyer et al., 1994; Laube et al., 1998; Kohr et al., 2003; Cull-Candy and Leszkiewicz, 2004; Furukawa et al., 2005; Ulbrich and Isacoff, 2008). $\chi 1$ (NR3A) subunits alone cannot form functional receptors, but can coassemble with the NR1/NR2 complex (Das et al., 1998; Perez-Otano et al., 2001). The notion that there is a developmental increase in the NR2A/NR2B ratio of synaptic NMDARs in principal/excitatory neurons is supported by robust experimental evidence (Hestrin, 1992; Watanabe et al., 1992; Monyer et al., 1994; Sheng et al., 1994; Li et al., 1998; Stocca and Vicini, 1998; Nase et al., 1999; Liu et al., 2004b). The change in NR2A/2B ratio is concomitant with the development of neural circuits and is regulated by sensory experiences (Carmignoto and Vicini, 1992; Flint et al., 1997; Ramoa and Prusky, 1997; Quinlan et al.,

*neuron@uwyo.edu, telephone 307 766 5602, fax 307 766 5526.

1999a; Quinlan et al., 1999b; Roberts and Ramoa, 1999; Chen et al., 2000; Philpot et al., 2001a; Philpot et al., 2001b; Fagiolini et al., 2003; Franks and Isaacson, 2005). It has been postulated that the NMDAR mediated long-term plasticity may underlie the formation of the CP (Kirkwood and Bear, 1994; Feldman et al., 1999; Barth and Malenka, 2001; Philpot et al., 2001a; Erisir and Harris, 2003; Daw et al., 2007). However, changes in NMDAR subunit composition may not be necessary for the role of NMDARs during CP plasticity (Lu et al., 2001; Medina et al., 2001).

It has been postulated that cortical GABAergic cells are prime targets for NMDA hypofunction in schizophrenia (Olney and Farber, 1995; Grunze et al., 1996). Several lines of evidence also link NMDA function with GABAergic cells. First, the NMDA receptors located on GABAergic interneurons are more sensitive to NMDA receptor antagonists than the NMDA receptors on pyramidal neurons (Grunze et al., 1996; Li et al., 2002). Second, chronic administration of NMDA antagonists decrease GAD expression and GABAergic inhibition (Behrens et al., 2007; Zhang et al., 2008; Lodge et al., 2009). Genetic deletion of NMDAR from Ppp1r2-Cre positive neurons (which randomly labels corticolimbic GABAergic cells) from birth decreases the number of GABAergic cells accompanied by disinhibition in pyramidal neurons and produced schizophrenia-related behaviors in mice (Belforte et al., 2010). Despite the importance of NMDAR to GABAergic interneurons, little is known regarding 1) developmental regulation of the molecular composition of NMDAR subunits, 2) the contribution of specific NMDAR subunits to the development of interneurons. Therefore the main goal of this study is to experimentally examine these two questions, which will provide insights into the mechanisms underlying experience-dependent maturation of inhibition.

GABA-mediated inhibition plays an important role in CP plasticity in mouse visual cortex (Komatsu, 1994; Fagiolini and Hensch, 2000). In the barrel cortex, it has been demonstrated that GABA-immunopositive synaptic populations increase abruptly between P10 and P15 (Micheva and Beaulieu, 1996), which is coincident with the CP for the formation of receptive fields in layer 2/3 barrel cortex (Stern et al., 2001). In addition, the maturation of GABA synapses (Jiao et al., 2006) and intrinsic firing properties of fast-spiking interneurons are experience-dependent (Sun, 2009). Although the involvement of glutamatergic transmission has been demonstrated (Sun et al., 2009), it is unclear whether the change in the molecular composition of NMDARs plays any role during CP. The main obstacle in studying the GABAergic cells in cortical circuits is that they occupy 10–20% of total neurons within the neocortex (Defelipe, 1993; Cauli et al., 1997; Kawaguchi and Kubota, 1997; Gupta et al., 2000; Defelipe, 2002). Most widely used techniques (e.g. western blot, in situ hybridization) in analyzing receptor molecular composition could not provide quantitative information regarding receptors involved in synaptic transmission in this small group of cells. By using whole-cell patch clamp recording from GAD67 GFP-positive neurons, for the first time, we demonstrate the change in NR2A/NR2B ratio in cortical interneurons during CP development. We show that blocking NR2A from early postnatal period impaired the expression of PV but not other calcium binding proteins, and selectively impaired inhibitory transmission from FS cells. This evidence support the hypothesis that developmental shift in NMDARs NR2 subunits is involved in the developmental maturation of specific GABAergic circuits during CP.

Materials and Methods

The use of animals was based on protocols approved by Institutional Animal Use Committee of Univ. Wyoming. **Transgenic GAD67-GFP (Δ neo) mice.** The selective expression of GFP is under the control of endogenous GAD67 gene promoter (Tamamaki et al., 2003; Jiao et al., 2006). In this strain, GFP is expressed in virtually all (~95%) GABAergic interneurons.

In layer 4 barrel cortex, ~80% of the GFP positive neurons are PV-positive fast-spiking (FS) basket cells (Sun, 2009). In this study, ~50% of the GFP positive neurons in layer 2/3 barrel cortex were FS cells, the rest of GFP positive neurons were predominantly regular spiking non-pyramidal (RSNP) neurons (data not shown). We divided GAD67 GFP-positive mice into the following two age groups: P6–10 as a pre-CP group and P20–40 as a post-CP group. The rationale for choosing appropriate age groups are as follows: in the barrel cortex layer 2/3, the development of receptive field properties exhibits a very narrow CP between P12–P14 (Stern et al., 2001). The major ascending projection connecting L4 and L2/3 barrel cortex is not fully developed until P15 (Micheva and Beaulieu, 1996; Stern et al., 2001; Bender et al., 2003; Bureau et al., 2004). Meanwhile, the expression of NR2A subunits starts at P7 and reaches peak at P20 (Monyer et al., 1994). We further divided the preCP group into P6–7 and P8–10 subgroups, and postCP group into P20–30 and P31–40 subgroups (Fig. 1A1). In this study, the differences in the NMDAR compositions between the pre- and post-CP groups, as well as within the preCP and the postCP groups were analyzed. In addition, based on the firing patterns, the postCP interneurons can be further divided into FS and RSNP neurons (Kawaguchi and Kubota, 1997). However, the interneurons in the preCP group could not be separated into different subgroups based on the firing patterns (instead they were collectively called immature multiple-spiking neurons) (Massengill et al., 1997), morphological features (Fig. 1B1) or PV expression (Alcantara et al., 1993). Therefore, we only separated the interneurons in the postCP group and made comparisons between the preCP and postCP (FS+RSNP), the preCP and postCP (FS), as well as the preCP and postCP (RSNP).

Slice Preparation

GAD67-GFP-positive mice, ages ranging from P6 to P40, were deeply anesthetized with nembutal (40 mg/kg) and decapitated. Brains were quickly removed and transferred into cold (~4°C) oxygenated cutting solution containing the following (in mM): 2.5 KCl, 1.25 NaH₂PO₄, 10 MgSO₄, 0.5 CaCl₂, 11 glucose, and 23.4 sucrose. Thalamocortical (TC) slices were prepared according to methods described by Agmon and Connors (Agmon and Connors, 1991; Agmon et al., 1995). The reason why we use TC slices is because this preparation maximally preserves intracortical connections between layer 4 and layer 2/3 (Micheva and Beaulieu, 1996; Stern et al., 2001; Bender et al., 2003; Bureau et al., 2004). The slices (200 μ m) were cut using a vibratome (TPI, St. Louis, MO) and incubated in a holding chamber at 35°C for 1 h and subsequently at room temperature before being transferred to a recording chamber. The slices were fixed to a modified microscope stage, and allowed to equilibrate for at least 30 min before recording. The slices were minimally submerged and continuously superfused with oxygenated (95% O₂-5% CO₂) artificial CSF (aCSF) containing the following (in mM): 126 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 2 MgSO₄, 2 CaCl₂, 26 NaHCO₃, and 10 glucose, pH 7.4, at the rate of 4.0 ml/min.

Whole-cell Patch Recording

Recordings were obtained at 35 $\pm$ 1°C from GFP positive neurons in layer 2/3 barrel cortex (Fig. 1A2). Capillary glass pipette recording electrodes (1.5–2 μ m tip diameters, 3–6 M Ω) was filled with solution containing (in mM): 120 cesium gluconate, 10 phosphocreatine-Tris, 3 MgCl₂, 0.07 CaCl₂, 4 EGTA, 10 HEPES, 4 Na₂-ATP, and 1 Na-GTP, (pH 7.4 adjusted with Cs-OH, 280 mOsm). Neurobiotin (0.5%; Vector Labs, Burlingame, CA) was regularly added to patch pipette solution for morphological reconstruction of neurons. A sharpened bipolar tungsten electrode, placed in close proximity to the recorded neurons, was used to deliver synaptic stimulation at low frequency (0.1 Hz) (Fig. 1A2). The intensity of the stimulus was maintained at ~ 15% over the threshold of the postsynaptic responses. The thresholds were defined as a large proportion of failures (Dobrunz and Stevens, 1997). For example, during five consecutive recordings, the stimulation could induce the postsynaptic

response in only one or two of the recordings. In voltage clamp, the membrane potential was held at +40 mV to reveal EPSCs_{NMDAR}. EPSCs were recorded with a multiclamp 700B amplifier and pClamp software (Molecular Device, Sunnyvale, CA). Series resistance was continuously monitored. Experiments in which the resistance changed by >15% were rejected. EPSCs_{NMDAR} were evoked in the presence of 10 μ M 2,3-dihydro-6-nitro-7-sulfamoyl-benzo(F) quinoxaline (NBQX) (Tocris Bioscience, Ellisville, MO) to block non-NMDA receptors, and 50 μ M picrotoxin (PTX) (Tocris) to block GABA_A receptors. Monosynaptic inhibitory postsynaptic currents (IPSCs) were evoked in pyramidal neurons with the stimuli applied adjacently and was recorded at holding potential of 0 mV in the presence of glutamate antagonist NBQX (10 μ M) and D(-)-2-amino-5-phosphonopentanoic acid (D-APV) (Tocris) (100 μ M). Minimal stimulus intensity was defined based on methods described by Allen & Stevens (Allen and Stevens, 1994). The following chemicals were applied via a local perfusion system that allowed fast switching between media: 100 μ M D-APV (Tocris), 3 μ M ifenprodil (Tocris), 0.5 μ M Ro25-6981 [(α R, β S)- α -(4-hydroxyphenyl)- β -methyl-4-(phenylmethyl)-1-piperidinepropanol] (Sigma), 0.5 μ M (R)-[(S)-1-(4-bromophenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid (NVP-AAM077) (gift from Novartis, Switzerland), 0.5 μ M (2S*,3R*)-1-(Phenanthren-2-carbonyl) piperazine-2,3-dicarboxylic acid (PPDA) (Ascent Scientific, Princeton, NJ).

Chronic blockage of NR2A or NR2B

GAD67-GFP-positive mice from the same litter were randomly divided into three groups: saline-injected (control), NVP-AAM077 injected (1.2 mg/kg, i.p.) (Fox et al., 2006) and Ro 25-6981-injected (6.0 mg/kg, i.p.) (Chaperon et al., 2003; Fox et al., 2006). The injections started at P7 for 11 or 18 days. The injection was given at a similar time daily. At P18 or P25, mice were given a lethal injection of Nembutal and perfused intracardially with 0.9% sodium chloride, followed by 4% paraformaldehyde. The brain was then removed, TC sections were prepared as previously described (Agmon and Connors, 1991).

Fluorescent labeling

TC sections (40 μ m) were incubated in 0.6% H₂O₂ for 30 min, PBS washed, switched to 50% alcohol for 10 min, PBS washed, and then incubated in TBS with 0.5% Triton X-100, 2% BSA, and 10% normal goat serum for 2 h at room temperature, and incubated in primary antibodies directed against PV (1:1000; Calbiochem) and VGluT2 (1:250; Chemicon) at 4 °C overnight. The next day, after PBS washed, sections were incubated in Alexa Fluor 594 and goat anti-rabbit IgG (heavy and light chains; 1:1000; Invitrogen, Carlsbad, CA) and Alexa Fluor 350 and goat anti-mouse IgG (heavy and light chains; 1:1000; Invitrogen, Carlsbad, CA) for PV and VGluT2 for 3 h and then washed, mounted, and coverslipped. The immunofluorescent specimens were examined using an epifluorescence microscope (Zeiss, Thornwood, NY) equipped with AxioCam digital color camera. Layer 2/3, layer 4 and layer 5 barrel cortex were identified by the VGluT2 staining. The outline areas of the layer 2/3, 4 and 5 were manually defined and cell numbers within the outline areas were counted by using interactive measurement function in Axiovision 4.6 (Zeiss, Thornwood, NY). Cell density was calculated as previously described (Jiao et al., 2006). The other primary antibodies used were a polyclonal rabbit anti-calbindin (1:1000; Sigma), a polyclonal rabbit anti-calretinin (1:500; Sigma), and a monoclonal mouse anti-GAD67 (1:1000; Chemicon).

Fixation, Immunocytochemistry and Histology

After recording, slices were fixed in 100 mM phosphate-buffered solution, pH 7.4, containing 4% paraformaldehyde at 4°C for at least 24 h. Endogenous peroxidase were blocked by incubating the slices in 1% H₂O₂ for 15–20 min. After several rinses in PB solution, the slices were transferred to 1% avidin-biotinylated horseradish peroxidase

complex containing 0.1% Triton X-100 in PB (0.1 M, pH 7.4; ABC-Elite Camon, Wiesbaden, Germany) and left overnight at 4°C while being shaken lightly. Slices were then reacted using 3, 3-diaminobenzidine (DAB; Sigma) and 0.01% H₂O₂ until dendrites and axonal arbors were clearly visible (approximately 2–5 min). Slices were mounted on glass slides, embedded in DPX-mounting media (Aldrich, Milwaukee), and coverslipped for image analysis.

Data Analysis

Traces shown in the figures are the average of 10 to 20 consecutive EPSCs and all values are expressed as mean $\pm$ SEM. One-way ANOVA was used for multiple group comparisons and Bonferroni post-hoc test was used for the comparisons within groups. Two-tailed Student's *t* test was performed for two group comparisons. Significance was placed at $P < 0.05$. The rise time constants for EPSCs were calculated from a standard single-exponential fit of averaged recordings using Clampfit (Molecular Device, Sunnyvale, CA). The decay time constant was fitted by a standard double exponential function or a standard single-exponential function (Clampfit). The conductance-voltage (*g*-*V*) curve for each neuron was calculated as described by Kumar (Kumar and Huguenard, 2003) using the following equation:

$$g = I / (V - E_{rev}) \quad (1)$$

Where *I* is the averaged peak amplitude of 10 consecutive EPSCs while holding the membrane potential at a constant voltage. *V* is the holding potential. E_{rev} is the reversal potential for each neuron. The maximum conductance (g_{max}) for each neuron was calculated by fitting the individual *g*-*V* curve with Boltzmann fit using Origin 6.1 (Microcal Software, Northampton, MA) with the following equation:

$$g = \{1 + \exp[(v - v_{1/2})/k]\}^{-1} \quad (2)$$

Results

Isolation of EPSCs_{NMDAR} in preCP and postCP GFP-positive GABAergic interneurons in layer 2/3 barrel cortex

Whole-cell patch clamp recordings were obtained from visually identified GFP-positive GABAergic interneurons in layer 2/3 barrel cortex of GAD67-GFP mice. Interneurons were divided into two groups: preCP (postnatal days [P] 6–10, mean age = 7.9 ± 0.2 day, *n*=65) and postCP (P20–40, mean age = 26.6 ± 0.7 day, *n*=65). EPSCs were evoked by tungsten bipolar electrode located in close proximity to the recorded cells (Fig. 1A2). Neurons in early postnatal period had fewer dendritic arbors and shorter dendritic processes (Fig. 1B1 vs. B2). The neurons in layer 2/3 barrel cortex receive sensory inputs from layer 4 ascending projections rather than thalamus (Micheva and Beaulieu, 1996; Stern et al., 2001; Bender et al., 2003; Bureau et al., 2004), thus the EPSCs were predominantly evoked intracortically from layer 4 to 2/3 projections, as well as excitatory synapses from the same layer pyramidal cells (Holmgren et al., 2003). In each recording, the stimulation intensity was adjusted to ~15% over the threshold to reliably evoke a single EPSC without failures (Fig. 1C). To isolate EPSCs_{NMDAR}, the neurons' membrane potential was held at +40 mV in the presence of 50 μ M PTX to block GABA_A receptors and 10 μ M NBQX to block non-NMDA receptors. At P6, less than 15% (8/55) of the recorded interneurons had evoked EPSCs_{NMDAR}; at P7, ~40% (20/53); at P8–10, more than 85% (37/43); at P20–40, 94% (65/69). The synaptic EPSCs_{NMDAR} could not be evoked from P5 or younger neurons, and in rare cases when there were evoked EPSCs, they were very unstable and difficult to

perform statistical analysis or pharmacological study upon. An example of the input/output relationship curve for EPSCs_{NMDAR} is shown in Fig. 1C&D. Compared with the postCP group, the preCP neurons usually required a higher stimulation intensity to evoke reliable EPSCs_{NMDAR} (Fig. 1D) and the peak amplitude of the evoked EPSCs_{NMDAR} was generally smaller (e.g. Fig. 1D). Detailed comparisons between these two groups are shown in subsequent sections.

Differences in voltage-dependent properties of EPSCs_{NMDAR} in the preCP and postCP groups

Voltage-dependent properties of EPSCs_{NMDAR} in preCP and postCP GFP-positive GABAergic interneurons were examined by measuring the peak amplitude of EPSCs_{NMDAR} at different holding potentials from -80 to $+40$ mV. Representative traces for the two age groups were shown in Fig. 2A1& A2. For both preCP and postCP groups, the average reversal potentials (E_{rev}) were close to 0 mV (average E_{rev} was 1.3 ± 1.4 mV for preCP, $n=6$ and -2.6 ± 2.2 mV for postCP, $n=13$). The I - V curves showed prominent regions of inward rectification in I/V slopes in both age groups, however, the inward currents of the two groups peaked at slightly different holding potentials (-35 ± 3.1 mV in postCP and -30 ± 3.7 mV in preCP, $p>0.05$) (Fig. 2B). The conductance-voltage (g - V) relationship for each neuron was calculated from individual I - V curves for preCP ($n=6$) and postCP ($n=13$) groups. To quantify the voltage-dependent differences in the two groups, g - V relationships for each neuron were normalized to their respective maximum conductance (g_{max}) calculated from each individual Boltzmann fit, and the averaged g/g_{max} relationship was shown in Fig. 2C. The average half-maximal membrane potential ($g/g_{max}=0.5$) for the postCP group (-20.3 ± 2.0 mV) was significantly more hyperpolarized than preCP group (-11.1 ± 1.7 mV, $p<0.05$). This suggests that the voltage-dependent properties of the EPSCs_{NMDAR} are different between the two groups.

Difference in other properties of EPSCs_{NMDAR}

The postCP interneuron subtypes were further separated into FS and RSNP groups. FS and RSNP cells exhibit clear differences in expression of GABA_A receptors (Bacci et al., 2003), gap junctions (Beierlein et al., 2003), metabotropic glutamate receptor properties (Sun et al., 2009) and sensitivity to sensory deprivation (Sun, 2009). Under voltage-clamp mode, prior to break-in, we applied small amount of current to gain electrical access to recorded cells. We adjusted the amount of injected currents to avoid break-in and formation of whole-cell mode. Under this condition, the access resistance ranged from 400 – 700 M Ω , which was sufficient to record truncated inward sodium currents underlying action potentials (APs) from interneuron (e.g. Fig. 3A). The holding potentials were kept at a negative value of -60 mV to prevent Cs⁺ from diffusing into the cytoplasm. Under these conditions, the sodium currents underlying AP firing patterns from both the preCP and postCP interneurons were recorded and analyzed. In the postCP group, the AP firing patterns were different (Fig. 3A) and clearly consistent with those of FS and RSNP groups as previously defined (Kawaguchi et al., 1995; Sun, 2009). In this study, cells were defined as FS neurons only when their AP properties met the following three criteria: the $\tau_{decay}<2.5$ ms, half-width <2.0 ms, firing frequency >100 Hz (Fig. 3B1&B2). The interneurons were defined as RSNP neurons only when their AP properties met the three criteria: the $\tau_{decay}>3.5$ ms, half-width >2.5 ms, firing frequency <100 Hz (Fig. 3B1&B2). The neurons that did not meet the above criteria were discarded from RSNP or FS groups (but were kept as part of the postCP group). In addition, the reconstruction of neurobiotin-filled neurons could also provide useful morphological information for the postCP group (supplemental Fig. 1A&B). Based on the electrophysiological analysis, we divided the 65 recorded postCP interneurons into FS ($n=22$) and RSNP ($n=20$) groups. Because the preCP interneurons were not fully differentiated, they exhibited similar firing patterns (Massengill et al., 1997) and

morphological features. The reconstruction of neurobiotin-filled neurons could not provide useful morphological information for the preCP group (Fig. 1B1).

The properties of EPSC_SNMDAR in preCP and postCP GFP-positive GABAergic interneurons were tested at holding potential +40 mV and characterized by 5 parameters: the peak amplitude, the mean *coefficient of variation* (CV) of the peak amplitude, the half-width (widths at half-maximum amplitude, HWs), rise time constant (τ_{rise}) and decay time constant (τ_{decay}). The mean CV of the EPSC_SNMDAR in the preCP group neurons was significantly larger than the postCP RSNP ($p < 0.01$) and postCP FS groups ($p < 0.001$; Fig. 3C1&C2), respectively. The peak amplitude of EPSC_SNMDAR of the preCP group neurons was significantly smaller than the postCP RSNP ($p < 0.001$) and postCP FS groups ($p < 0.001$, Fig. 4A&B), respectively. HWs of the EPSC_SNMDAR in the preCP group neurons were significantly larger than the postCP RSNP ($p < 0.001$) and postCP FS groups ($p < 0.001$, Fig. 4C), respectively. The τ_{rise} of EPSC_SNMDAR in preCP group was significantly slower than postCP RSNP ($p < 0.01$) and postCP FS groups ($p < 0.01$, Fig. 4D), respectively. Based on the results from double exponential fit ($\tau_{\text{decay-Fast}}$ and $\tau_{\text{decay-Slow}}$), the EPSC_SNMDAR in preCP group decayed at a significantly slower rate than the postCP RSNP and postCP FS groups (Fig. 4A1, A2&E), respectively. Using single exponential fit, τ_{decay} of preCP group (145.4 ± 6.2 ms) was also significantly slower than the postCP group (63.0 ± 2.9 ms, $p < 0.001$, supplemental Fig. 2A1–A3). Within the postCP group, there were no significant differences between P20–30 and P31–40 subgroups in both decay time and half-width in the same cell type (FS or RSNP, see supplemental Fig 2). This suggests that developmental changes in these properties occurred during the CP.

No significant differences in the CV ($p = 0.7$), peak amplitude ($p = 0.2$), τ_{rise} ($p = 0.7$) and $\tau_{\text{decay-Slow}}$ ($p = 0.2$) between RSNP and FS cells of the postCP group (Fig. 4B, D&E). However, the RSNP neurons had significant longer half-width ($p < 0.001$) and $\tau_{\text{decay-Fast}}$ ($p < 0.001$) than the FS (Fig. 4C&E). These data showed that there were a number of differences in the EPSC_SNMDAR properties, which may be due, at least in part, to the differences in the subunit composition of the NMDARs.

Since the NR2A and NR2B mediate different phase of EPSC_SNMDAR (Monyer et al., 1994; Sheng et al., 1994), we also calculated the area of the EPSC_SNMDAR. We compared the areas between control groups (preCP, postCP RSNP and postCP FS) and found that there was no significant difference (one-way ANOVA, $p > 0.05$; supplemental Fig. 3A). This lack of differences in area of EPSC_SNMDAR can be explained by the opposite developmental changes in the peak amplitude and τ_{decay} . The neurons in preCP group had the smallest peak amplitude and the longest τ_{decay} ; the postCP FS neurons had the largest peak amplitude and shortest τ_{decay} (Fig. 4). As a result, the areas (amplitude*duration) measured in the preCP and postCP FS were similar.

To examine whether different stimulation intensities could change the outcome of our results, we analyzed the responses of neurons under minimal and 15% over minimal stimulation conditions (supplemental Fig. 1C&D). Although the evoked responses were smaller under minimal stimulation vs. 15% over minimal stimulation, the trends of the developmental changes were the same. This suggests that the developmental differences in NMDAR properties demonstrated here do not depend on the stimulation paradigms used.

Developmental shift in NR2A/NR2B ratio

We next examined if there were any changes in pharmacological properties of EPSC_SNMDAR in GABAergic interneurons. Local perfusion of 0.5 μM NVP-AAM077 (a selective NR2A subunit antagonist, see next section for detailed pharmacological characterizations) (Auberson et al., 2002; Liu et al., 2004a; Gerkin et al., 2007) or 0.5 μM

Ro25-6981 (an ifenprodil derivative that has high affinity and selectivity as an activity-dependent, voltage-independent, non-competitive NR2B subunit antagonist) (Mutel et al., 1998; Lynch and Guttman, 2001), followed by the mixture (i.e. NVP-AAM077 plus Ro 25-6981) were used to examine the subunit composition for NMDARs. If the EPSC_{NMDAR} were mediated solely by NMDARs composed of NR1/NR2A or NR1/NR2B subunits, the application of the mixture (NVP-AAM077 plus Ro25-6981) would eliminate the EPSC_{NMDAR}. In cells where EPSC_{NMDAR} were not completely blocked by the mixture, PPDA (selective NR2C/D antagonist, 0.5 μ M) (Feng et al., 2004; Morley et al., 2005; Harney et al., 2008) were used to test whether the EPSCs were mediated by NR2C/NR2D. APV (100 μ M) was used to confirm whether the EPSCs were mediated by NMDARs. The representative traces recorded in the absence and presence of each antagonist, or the mixture were shown in Fig 5A & B. The typical time courses of all antagonists' actions were shown in Fig. 6A1–A3 & B1–B3.

Local perfusion of NVP-AAM077 (0.5 μ M) blocked 54.2 ± 13.4 % of EPSC_{NMDAR} at P6–7; 62.8 ± 5.9 % at P8–10; 81.1 ± 6.1 % at P20–30; 73.5 ± 7.4 % at P31–40. Application of Ro25–6981 (0.5 μ M) blocked 39.1 ± 8.7 % of EPSC_{NMDAR} at P6–7; 37.9 ± 7.7 % at P8–10; 24.9 ± 9.9 % at P20–30; 21.6 ± 7.2 % at P31–40 (Fig 5C). Next, we examined the effects of another NR2B antagonist, ifenprodil (Tovar and Westbrook, 1999; Cull-Candy et al., 2001). The results obtained from ifenprodil (3 μ M) application were very similar to the effects of Ro25–6981. Ifenprodil blocked 43.2 ± 12.9 % of EPSC_{NMDAR} at P6–7; 30.4 ± 8.1 % at P8–10; 21.2 ± 4.0 % at P20–30; 10.4 ± 2.6 % at P31–40 (Fig. 5C). The typical traces representing the effects of NVP-AAM077, Ro 25–6981 and ifenprodil on the amplitudes and τ_{decay} of EPSCs for preCP and postCP groups were shown in Fig 5A1–A3 & B1–B3. If these pharmacological agents were receptor subunit selective as previously demonstrated, these pharmacological data demonstrated that there was a developmental shift in pharmacological sensitivities of the EPSC_{NMDAR} during development: while postCP EPSC_{NMDAR} became less sensitive to Ro 25–6981 or ifenprodil, they became more sensitive to NVP-AAM077. In addition, we analyzed the effects of NVP-AAM077 (0.5 μ M) and ifenprodil (3 μ M) on the peak amplitude of RSNP and FS neurons at P20–30 and P31–40. NVP-AAM077 blocked 67.5 ± 6.6 % of EPSC_{NMDAR} in RSNP and 79.1 ± 4.8 % in FS at P20–30; 64.4 ± 11.3 % in RSNP and 80.1 ± 8.5 % in FS at P31–40. No significant difference between RSNP and FS neurons (Fig. 5D). Ifenprodil blocked 20.4 ± 5.2 % of EPSC_{NMDAR} in RSNP and 20.4 ± 4.4 % in FS at P20–30; 21.4 ± 13.1 % in RSNP and 16.1 ± 6.6 % in FS at P31–40. No significant difference between RSNP and FS neurons (Fig. 5D). There were no significant differences in NR2A (NVP-AAM077) or NR2B (ifenprodil) antagonists mediated effects between P6–7 and P8–10 subgroups (Fig. 5C), or between P20–30 and P31–40 subgroups in the same cell types (FS or RSNP, Fig. 5D). This suggests that developmental changes in the pharmacological properties occurred during the CP.

The decay time is tens of milliseconds for the NR1/NR2A combination and hundreds of milliseconds for the NR1/NR2B and NR1/NR2C combination (Vicini et al., 1998), and seconds for NR1/NR2D channels (Monyer et al., 1994). Our earlier results indicated that young EPSC_{NMDAR} had a much slower τ_{decay} (Fig. 4E). If the pharmacological agents are as selective as suggested, the application of these antagonists would be expected to have different effects on τ_{decay} . This was indeed the case. The τ_{decay} not only increased after NVP-AAM077 application, but the increase in τ_{decay} by NVP-AAM077 was also different in the two age groups. In the preCP group (n=16), the $\tau_{\text{decay-Fast}}$ increased 10.9 ± 17.3 ms (p= 0.3) and the $\tau_{\text{decay-Slow}}$ increased 298.9 ± 141.6 ms (p<0.05). In the postCP group (n=11), the $\tau_{\text{decay-Fast}}$ increased 35.6 ± 18.7 ms (p<0.05) and the $\tau_{\text{decay-Slow}}$ increased 168.3 ± 81.0 ms (p<0.05) (Table 1). Ro25–6981 and ifenprodil had the opposite effect on τ_{decay} : i.e., induced a decrease in τ_{decay} . The decrease in τ_{decay} was also different between the two age groups. In the preCP group (n=14), the $\tau_{\text{decay-Fast}}$ decreased 63.4 ± 20.1 ms (p<0.01) and

the $\tau_{\text{decay-Slow}}$ decreased 289.6 ± 65.3 ms ($p < 0.0001$). In the postCP group ($n=12$), the $\tau_{\text{decay-Fast}}$ decreased 6.5 ± 4.1 ms ($p=0.1$) and the $\tau_{\text{decay-Slow}}$ decreased 33.5 ± 72.5 ms ($p=0.7$) (Table 1). The effect of ifenprodil was similar to Ro 25–6981. In the preCP group ($n=20$), the $\tau_{\text{decay-Fast}}$ decreased 37.1 ± 8.8 ms ($p < 0.001$) and the $\tau_{\text{decay-Slow}}$ decreased 173.6 ± 51.8 ms ($p < 0.001$). In the postCP group ($n=35$), the $\tau_{\text{decay-Fast}}$ decreased 0.1 ± 1.9 ms ($p=1.0$) and the $\tau_{\text{decay-Slow}}$ decreased 72.2 ± 29.6 ms ($p < 0.05$) (Table 1). The changes in the fast τ_{decay} did not reach statistical significance in the postCP group after Ro 25–6981 or ifenprodil application. These results suggest that the $\tau_{\text{decay-Fast}}$ was less likely mediated by NR2B in the postCP group. In contrast, the change in the $\tau_{\text{decay-Fast}}$ did not reach statistical significance in the preCP group after NVP-AAM077 application. It may indicate that the $\tau_{\text{decay-Fast}}$ was less likely mediated by NR2A in the preCP group. Thus, our data with either ifenprodil (or Ro25–6981) alone, or NVP-AAM077 alone, showed significant differences between preCP vs. postCP group. The effects of pharmacological agents are thus consistent with the EPSC_{sNMDAR} τ_{decay} data, which suggest that there was a shift in the contribution of NR2B to NR2A subunits to EPSC_{sNMDAR} in GABAergic interneurons during development.

We also compared the inhibition levels calculated by area following the application of antagonists. The area is determined by both peak amplitude and duration of EPSC_{sNMDAR}. The NR1/NR2A combination has larger peak amplitude (Monyer et al., 1992) and shorter decay time course than NR1/NR2B (Vicini et al., 1998). In theory, when NMDARs are mainly composed of NR1/NR2A and/or NR1/NR2B, blocking of NR2A subunits will decrease the peak amplitude of EPSC_{sNMDAR} and increase the decay time of NMDAR_{EPSCs}. In contrast, blocking of NR2B subunits will decrease both the amplitude and decay time of EPSC_{sNMDAR}. To see whether this was the case, we compared the results calculated by peak amplitude with the results calculated by area. We found that the percentage of EPSC_{sNMDAR} blocked by NVP-AAM077 (NR2A antagonist) calculated by area was smaller than that by peak amplitude. In contrast, the percentage of EPSC_{sNMDAR} blocked by ifenprodil or Ro25–6981 (NR2B antagonists) calculated by area was bigger than that by peak amplitude (Fig. 5C vs. supplemental Fig. 3B; Fig. 5D vs. supplemental Fig. 3C). These results demonstrate that NR2A and NR2B subunit-mediated EPSC_{sNMDAR} exhibit distinct characteristics.

In addition, within the same age group (such as preCP and postCP), there was variability in the amplitude of evoked EPSC_{sNMDAR} under 15% over minimal stimulation intensity. To examine whether the variability in the amplitude of EPSC_{sNMDAR} affected the effect of antagonists, we also analyzed the relationship between the effect of ifenprodil (NR2B antagonist) and the amplitude of EPSC_{sNMDAR}. For each age group, the neurons were further divided into two subgroups based on their peak amplitude. Although there was a significant difference in the evoked amplitude of EPSC_{sNMDAR} between the groups (supplemental Fig. 1E), there was no significant difference in the effect of ifenprodil (supplemental Fig. 1F). These results indicate that the evoked amplitude of EPSC_{sNMDAR} has no significant impact on the effect of antagonists.

The specificity of NMDAR antagonists

Although NVP-AAM077 is reported to have very high affinity for NR2A subunits (Auberson et al., 2002; Liu et al., 2004a; Gerkin et al., 2007), it is also reported that NVP-AAM077 discriminates poorly (~10 fold) between NR2A- and NR2B-containing receptors in rodent NMDARs expressed in *Xenopus* oocytes examined by the ability of NVP-AAM077 to inhibit current responses induced by application of glutamate plus glycine (Neyton and Paoletti, 2006; de Marchena et al., 2008). To examine whether the concentrations of the antagonists used in this experiment were sufficient to distinguish different receptor subtypes underlying synaptically activated EPSC_{sNMDAR}, we next tested the specificity of NMDAR antagonists. In our dose-response experiments, we tested

different concentrations of NVP-AAM077 (0.01, 0.05, 0.1, 0.5, and 5 μ M) with or without ifenprodil (3 μ M) in the bath perfusion solution (Fig. 6C) in P8–10 (n=6) and P20–21 (n=6) interneurons. As indicated in the dose-response curve, 0.5 μ M NVP-AAM077 blocked $95.7 \pm 3\%$ of the NMDAR EPSCs with 3 μ M ifenprodil in the bath perfusion, and $72.9 \pm 5\%$ of EPSC_{NMDAR} without ifenprodil. 3 μ M ifenprodil alone blocked $28.6 \pm 3.4\%$ (n=55) of EPSC_{NMDAR}, which was very similar to the differences between the mixture (i.e. NVP-AAM077 plus ifenprodil) and NVP-AAM077 alone ($95.7\% - 72.9\% = 24\%$). In addition, there were no significant differences in the IC₅₀ values for NVP-AAM077 in the presence or absence of ifenprodil (Fig. 6C). These results suggested that 0.5 μ M NVP-AAM077 was an appropriate concentration to be used for pharmacological separation of NR2A vs. NR2B mediated EPSC_{NMDAR} in brain slices used in similar experiment conditions.

The specificity of antagonists to different subunits should be independent of animals' age. We found that this was indeed true. When we combined the data from both age groups, NVP-AAM077 (0.5 μ M) alone blocked $70.3 \pm 3.7\%$ of EPSC_{NMDAR} (n=27); Ro25-6981 (0.5 μ M) alone blocked $31.5 \pm 4.3\%$ (n=26), and ifenprodil (3 μ M) alone blocked $28.6 \pm 3.4\%$ (n=55) (Fig. 6D). If the concentrations we used were too high or too low, when we added the effects of NVP-AAM077 alone plus Ro25-6981 alone (or NVP-AAM077 alone plus ifenprodil alone), the percentage would be much larger or lower than 100%, respectively. We found that none of the above scenario appeared to be true. When the effects of NVP-AAM077 alone and Ro 25-6981 alone, or NVP-AAM077 alone and ifenprodil alone were added together, they were very close to 100% (see Fig. 6D & E). In addition, the application of mixtures (NVP-AAM077 plus Ro 25-8198 or ifenprodil) blocked $98.4 \pm 0.3\%$ of EPSC_{NMDAR} when NVP-AAM077 was applied first, $99.8 \pm 0.1\%$ when Ro 25-6981 was applied first, and $92.7 \pm 2.1\%$ when ifenprodil was applied first (Fig. 6D). After the mixtures were applied, the application of APV (100 μ M) completely eliminated the residual of EPSC_{NMDAR} in NVP-AAM077 (applied first) or Ro 25-6981 (applied first) groups, and inhibited $99.8 \pm 0.2\%$ of the residual of EPSC_{NMDAR} in ifenprodil (applied first) group (Fig. 6D). These data suggested that at the current concentration used in our experiments, these antagonists (NVP-AAM077, Ro 25-8198, and ifenprodil) selectively blocked distinct groups of NMDARs. Together these data suggested that the NMDARs in GABAergic interneurons are mainly composed of NR1, NR2A and NR2B subunits.

If the NMDARs were mainly composed of NR1, NR2A and NR2B subunits, after the application of NR2A specific antagonist, the remaining EPSC_{NMDAR} would be mediated by NR2B subunits, and *vice versa*. For additional confirmation, we calculated the remaining NR2A- and NR2B-mediated EPSCs by the following equations:

$$NR2B\ EPSC_{remaining} = (NVP - AAM077 + Ro\ 25 - 6981) - NVP - AAM077. \quad (3)$$

$$NR2A\ EPSC_{remaining\ 1} = (NVP - AAM077 + Ro\ 25 - 6981) - Ro\ 25 - 6981 \quad (4)$$

and

$$NR2A\ EPSC_{remaining\ 2} = (NVP - AAM077 + ifenprodil) - ifenprodil \quad (5)$$

The average remaining NR2B EPSCs calculated from the equation (3) was $29.4 \pm 3.7\%$, (n=27), which was not statistically different ($p > 0.05$) from the effects produced by Ro 25-6981 alone ($31.5 \pm 4.3\%$, n=26) or by ifenprodil alone ($28.6 \pm 3.4\%$, n=55). The average

remaining NR2A-mediated EPSCs calculated from equations (4) and (5) were $68.4 \pm 4.3\%$ ($n=26$) and $64.2 \pm 4.2\%$ ($n=55$), respectively, which were not statistically different ($p>0.05$) from the percentage of inhibition produced by NVP-AAM077 alone ($70.3 \pm 3.7\%$, $n=28$). In addition, there was no statistical significance between NR2A EPSCs remaining 1 and 2 ($p>0.05$) (Fig. 6E). The results calculated by area were similar to that calculated by the peak amplitude (supplemental Fig. 3D&E vs. Fig. 6D&E). The difference in the selectivity of NVP-AAM077 between pyramidal neurons and interneurons may be due to the difference in the NMDAR components. Our results suggested that in interneurons NR2A and NR2B subunit form distinct receptors (see further details below). This may not be the case in principal neurons.

NR1/NR2A/NR2B and/or NR1/NR2C/NR2D mediated currents in preCP and postCP groups

In the preCP group ($n=65$), local perfusion of the mixture (NVP-AAM077 plus ifenprodil, or NVP-AAM077 plus Ro 25-6981) eliminated $99.7 \pm 0.2\%$ of the EPSCs_{NMDAR} in 64 out of 65 neurons, and local perfusion of APV (100 μ M) completely eliminated the residual EPSCs_{NMDAR}. There was one neuron (P9), in which the mixture only eliminated 68.5% of EPSCs_{NMDAR}. The residual EPSCs_{NMDAR} was completely blocked by NR2C/D antagonist PPDA (0.5 μ M).

In the postCP group ($n=65$), the mixture (NVP-AAM077 plus ifenprodil, or NVP-AAM077 plus Ro 25-6981) blocked $99.7 \pm 0.1\%$ of EPSCs_{NMDAR} in 56 out of 65 neurons. The residual EPSCs_{NMDAR} were completely blocked by APV. There were 9 out of 65 neurons, in which the mixture only blocked $69.3 \pm 4.7\%$ of EPSCs_{NMDAR}, and in 2 (a P20 and a P21) out of the 9 neurons, the residual EPSCs_{NMDAR} were completely blocked by PPDA (21% and 53.2%, respectively). However, the PPDA had no effect on the residual EPSCs_{NMDAR} of the rest of 7 neurons. The residual EPSCs of the 7 neurons were blocked $98.5 \pm 1.2\%$ by APV.

These results indicate that in both the preCP and postCP groups, very small population of cells (1/65 in preCP and 2/65 cells in postCP) have NR2C/D-containing NMDARs, similar to spiny stellate neurons in layer 4 barrel cortex (Binshtok et al., 2006). In the postCP group (7/65) but not in the preCP group (0/65), NR1/NR2A/NR2B-containing NMDARs may contribute to EPSCs_{NMDAR} (there was a significant difference between the preCP and postCP groups, $p<0.01$). This was because the residual EPSCs_{NMDAR} were not blocked by NR2A, NR2B or NR2C/D antagonists in 7/65 cells tested in the postCP group, but were completely eliminated by APV, which indicates that the residual EPSCs_{NMDAR} may be mediated by NR1/NR2A/NR2B (Sheng et al., 1994; Kumar and Huguenard, 2003) which may have a reduced sensitivity to the antagonists (Hatton and Paoletti, 2005).

The inhibition of plasmalemmal glutamate transporter activity increased decay time constant of EPSCs_{NMDAR}

Although the τ_{decay} of EPSCs_{NMDAR} mainly depends on the NMDAR subunit composition (Monyer et al., 1992), changes in transmitter clearance can also cause changes in synaptic properties (Clements et al., 1992). Glutamate is not degraded in the synaptic cleft. The uptake of glutamate through glutamate transporters [GTs; (Gegelashvili and Schousboe, 1997), for a review, see (Danbolt, 2001)] is the most efficient way for removing glutamate from the extracellular space and maintaining low synaptic glutamate levels. Here we tested the effects of glutamate spillover on the τ_{decay} of NMDA channels. TBOA is a nonsubstrate glutamate transporter inhibitor (Shimamoto et al., 1998) and can prolong the decay time course of NMDAR channels (Diamond, 2001; Arnth-Jensen et al., 2002; Tsukada et al., 2005). The slowing of the decay of EPSCs_{NMDAR} would reflect the extent of glutamate spillover, which causes cooperation in the activation of neighboring postsynaptic or peri-

synaptic NMDARs (Carter and Regehr, 2000; Diamond, 2001; Arnth-Jensen et al., 2002; Lozovaya et al., 2004; Scimemi et al., 2004). To examine the contribution of plasmalemmal glutamate transporter to the τ_{decay} , we recorded the EPSCs_{NMDAR} in the absence and presence of 30 μM TBOA (Tocris) in the bath perfusion. The brain slices were allowed to equilibrate in bath solution (absence or presence of 30 μM TBOA) for 30 min before recording. Representative traces obtained from averaging 10 consecutive EPSCs were shown in Fig. 7A1 & B1. The application of TBOA had no significant effect on the τ_{rise} in both groups (Table 2). However, the τ_{decay} and HWs increased significantly in both groups after TBOA application (Table 2). Interestingly, the amplitude of EPSCs_{NMDAR} decreased significantly after TBOA application in both pre- and postCP groups (Table 2). The decrease in the amplitude could be due to the postsynaptic NMDAR desensitization, which may be resulted from calcium dependent (Legendre et al., 1993; Medina et al., 1995; Krupp et al., 1996; Krupp et al., 1999) and calcium independent (Sather et al., 1990; Tong and Jahr, 1994) weakening of glycine binding site affinity (Benveniste et al., 1990; Vyklicky, Jr. et al., 1990). The magnitude of desensitization is controlled by the key residues in the NR2 subunit (Krupp et al., 1998) and actively modulated by second messengers in the postsynaptic cell (Rosenmund et al., 1995; Tong et al., 1995; Raman et al., 1996). In addition, at holding potential +40 mV, the holding current slightly increased from 370.7 ± 20.2 pA to 453.3 ± 55.2 pA after TBOA application in the postCP group ($p=0.2$), and significantly increased in preCP group (132.9 ± 9.3 pA in control vs. 210.0 ± 25.9 pA in TBOA, $p=0.01$). The effects of TBOA on both HWs and τ_{decay} were much larger in the preCP group vs. post CP group, thus suggesting that TBOA-sensitive plasmalemmal glutamate transporter play important role in the clearance of glutamate and that their role are presumably taken over by TBOA-insensitive transporters as interneurons develop. However, it was also possible that the difference in the change in time constants could be due to a developmental difference in presynaptic release between preCP and postCP groups. To test whether it was the case, we compared the change in the CV before and after TBOA application in both preCP and postCP groups. The CV significantly increased in the preCP group after TBOA application, which indicates the decrease in presynaptic release probability in preCP neurons (Schulz et al., 1994; Choi and Lovinger, 1997; Kirischuk et al., 2002). There was no significant change in the CV in postCP neurons after TBOA application (Table 2). However, the τ_{decay} significantly changed in both preCP and postCP groups after TBOA admission, which implies that the change in the time constants is unlikely due to the developmental difference in presynaptic release probabilities.

Chronic blockage of NR2A from early postnatal age decrease the expression of PV in layer 2/3 and 4 barrel cortex in GAD67 GFP interneurons

If there were developmental increases in NR2A and decreases in NR2B mediated-EPSCs_{NMDAR} in interneurons, as we have demonstrated in this study, does this play any functional role in the maturation of interneurons during the CP of development? PV expression occurs at the CP in barrel and visual cortex (Alcantara and Ferrer, 1994; Yan et al., 1996; Czeiger and White, 1997; Maier and McCasland, 1997; Letinic and Kostovic, 1998; Hada et al., 1999; Moon et al., 2002). PV-cell network controls ocular dominance plasticity onset (Hensch, 2005; Sugiyama et al., 2008). Several groups (Itami et al., 2007; Liguz-Lecznar et al., 2009; Belforte et al., 2010), including ours (Jiao et al., 2006; Sun, 2009), have shown that the amount of PV expression is regulated by sensory activities during the CP. Thus, we examined the effects of the chronic blockage of NR2A or NR2B on PV expression in GAD67-GFP-positive interneurons. The density of PV-positive neurons significantly decreased in layer 2/3 ($170 \pm 10/\text{cm}^2$ in NVP-injected vs. $230 \pm 10/\text{cm}^2$ in control, $p<0.01$) and layer 4 ($310 \pm 20/\text{cm}^2$ in NVP-injected vs. $460 \pm 40/\text{cm}^2$ in control, $p<0.001$) barrel cortex in NVP-injected brain ($n=6$ mice) compared with control ($n=6$ mice, Fig. 8A1, B1&D1). We next tested whether the reduction in PV-positive cells is due to the

reduced proliferation/increased apoptosis of PV-type GABAergic cells or the down-regulation of PV expression in a subpopulation of GABAergic cells. By using the GAD67–GFP mouse, in which >95% of GABAergic cells express GFP under the promoter of GAD67 in motor cortex (Tamamaki et al., 2003) and in somatosensory cortex (Jiao et al., 2006), we counted the GFP-positive cells. The densities of GAD67–GFP-positive neurons in the NVP-AAM077-injected barrels were similar to control brains (Fig. 8A2, B2 & E1). In layer 2/3, $510 \pm 20/\text{cm}^2$ in NVP-injected vs. $510 \pm 20/\text{cm}^2$ in control ($p=0.9$); in layer 4, $610 \pm 40/\text{cm}^2$ in NVP-injected vs. $600 \pm 40/\text{cm}^2$ in control ($p=0.8$). The PV/GFP ratio showed a significant reduction in layer 2/3 and layer 4 in NVP-injected brains (Fig. 8A3, B3 & F1). In layer 2/3, 0.45 ± 0.02 in control vs. 0.34 ± 0.02 in NVP-injected ($p<0.01$); in layer 4, 0.76 ± 0.03 in control vs. 0.51 ± 0.03 in NVP-injected ($p<0.001$). However, the total number of GAD67–GFP neurons, which is similar to the total number of GABAergic neurons, remained overall unchanged. We concluded that chronic blockage of NR2A from early postnatal age (P7) can induce profound down-regulation of PV expression in layer 2/3 and 4 GABAergic interneurons. We also examined whether there was a sensitive period for the down-regulation of PV expression by the chronic blockage of NR2A. In the GAD67–GFP mice ($n=4$ mice for control and NVP-injected, respectively), in which the NVP-AAM077 injection stopped at P18, we did not find significant changes in the density of PV cells. In layer 2/3, $170 \pm 2/\text{cm}^2$ in control vs. $150 \pm 9/\text{cm}^2$ in NVP-AAM077-injected ($p=0.8$); in layer 4, $310 \pm 40/\text{cm}^2$ in control vs. $360 \pm 20/\text{cm}^2$ in NVP-AAM077-injected, ($p=0.3$, $n=12$ slices, respectively; Fig. 8D2–F2). Thus our results suggest that chronic blockage of NR2A from P7 to P25, but not from P7 to P18, impaired developmental expression of PV. Finally, we examined the effects of chronic blockage of NR2A on the expression of calbindin and calretinin in GAD67–GFP interneurons. No significant difference was found in the expression of calbindin and calretinin in control ($n=4$ mice) vs. NVP-AAM077-injected mice ($n=4$ mice, Table 3). In contrast, chronic blockage of NR2B via injection of Ro 25-6981 from P7 for the same period (i.e. to P25 or P18), did not induce significant changes in PV expression in GAD-67 GFP interneurons (Fig. 8), suggesting that NR2A, but not NR2B, is important for the developmental expression of PV.

Perisomatic inhibition is impaired after the chronic blockage of NR2A subunits

The properties of inhibitory synaptic transmission are associated with specific calcium channels located at the nerve terminals (Poncer et al., 1997; Poncer et al., 2000). In hippocampus or neocortex P/Q channels are predominantly expressed at GABAergic terminals of FS cells, whereas N-type calcium channels were predominantly expressed at GABAergic terminals of non-FS GABAergic cells (Poncer et al., 1997; Poncer et al., 2000; Ali and Nelson, 2006; Zaitsev et al., 2007). To further study the functional maturation of GABA transmission associated with FS and RSNP cells, we applied ω -agatoxin-IVA ($2 \mu\text{M}$) (Bachem Bioscience Inc., King of Prussia, PA), a selectively P/Q calcium channels antagonist (Poncer et al., 1997; Poncer et al., 2000; Sun and Dale, 1998), and analyzed both evoked IPSCs (eIPSCs) and spontaneous IPSCs (sIPSCs) in spiny neurons in layer 4 barrel cortex, because layer 4 PV+ cells were also significantly decreased in NVP-AAM077 injected mice (Fig. 8D1 & F1). As shown in Fig. 9, the amplitude of eIPSCs was significantly smaller in NVP-AAM077 injected neurons (Fig. 9A&B) and the half-width and τ_{decay} were significantly larger in NVP-AAM077 injected neurons (Fig. 9C&D). In addition, CV and paired-pulse ratio (IPSC2/IPSC1) were significantly larger in NVP-AAM077 injected neurons (Fig. 9E&F), indicating that the presynaptic release probability was lower in NVP-injected neurons. eIPSCs in both control and NVP-AAM077 injected neurons were almost entirely eliminated by bath application of ω -agatoxin-IVA ($2 \mu\text{M}$) (Fig. 9A&B), suggesting that eIPSCs onto the spiny neurons is produced almost exclusively by FS cells, as shown in previous studies (Poncer et al., 1997; Poncer et al., 2000; Ali and Nelson, 2006; Zaitsev et

al., 2007). Therefore the decrease in eIPSCs in NVP-AAM077 injected neurons suggests a down-regulation of inhibitory transmission from FS cells.

Next, we tested the effects of ω -agatoxin-IVA on sIPSCs. In control condition, the amplitude and frequency of IPSCs were reduced by agatoxin (Fig 10A1, B&C), suggesting that sIPSCs were mainly mediated via P/Q type calcium-dependent release from FS cells. There was no significant change in half-width and τ_{decay} (Fig. 10D&E). In NVP-AAM077 injected neurons, both the amplitude and frequency of sIPSCs were smaller than control (Fig 10A1 vs. A2, B&C). Agatoxin decreased the amplitude of sIPSCs (Fig. 10A2&B) and had no significant effect on the frequency, half-width and τ_{decay} of sIPSCs (Fig 10C, D&E), supporting results with eIPSCs.

Chronic blockage of NR2A from P7 to P25 increased the NR2B-mediated EPSCs_{NMDAR}

To further test whether the chronic blockade of NR2A activity during P7–25 affects the development changes of NMDA receptor subunit compositions in the interneuron, we analyzed the proportion of NR2B-mediated EPSCs_{NMDAR} in layer 2/3 interneurons from both control and NVP-AAM077-injected mice. Because RSNP and FS showed similar developmental changes in NR2A/NR2B ratio in naïve animals (Fig 4), we therefore treated the interneurons as one group and compared the properties of EPSCs_{NMDAR} and the percentage of NR2B-mediated currents in control and NVP-AAM077 injected mice. We found that there were a decrease in both the amplitude and area of EPSCs_{NMDAR} and an increase in CV in NVP-AAM077 injected neurons. This result suggested that there were reductions in presynaptic release probability and changes in postsynaptic properties induced by NVP-AAM077. The half-width and $\tau_{\text{decay-fast}}$ and $\tau_{\text{decay-slow}}$ increased in NVP-AAM077 injected neurons, which suggested an increase of NR2B-mediated currents (Table 4). The application ifenprodil (3 μ M) blocked $44.0 \pm 7.9\%$ of the peak amplitude of EPSCs_{NMDAR} in NVP-injected neurons, which was significantly larger than control ($20.2 \pm 3.4\%$, $p < 0.05$) (Fig. 11A&B). In addition, the application ifenprodil (3 μ M) blocked $56.8 \pm 8.4\%$ of the area of EPSCs_{NMDAR} in NVP-injected neurons, which was significantly larger than control ($27.2 \pm 4.2\%$, $p < 0.01$) (Fig. 11C). The increases in the percentage of EPSCs_{NMDAR} blocked by ifenprodil, as well as the increase in half-width and τ_{decay} , together indicated that the NR2B-mediated EPSCs_{NMDAR} increased after the chronic blockage of NR2A from P7–25. These results also support the notion that NR2A subunits, not the amount of NMDA currents carried by NR2A subunits per se, play an important role in the maturation of FS GABAergic cells.

Discussion

Developmental shift in the contribution of NR2A vs. NR2B subunits to EPSCs_{NMDAR}

In this study, by using whole cell patch clamp recording with application of subunit-specific antagonists, we demonstrated that there were developmental changes in the molecular composition of NMDAR in GABAergic interneurons in layer 2/3 barrel cortex. We found that NR2B-subunit-specific antagonists had larger effects on EPSCs_{NMDAR} in the preCP vs. postCP group. An opposite effect was obtained with NR2A-subunit-specific antagonist, NVP-AAM077, which had larger effect on EPSCs_{NMDAR} in the postCP vs. preCP group. The effects of NVP-AAM077 and ifenprodil (or Ro25-6981) were complementary: while there was roughly 20% increase in NVP-AAM077-sensitive currents (i.e. NR2A), there was a similar amount of decrease in ifenprodil-sensitive currents (i.e. NR2B) in the postCP vs. preCP group (Fig. 5). A similar decrease in the sensitivity of EPSCs_{NMDAR} to NR2B antagonist during postnatal development has also been found in the hippocampal pyramidal neurons (Kirson and Yaari, 1996).

Compared with NR2B-containing receptor channels, NR2A-containing receptor channels have considerably faster rising and decaying currents and larger peak amplitude of EPSCs (Carmignoto and Vicini, 1992; Hestrin, 1992; Monyer et al., 1992; Kutsuwada et al., 1992; Monyer et al., 1994; Kirson and Yaari, 1996; Flint et al., 1997; Stocca and Vicini, 1998; Chen et al., 1999; Roberts and Ramoa, 1999; Cathala et al., 2000; Lei and McBain, 2002; Prybylowski et al., 2002), as well as much lower affinity for ifenprodil (Williams, 1993). In this study, the postCP (RSNP and FS) interneurons have larger peak amplitude, faster rising and decaying currents (Fig 4 & Table 1), which are consistent with a larger contribution from NR2A.

The NMDAR components are highly variable among interneurons (Sah et al., 1990; Lei and McBain, 2002; Maccaferri and Dingledine, 2002). Our results are consistent with these earlier findings. We found that the preCP neurons have the longest half-width and τ_{decay} ; the postCP FS neurons have the shortest half-width and τ_{decay} ; the postCP RSNP neurons have the intermediate half-width and τ_{decay} (Fig. 4). These data not only indicate that there are differences in the molecular composition of NMDARs between the pre- and postCP (RSNP + FS), but also imply that the postCP FS neurons may have the most NR2A component. This result is consistent with an early study from cultured interneuron (Kinney et al., 2006). It is also possible that RSNP neurons have more NR1/NR2A/NR2B tri-heteromeric channels, which exhibit intermediate decay time courses between the NR1/NR2A and NR1/NR2B channel types (Vicini et al., 1998). Although the overwhelming evidence that shows developmental switch in NR2A over NR2B in pyramidal neurons (Hestrin, 1992; Watanabe et al., 1992; Monyer et al., 1994; Sheng et al., 1994; Li et al., 1998; Stocca and Vicini, 1998; Nase et al., 1999; Liu et al., 2004b), here we provide the first evidence (to the best of our knowledge), to demonstrate that there is a similar developmental shift in the NR2A/NR2B ratio in GABAergic interneurons and this shift occurs exclusively during the 2 day CP (Fig 5 & Supplemental Fig 2).

In addition to the differences in the contribution of NR2A vs. NR2B to EPSCs_{NMDAR}, we also demonstrate several differences in glutamatergic synapses that are not related to postsynaptic expression of NMDARs. For example, the synaptic densities are low at early postnatal age (Micheva and Beaulieu, 1996; Washbourne et al., 2004), NMDARs are expressed on the surface of the dendrites and actively exo/endocytosed through the dendritic membrane (Washbourne et al., 2004), which could cause the smaller amplitude of EPSCs_{NMDAR} in preCP. The CV reflects both the number of release sites (N) and the release probability (p). Lower neurotransmitter release probability (Chuhma and Ohmori, 1998) and fewer release site (Iwasaki and Takahashi, 2001) in the preCP group may also be responsible for the larger CV in the preCP group. Interestingly, some of these features remained immature in chronic NVP-AAM077 treated animals (table 4).

The pharmacological data indicate that the τ_{decay} was increased by acute administering NVP-AAM077 and decreased by Ro25-6981 or ifenprodil in both preCP and post CP (RSNP and FS) groups. Interestingly, the changes in the τ_{decay} observed in the presence of NR2A or NR2B antagonists were different between the preCP vs. postCP neurons (Table 1), which may be due to the differences in phosphorylation state (Lieberman and Mody, 1994; Tong et al., 1995) and glutamate uptake (Carter and Regehr, 2000; Diamond, 2001; Arnth-Jensen et al., 2002; Lozovaya et al., 2004; Scimemi et al., 2004).

The contribution of NR1/NR2A/NR2B to the EPSCs_{NMDAR}

The g/g_{max} curve was leftward shifted in the postCP group, which might have resulted from the different affinity of NMDAR channels for Mg^{2+} (Chen and Huang, 1992; Kato and Yoshimura, 1993; Mayer et al., 1989). The NMDARs activated at more hyperpolarized holding potential would be expected to have a lower affinity for Mg^{2+} and less voltage-

dependent Mg^{2+} blockage (Kumar and Huguenard, 2003). However, difference in affinity for Mg^{2+} has not been related to NR2A and NR2B receptors (Mayer et al., 1984; Nowak et al., 1984), but may be related to NR2C and NR2D (Kutsuwada et al., 1992; Binshtok et al., 2006). NR2C subunits have similar developmental patterns as NR2A subunits and are expressed mainly in cerebellum. NR2D subunits already present at birth and are located predominantly at extrasynaptic area at midbrain structures (Cull-Candy et al., 1998; Misra et al., 2000; Momiyama, 2000). NR2C and NR2D subunits are also expressed in dispersed interneurons at hippocampus (Monyer et al., 1994) and neocortex (Cauli et al., 1997) and in spiny stellate cells in layer 4 barrel cortex (Binshtok et al., 2006). However, the proportions of NR2C/2D-containing receptors are similar between pre- and postCP groups (1/65 vs. 2/65), in this case, it should not be responsible for the left-shift of g/g_{max} curve. Triheteromeric NMDARs, containing both NR2A and NR2B subunits have also been identified (Sheng et al., 1994; Mirshahi and Woodward, 1995), but their kinetic properties have not been determined due to lack of selective antagonists (Hatton and Paoletti, 2005). Our results suggest that an increase in NR1/NR2A/NR2B-containing NMDARs in 10% of postCP interneurons may be responsible for the difference in the conductance between pre- and postCP groups.

Postnatal chronic blockage of NR2A, but not NR2B, down-regulated the density of PV-positive interneurons and inhibitory synaptic transmission from FS cells

GABAergic interneurons not only participate in broad range of physiologically relevant process in the mammalian CNS (Toth and McBain, 2000; McBain and Fisahn, 2001; Klausberger and Somogyi, 2008; Gao and Strowbridge, 2009; McBain and Kauer, 2009; Wulff et al., 2009), but are also involved in neurological diseases. PV is a calcium binding protein that can bind the cytoplasmic Ca^{2+} and prevent the activation of Ca^{2+} activated K^{+} current, thus shorten the refractory period of action potential (Celio, 1986). PV-positive interneurons fire high-frequency action potential and synapse on the soma or axon initial segment of glutamatergic neurons, which enable them to potently regulate pyramidal neuron output (van Brederode et al., 1991). The loss of PV-positive interneurons is functionally associated with epilepsy (Marco et al., 1997; Arellano et al., 2004), schizophrenia (Woo et al., 1998; Zhang and Reynolds, 2002; Lewis et al., 2005; Lodge et al., 2009) and autism (Selby et al., 2007). Intriguingly, the treatment of NMDAR antagonists can produce a similar decrease in PV expression (Keilhoff et al., 2004; Kinney et al., 2006; Abekawa et al., 2007; Behrens et al., 2007). Genetic deletion of NR1 from interneurons produced similar results (Belforte et al., 2010). However, evidence linking the function of NR2A and NR2B receptor with PV neuron development *in vivo* is lacking.

In this experiment, chronic blockage of NR2A, but not NR2B, from P7 to P25, reduced the density of PV-positive interneurons in layer 2/3 and layer 4 barrel cortex (Fig. 8). The chronic administration of NR2A and NR2B antagonists will affect both pyramidal neurons and interneurons. NR2A-containing NMDARs increase the surface expression of GluR1 subunit of AMPA receptors in the pyramidal neurons (Kim et al., 2005). The decrease in the PV expression by chronic blockage of NR2A could be caused by a decrease in the glutamatergic transmission from the pyramidal neurons. However, because PV interneurons exhibit a higher NR2A/NR2B ratio than pyramidal neurons (Kinney et al., 2006), it is more likely that the decrease in the PV expression was caused by direct effects in interneurons. By measuring the eIPSCs and sIPSCs in layer 4 spiny cells, we found that the perisomatic inhibition, which was mainly mediated by PV-positive FS interneuron, was impaired after blocking NR2A activity (Fig. 9&10). Also, the NR2B-mediated EPSC_{NMDAR} in layer 2/3 interneurons increased after the chronic blockage of NR2A from P7 to P25 (Fig. 11 and table 4). For the first time, we demonstrated that the NR2A receptor can affect the expression of calcium binding proteins and synaptic transmission from a specific group of

interneurons (FS cells). However, little is known about the mechanisms underlying the role of NR2A in the maturation of PV-positive cells, which warrants future more detailed study of the NR2A-mediated signaling in PV interneurons *in vivo*.

The chronic blockage of NR2B had no effect on the expression of PV in the barrel cortex (Fig. 8). Our results obtained *in vivo* are similar to an earlier study, where NR2A, but not NR2B receptors, are shown to play a pivotal role in the maintenance of the GABAergic function of PV interneurons in culture (Kinney et al., 2006). In both studies, the application of NVP-AAM077, but not Ro25-6981, affected PV expressions. On surface, this result appears to link PV expression with NR2A receptor only. However, the interpretation of this result needs some caution. For example, NVP-AAM077 provides a much stronger inhibition of EPSCs_{NMDAR} than Ro 25-6981 does. The reduction in PV-containing interneurons after NVP-AAM077 injection could simply be due to the stronger inhibition provided by NVP-AAM077, rather than its subunit specificity. However, our results regarding increase in NR2B mediated currents in chronic NVP-AAM077 treated mice (see Fig. 11) suggest that it is NR2A subunits, but not the amount of NMDA currents carried by NR2A per se, appears to be responsible for developmental maturation of GABAergic neurons. Additionally, chronic blockage of NR2A from P7 to P18 did not induce significant change in PV-expression; this could have been due to the difference in the length of treatments, as opposed to a sensitive period effects. We do not know the expected *in vivo* half-life for NVP-AAM077 and Ro25-6981 in the brain. Although Ro 25-6981 has a very slow half-life of dissociation (>5 h) at 4°C *in vitro* (Mutel et al., 1998), it is difficult to predict how long the effectiveness of NVP-AAM077 or Ro25-6981 can last in the brain *in vivo*. Under this circumstance, there are two factors that will determine whether the treatment will be effective or not. One is the cumulative effect of the chemicals (NVP-AAM077 or Ro25-6981) *in vivo* and the other is the length of the treatment.

In summary, we recorded synaptically evoked EPSCs_{NMDAR} from an early postnatal group (preCP) and a juvenile group (postCP) of GABAergic interneurons in layer2/3 barrel cortex. The differences in EPSCs_{NMDAR} properties and sensitivity to NR2 subunit-specific antagonists indicate that the molecular composition of NMDARs in GABAergic interneurons alters during critical developmental stages. The results from this research suggest that NR2A containing NMDARs play a pivotal role in the maturation of PV-positive cells *in vivo*. These results raise possibilities regarding involvement of specific NR2 subunits in experience-dependent plasticity of cortical inhibitory networks.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Reference List

Abekawa T, Ito K, Nakagawa S, Koyama T. Prenatal exposure to an NMDA receptor antagonist, MK-801 reduces density of parvalbumin-immunoreactive GABAergic neurons in the medial prefrontal cortex and enhances phencyclidine-induced hyperlocomotion but not behavioral

- sensitization to methamphetamine in postpubertal rats. *Psychopharmacology (Berl)*. 2007; 192:303–316. [PubMed: 17340116]
- Agmon A, Connors BW. Thalamocortical responses of mouse somatosensory (barrel) cortex in vitro. *Neuroscience*. 1991; 41:365–379. [PubMed: 1870696]
- Agmon A, Yang LT, Jones EG, O'Dowd DK. Topological precision in the thalamic projection to neonatal mouse barrel cortex. *J Neurosci*. 1995; 15:549–561. [PubMed: 7823163]
- Alcantara S, Ferrer I. Postnatal development of parvalbumin immunoreactivity in the cerebral cortex of the cat. *J Comp Neurol*. 1994; 348:133–149. [PubMed: 7814682]
- Alcantara S, Ferrer I, Soriano E. Postnatal development of parvalbumin and calbindin D28K immunoreactivities in the cerebral cortex of the rat. *Anat Embryol (Berl)*. 1993; 188:63–73. [PubMed: 8214625]
- Ali AB, Nelson C. Distinct Ca²⁺ channels mediate transmitter release at excitatory synapses displaying different dynamic properties in rat neocortex. *Cereb Cortex*. 2006; 16:386–393. [PubMed: 15917483]
- Allen C, Stevens CF. An evaluation of causes for unreliability of synaptic transmission. *Proc Natl Acad Sci U S A*. 1994; 91:10380–10383. [PubMed: 7937958]
- Arellano JI, Munoz A, Ballesteros-Yanez I, Sola RG, Defelipe J. Histopathology and reorganization of chandelier cells in the human epileptic sclerotic hippocampus. *Brain*. 2004; 127:45–64. [PubMed: 14534159]
- Arnth-Jensen N, Jabaudon D, Scanziani M. Cooperation between independent hippocampal synapses is controlled by glutamate uptake. *Nat Neurosci*. 2002; 5:325–331. [PubMed: 11896395]
- Auberson YP, Allgeier H, Bischoff S, Lingenhoehl K, Moretti R, Schmutz M. 5-Phosphonomethylquinoxalinediones as competitive NMDA receptor antagonists with a preference for the human 1A/2A, rather than 1A/2B receptor composition. *Bioorg Med Chem Lett*. 2002; 12:1099–1102. [PubMed: 11909726]
- Bacci A, Rudolph U, Huguenard JR, Prince DA. Major differences in inhibitory synaptic transmission onto two neocortical interneuron subclasses. *J Neurosci*. 2003; 23:9664–9674. [PubMed: 14573546]
- Barth AL, Malenka RC. NMDAR EPSC kinetics do not regulate the critical period for LTP at thalamocortical synapses. *Nat Neurosci*. 2001; 4:235–236. [PubMed: 11224537]
- Behrens MM, Ali SS, Dao DN, Lucero J, Shekhtman G, Quick KL, Dugan LL. Ketamine-induced loss of phenotype of fast-spiking interneurons is mediated by NADPH-oxidase. *Science*. 2007; 318:1645–1647. [PubMed: 18063801]
- Beierlein M, Gibson JR, Connors BW. Two dynamically distinct inhibitory networks in layer 4 of the neocortex. *J Neurophysiol*. 2003; 90:2987–3000. [PubMed: 12815025]
- Belforte JE, Zsiros V, Sklar ER, Jiang Z, Yu G, Li Y, Quinlan EM, Nakazawa K. Postnatal NMDA receptor ablation in corticolimbic interneurons confers schizophrenia-like phenotypes. *Nat Neurosci*. 2010; 13:76–83. [PubMed: 19915563]
- Bender KJ, Rangel J, Feldman DE. Development of columnar topography in the excitatory layer 4 to layer 2/3 projection in rat barrel cortex. *J Neurosci*. 2003; 23:8759–8770. [PubMed: 14507976]
- Benveniste M, Clements J, Vyklicky L Jr, Mayer ML. A kinetic analysis of the modulation of N-methyl-D-aspartic acid receptors by glycine in mouse cultured hippocampal neurones. *J Physiol*. 1990; 428:333–357. [PubMed: 2146385]
- Binshok AM, Fleidervish IA, Sprengel R, Gutnick MJ. NMDA receptors in layer 4 spiny stellate cells of the mouse barrel cortex contain the NR2C subunit. *J Neurosci*. 2006; 26:708–715. [PubMed: 16407568]
- Bureau I, Shepherd GM, Svoboda K. Precise development of functional and anatomical columns in the neocortex. *Neuron*. 2004; 42:789–801. [PubMed: 15182718]
- Carmignoto G, Vicini S. Activity-dependent decrease in NMDA receptor responses during development of the visual cortex. *Science*. 1992; 258:1007–1011. [PubMed: 1279803]
- Carter AG, Regehr WG. Prolonged synaptic currents and glutamate spillover at the parallel fiber to stellate cell synapse. *J Neurosci*. 2000; 20:4423–4434. [PubMed: 10844011]

- Cathala L, Misra C, Cull-Candy S. Developmental profile of the changing properties of NMDA receptors at cerebellar mossy fiber-granule cell synapses. *J Neurosci*. 2000; 20:5899–5905. [PubMed: 10934236]
- Cauli B, Audinat E, Lambolez B, Angulo MC, Ropert N, Tsuzuki K, Hestrin S, Rossier J. Molecular and physiological diversity of cortical nonpyramidal cells. *J Neurosci*. 1997; 17:3894–3906. [PubMed: 9133407]
- Celio MR. Parvalbumin in most gamma-aminobutyric acid-containing neurons of the rat cerebral cortex. *Science*. 1986; 231:995–997. [PubMed: 3945815]
- Chaperon F, Muller W, Auberson YP, Tricklebank MD, Neijt HC. Substitution for PCP, disruption of prepulse inhibition and hyperactivity induced by N-methyl-D-aspartate receptor antagonists: preferential involvement of the NR2B rather than NR2A subunit. *Behav Pharmacol*. 2003; 14:477–487. [PubMed: 14501261]
- Chen L, Cooper NG, Mower GD. Developmental changes in the expression of NMDA receptor subunits (NR1, NR2A, NR2B) in the cat visual cortex and the effects of dark rearing. *Brain Res Mol Brain Res*. 2000; 78:196–200. [PubMed: 10891601]
- Chen L, Huang LY. Protein kinase C reduces Mg²⁺ block of NMDA-receptor channels as a mechanism of modulation. *Nature*. 1992; 356:521–523. [PubMed: 1373227]
- Chen N, Luo T, Raymond LA. Subtype-dependence of NMDA receptor channel open probability. *J Neurosci*. 1999; 19:6844–6854. [PubMed: 10436042]
- Choi S, Lovinger DM. Decreased probability of neurotransmitter release underlies striatal long-term depression and postnatal development of corticostriatal synapses. *Proc Natl Acad Sci U S A*. 1997; 94:2665–2670. [PubMed: 9122253]
- Chuhma N, Ohmori H. Postnatal development of phase-locked high-fidelity synaptic transmission in the medial nucleus of the trapezoid body of the rat. *J Neurosci*. 1998; 18:512–520. [PubMed: 9412527]
- Clements JD, Lester RA, Tong G, Jahr CE, Westbrook GL. The time course of glutamate in the synaptic cleft. *Science*. 1992; 258:1498–1501. [PubMed: 1359647]
- Cull-Candy S, Brickley S, Farrant M. NMDA receptor subunits: diversity, development and disease. *Curr Opin Neurobiol*. 2001; 11:327–335. [PubMed: 11399431]
- Cull-Candy SG, Brickley SG, Misra C, Feldmeyer D, Momiyama A, Farrant M. NMDA receptor diversity in the cerebellum: identification of subunits contributing to functional receptors. *Neuropharmacology*. 1998; 37:1369–1380. [PubMed: 9849672]
- Cull-Candy SG, Leszkiewicz DN. Role of distinct NMDA receptor subtypes at central synapses. *Sci STKE*. 2004; re16. [PubMed: 15494561]
- Czeiger D, White EL. Comparison of the distribution of parvalbumin-immunoreactive and other synapses onto the somata of callosal projection neurons in mouse visual and somatosensory cortex. *J Comp Neurol*. 1997; 379:198–210. [PubMed: 9050785]
- Danbolt NC. Glutamate uptake. *Prog Neurobiol*. 2001; 65:1–105. [PubMed: 11369436]
- Das S, Sasaki YF, Rothe T, Premkumar LS, Takasu M, Crandall JE, Dikkes P, Conner DA, Rayudu PV, Cheung W, Chen HS, Lipton SA, Nakanishi N. Increased NMDA current and spine density in mice lacking the NMDA receptor subunit NR3A. *Nature*. 1998; 393:377–381. [PubMed: 9620802]
- Daw MI, Scott HL, Isaac JT. Developmental synaptic plasticity at the thalamocortical input to barrel cortex: mechanisms and roles. *Mol Cell Neurosci*. 2007; 34:493–502. [PubMed: 17329121]
- de Marchena J, Roberts AC, Middlebrooks PG, Valakh V, Yashiro K, Wilfley LR, Philpot BD. NMDA receptor antagonists reveal age-dependent differences in the properties of visual cortical plasticity. *J Neurophysiol*. 2008; 100:1936–1948. [PubMed: 18667547]
- Defelipe J. Neocortical neuronal diversity: chemical heterogeneity revealed by colocalization studies of classic neurotransmitters, neuropeptides, calcium-binding proteins, and cell surface molecules. *Cereb Cortex*. 1993; 3:273–289. [PubMed: 8104567]
- Defelipe J. Cortical interneurons: from Cajal to 2001. *Prog Brain Res*. 2002; 136:215–238. [PubMed: 12143384]
- Diamond JS. Neuronal glutamate transporters limit activation of NMDA receptors by neurotransmitter spillover on CA1 pyramidal cells. *J Neurosci*. 2001; 21:8328–8338. [PubMed: 11606620]

- Dobrunz LE, Stevens CF. Heterogeneity of release probability, facilitation, and depletion at central synapses. *Neuron*. 1997; 18:995–1008. [PubMed: 9208866]
- Erisir A, Harris JL. Decline of the critical period of visual plasticity is concurrent with the reduction of NR2B subunit of the synaptic NMDA receptor in layer 4. *J Neurosci*. 2003; 23:5208–5218. [PubMed: 12832545]
- Fagiolini M, Hensch TK. Inhibitory threshold for critical-period activation in primary visual cortex. *Nature*. 2000; 404:183–186. [PubMed: 10724170]
- Fagiolini M, Katagiri H, Miyamoto H, Mori H, Grant SG, Mishina M, Hensch TK. Separable features of visual cortical plasticity revealed by N-methyl-D-aspartate receptor 2A signaling. *Proc Natl Acad Sci U S A*. 2003; 100:2854–2859. [PubMed: 12591944]
- Feldman DE, Nicoll RA, Malenka RC. Synaptic plasticity at thalamocortical synapses in developing rat somatosensory cortex: LTP, LTD, and silent synapses. *J Neurobiol*. 1999; 41:92–101. [PubMed: 10504196]
- Feng B, Tse HW, Skifter DA, Morley R, Jane DE, Monaghan DT. Structure-activity analysis of a novel NR2C/NR2D-preferring NMDA receptor antagonist: 1-(phenanthrene-2-carbonyl) piperazine-2,3-dicarboxylic acid. *Br J Pharmacol*. 2004; 141:508–516. [PubMed: 14718249]
- Flint AC, Maisch US, Weishaupt JH, Kriegstein AR, Monyer H. NR2A subunit expression shortens NMDA receptor synaptic currents in developing neocortex. *J Neurosci*. 1997; 17:2469–2476. [PubMed: 9065507]
- Fox CJ, Russell KI, Wang YT, Christie BR. Contribution of NR2A and NR2B NMDA subunits to bidirectional synaptic plasticity in the hippocampus in vivo. *Hippocampus*. 2006; 16:907–915. [PubMed: 17024679]
- Franks KM, Isaacson JS. Synapse-specific downregulation of NMDA receptors by early experience: a critical period for plasticity of sensory input to olfactory cortex. *Neuron*. 2005; 47:101–114. [PubMed: 15996551]
- Furukawa H, Singh SK, Mancusso R, Gouaux E. Subunit arrangement and function in NMDA receptors. *Nature*. 2005; 438:185–192. [PubMed: 16281028]
- Gao Y, Strowbridge BW. Long-term plasticity of excitatory inputs to granule cells in the rat olfactory bulb. *Nat Neurosci*. 2009; 12:731–733. [PubMed: 19412165]
- Gegelashvili G, Schousboe A. High affinity glutamate transporters: regulation of expression and activity. *Mol Pharmacol*. 1997; 52:6–15. [PubMed: 9224806]
- Gerkin RC, Lau PM, Nauen DW, Wang YT, Bi GQ. Modular competition driven by NMDA receptor subtypes in spike-timing-dependent plasticity. *J Neurophysiol*. 2007; 97:2851–2862. [PubMed: 17267756]
- Grunze HC, Rainnie DG, Hasselmo ME, Barkai E, Hearn EF, McCarley RW, Greene RW. NMDA-dependent modulation of CA1 local circuit inhibition. *J Neurosci*. 1996; 16:2034–2043. [PubMed: 8604048]
- Gupta A, Wang Y, Markram H. Organizing principles for a diversity of GABAergic interneurons and synapses in the neocortex. *Science*. 2000; 287:273–278. [PubMed: 10634775]
- Hada Y, Yamada Y, Imamura K, Mataga N, Watanabe Y, Yamamoto M. Effects of monocular enucleation on parvalbumin in rat visual system during postnatal development. *Invest Ophthalmol Vis Sci*. 1999; 40:2535–2545. [PubMed: 10509647]
- Harney SC, Jane DE, Anwyl R. Extrasynaptic NR2D-containing NMDARs are recruited to the synapse during LTP of NMDAR-EPSCs. *J Neurosci*. 2008; 28:11685–11694. [PubMed: 18987204]
- Hatton CJ, Paoletti P. Modulation of triheteromeric NMDA receptors by N-terminal domain ligands. *Neuron*. 2005; 46:261–274. [PubMed: 15848804]
- Hensch TK. Critical period plasticity in local cortical circuits. *Nat Rev Neurosci*. 2005; 6:877–888. [PubMed: 16261181]
- Hestrin S. Developmental regulation of NMDA receptor-mediated synaptic currents at a central synapse. *Nature*. 1992; 357:686–689. [PubMed: 1377360]
- Holmgren C, Harkany T, Svennenfors B, Zilberter Y. Pyramidal cell communication within local networks in layer 2/3 of rat neocortex. *J Physiol*. 2003; 551:139–153. [PubMed: 12813147]

- Itami C, Kimura F, Nakamura S. Brain-derived neurotrophic factor regulates the maturation of layer 4 fast-spiking cells after the second postnatal week in the developing barrel cortex. *J Neurosci*. 2007; 27:2241–2252. [PubMed: 17329421]
- Iwasaki S, Takahashi T. Developmental regulation of transmitter release at the calyx of Held in rat auditory brainstem. *J Physiol*. 2001; 534:861–871. [PubMed: 11483715]
- Jiao Y, Zhang C, Yanagawa Y, Sun QQ. Major effects of sensory experiences on the neocortical inhibitory circuits. *J Neurosci*. 2006; 26:8691–8701. [PubMed: 16928857]
- Kato N, Yoshimura H. Reduced Mg²⁺ block of N-methyl-D-aspartate receptor-mediated synaptic potentials in developing visual cortex. *Proc Natl Acad Sci U S A*. 1993; 90:7114–7118. [PubMed: 8394010]
- Kawaguchi Y, Kubota Y. GABAergic cell subtypes and their synaptic connections in rat frontal cortex. *Cereb Cortex*. 1997; 7:476–486. [PubMed: 9276173]
- Kawaguchi Y, Wilson CJ, Augood SJ, Emson PC. Striatal interneurons: chemical, physiological and morphological characterization. *Trends Neurosci*. 1995; 18:527–535. [PubMed: 8638293]
- Keilhoff G, Becker A, Grecksch G, Wolf G, Bernstein HG. Repeated application of ketamine to rats induces changes in the hippocampal expression of parvalbumin, neuronal nitric oxide synthase and cFOS similar to those found in human schizophrenia. *Neuroscience*. 2004; 126:591–598. [PubMed: 15183509]
- Kim MJ, Dunah AW, Wang YT, Sheng M. Differential roles of. *Neuron*. 2005; 46:745–760. [PubMed: 15924861]
- Kinney JW, Davis CN, Tabarean I, Conti B, Bartfai T, Behrens MM. A specific role for NR2A-containing NMDA receptors in the maintenance of parvalbumin and GAD67 immunoreactivity in cultured interneurons. *J Neurosci*. 2006; 26:1604–1615. [PubMed: 16452684]
- Kirischuk S, Clements JD, Grantyn R. Presynaptic and postsynaptic mechanisms underlie paired pulse depression at single GABAergic boutons in rat collicular cultures. *J Physiol*. 2002; 543:99–116. [PubMed: 12181284]
- Kirkwood A, Bear MF. Hebbian synapses in visual cortex. *J Neurosci*. 1994; 14:1634–1645. [PubMed: 8126560]
- Kirson ED, Yaari Y. Synaptic NMDA receptors in developing mouse hippocampal neurones: functional properties and sensitivity to ifenprodil. *J Physiol*. 1996; 497 (Pt 2):437–455. [PubMed: 8961186]
- Klausberger T, Somogyi P. Neuronal diversity and temporal dynamics: the unity of hippocampal circuit operations. *Science*. 2008; 321:53–57. [PubMed: 18599766]
- Kohr G, Jensen V, Koester HJ, Mihaljevic AL, Utvik JK, Kvellø A, Ottersen OP, Seeburg PH, Sprengel R, Hvalby O. Intracellular domains of NMDA receptor subtypes are determinants for long-term potentiation induction. *J Neurosci*. 2003; 23:10791–10799. [PubMed: 14645471]
- Komatsu Y. Age-dependent long-term potentiation of inhibitory synaptic transmission in rat visual cortex. *J Neurosci*. 1994; 14:6488–6499. [PubMed: 7965053]
- Krupp JJ, Vissel B, Heinemann SF, Westbrook GL. N-terminal domains in the NR2 subunit control desensitization of NMDA receptors. *Neuron*. 1998; 20:317–327. [PubMed: 9491992]
- Krupp JJ, Vissel B, Heinemann SF, Westbrook GL. Calcium-dependent inactivation of recombinant N-methyl-D-aspartate receptors is NR2 subunit specific. *Mol Pharmacol*. 1996; 50:1680–1688. [PubMed: 8967993]
- Krupp JJ, Vissel B, Thomas CG, Heinemann SF, Westbrook GL. Interactions of calmodulin and alpha-actinin with the NR1 subunit modulate Ca²⁺-dependent inactivation of NMDA receptors. *J Neurosci*. 1999; 19:1165–1178. [PubMed: 9952395]
- Kumar SS, Huguenard JR. Pathway-specific differences in subunit composition of synaptic NMDA receptors on pyramidal neurons in neocortex. *J Neurosci*. 2003; 23:10074–10083. [PubMed: 14602822]
- Kutsuwada T, Kashiwabuchi N, Mori H, Sakimura K, Kushiya E, Araki K, Meguro H, Masaki H, Kumanishi T, Arakawa M. Molecular diversity of the NMDA receptor channel. *Nature*. 1992; 358:36–41. [PubMed: 1377365]
- Laube B, Kuhse J, Betz H. Evidence for a tetrameric structure of recombinant NMDA receptors. *J Neurosci*. 1998; 18:2954–2961. [PubMed: 9526012]

- Legendre P, Rosenmund C, Westbrook GL. Inactivation of NMDA channels in cultured hippocampal neurons by intracellular calcium. *J Neurosci.* 1993; 13:674–684. [PubMed: 7678859]
- Lei S, McBain CJ. Distinct NMDA receptors provide differential modes of transmission at mossy fiber-interneuron synapses. *Neuron.* 2002; 33:921–933. [PubMed: 11906698]
- Letinic K, Kostovic I. Postnatal development of calcium-binding proteins calbindin and parvalbumin in human visual cortex. *Cereb Cortex.* 1998; 8:660–669. [PubMed: 9823487]
- Lewis DA, Hashimoto T, Volk DW. Cortical inhibitory neurons and schizophrenia. *Nat Rev Neurosci.* 2005; 6:312–324. [PubMed: 15803162]
- Li JH, Wang YH, Wolfe BB, Krueger KE, Corsi L, Stocca G, Vicini S. Developmental changes in localization of NMDA receptor subunits in primary cultures of cortical neurons. *Eur J Neurosci.* 1998; 10:1704–1715. [PubMed: 9751142]
- Li Q, Clark S, Lewis DV, Wilson WA. NMDA receptor antagonists disinhibit rat posterior cingulate and retrosplenial cortices: a potential mechanism of neurotoxicity. *J Neurosci.* 2002; 22:3070–3080. [PubMed: 11943810]
- Lieberman DN, Mody I. Regulation of NMDA channel function by endogenous Ca(2+)-dependent phosphatase. *Nature.* 1994; 369:235–239. [PubMed: 7514273]
- Liguz-Leczna M, Waleszczyk WJ, Zakrzewska R, Skangiel-Kramska J, Kossut M. Associative pairing involving monocular stimulation selectively mobilizes a subclass of GABAergic interneurons in the mouse visual cortex. *J Comp Neurol.* 2009; 516:482–492. [PubMed: 19672986]
- Liu L, Wong TP, Pozza MF, Lingenhoehl K, Wang Y, Sheng M, Auberson YP, Wang YT. Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity. *Science.* 2004a; 304:1021–1024. [PubMed: 15143284]
- Liu XB, Murray KD, Jones EG. Switching of NMDA receptor 2A and 2B subunits at thalamic and cortical synapses during early postnatal development. *J Neurosci.* 2004b; 24:8885–8895. [PubMed: 15470155]
- Lodge DJ, Behrens MM, Grace AA. A loss of parvalbumin-containing interneurons is associated with diminished oscillatory activity in an animal model of schizophrenia. *J Neurosci.* 2009; 29:2344–2354. [PubMed: 19244511]
- Lozovaya NA, Grebenyuk SE, Tsintsadze TS, Feng B, Monaghan DT, Krishtal OA. Extrasynaptic NR2B and NR2D subunits of NMDA receptors shape 'superslow' afterburst EPSC in rat hippocampus. *J Physiol.* 2004; 558:451–463. [PubMed: 15146049]
- Lu HC, Gonzalez E, Crair MC. Barrel cortex critical period plasticity is independent of changes in NMDA receptor subunit composition. *Neuron.* 2001; 32:619–634. [PubMed: 11719203]
- Lynch DR, Guttman RP. NMDA receptor pharmacology: perspectives from molecular biology. *Curr Drug Targets.* 2001; 2:215–231. [PubMed: 11554549]
- Maccaferri G, Dingledine R. Complex effects of CNQX on CA1 interneurons of the developing rat hippocampus. *Neuropharmacology.* 2002; 43:523–529. [PubMed: 12367599]
- Maier DL, McCasland JS. Calcium-binding protein phenotype defines metabolically distinct groups of neurons in barrel cortex of behaving hamsters. *Exp Neurol.* 1997; 145:71–80. [PubMed: 9184110]
- Marco P, Sola RG, Cajal S, Defelipe J. Loss of inhibitory synapses on the soma and axon initial segment of pyramidal cells in human epileptic peritumoural neocortex: implications for epilepsy. *Brain Res Bull.* 1997; 44:47–66. [PubMed: 9288831]
- Massengill JL, Smith MA, Son DI, O'Dowd DK. Differential expression of K4-AP currents and Kv3.1 potassium channel transcripts in cortical neurons that develop distinct firing phenotypes. *J Neurosci.* 1997; 17:3136–3147. [PubMed: 9096148]
- Mayer ML, Vyklicky L Jr, Clements J. Regulation of NMDA receptor desensitization in mouse hippocampal neurons by glycine. *Nature.* 1989; 338:425–427. [PubMed: 2538755]
- Mayer ML, Westbrook GL, Guthrie PB. Voltage-dependent block by Mg²⁺ of NMDA responses in spinal cord neurones. *Nature.* 1984; 309:261–263. [PubMed: 6325946]
- McBain CJ, Fisahn A. Interneurons unbound. *Nat Rev Neurosci.* 2001; 2:11–23. [PubMed: 11253355]
- McBain CJ, Kauer JA. Presynaptic plasticity: targeted control of inhibitory networks. *Curr Opin Neurobiol.* 2009; 19:254–262. [PubMed: 19581079]

- Medina AE, Liao DS, Mower AF, Ramoa AS. Do NMDA receptor kinetics regulate the end of critical periods of plasticity? *Neuron*. 2001; 32:553–555. [PubMed: 11719195]
- Medina I, Filippova N, Charton G, Rougeole S, Ben-Ari Y, Khrestchatsky M, Bregestovski P. Calcium-dependent inactivation of heteromeric NMDA receptor-channels expressed in human embryonic kidney cells. *J Physiol*. 1995; 482 (Pt 3):567–573. [PubMed: 7537819]
- Micheva KD, Beaulieu C. Quantitative aspects of synaptogenesis in the rat barrel field cortex with special reference to GABA circuitry. *J Comp Neurol*. 1996; 373:340–354. [PubMed: 8889932]
- Mirshahi T, Woodward JJ. Ethanol sensitivity of heteromeric NMDA receptors: effects of subunit assembly, glycine and NMDAR1 Mg(2+)-insensitive mutants. *Neuropharmacology*. 1995; 34:347–355. [PubMed: 7630488]
- Misra C, Brickley SG, Wyllie DJ, Cull-Candy SG. Slow deactivation kinetics of NMDA receptors containing NR1 and NR2D subunits in rat cerebellar Purkinje cells. *J Physiol*. 2000; 525(Pt 2): 299–305. [PubMed: 10835034]
- Momiyama A. Distinct synaptic and extrasynaptic NMDA receptors identified in dorsal horn neurones of the adult rat spinal cord. *J Physiol*. 2000; 523(Pt 3):621–628. [PubMed: 10718742]
- Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron*. 1994; 12:529–540. [PubMed: 7512349]
- Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N, Sakmann B, Seeburg PH. Heteromeric NMDA receptors: molecular and functional distinction of subtypes. *Science*. 1992; 256:1217–1221. [PubMed: 1350383]
- Moon JS, Kim JJ, Chang IY, Chung YY, Jun JY, You HJ, Yoon SP. Postnatal development of parvalbumin and calbindin D-28k immunoreactivities in the canine anterior cingulate cortex: transient expression in layer V pyramidal cells. *Int J Dev Neurosci*. 2002; 20:511. [PubMed: 12392755]
- Morley RM, Tse HW, Feng B, Miller JC, Monaghan DT, Jane DE. Synthesis and pharmacology of N1-substituted piperazine-2,3-dicarboxylic acid derivatives acting as NMDA receptor antagonists. *J Med Chem*. 2005; 48:2627–2637. [PubMed: 15801853]
- Mutel V, Buchy D, Klingelschmidt A, Messer J, Bleuel Z, Kemp JA, Richards JG. In vitro binding properties in rat brain of [3H]Ro 25–6981, a potent and selective antagonist of NMDA receptors containing NR2B subunits. *J Neurochem*. 1998; 70:2147–2155. [PubMed: 9572302]
- Nase G, Weishaupt J, Stern P, Singer W, Monyer H. Genetic and epigenetic regulation of NMDA receptor expression in the rat visual cortex. *Eur J Neurosci*. 1999; 11:4320–4326. [PubMed: 10594657]
- Neyton J, Paoletti P. Relating NMDA receptor function to receptor subunit composition: limitations of the pharmacological approach. *J Neurosci*. 2006; 26:1331–1333. [PubMed: 16452656]
- Nowak L, Bregestovski P, Ascher P, Herbert A, Prochiantz A. Magnesium gates glutamate-activated channels in mouse central neurones. *Nature*. 1984; 307:462–465. [PubMed: 6320006]
- Olney JW, Farber NB. NMDA antagonists as neurotherapeutic drugs, psychotogens, neurotoxins, and research tools for studying schizophrenia. *Neuropsychopharmacology*. 1995; 13:335–345. [PubMed: 8747758]
- Perez-Otano I, Schulteis CT, Contractor A, Lipton SA, Trimmer JS, Sucher NJ, Heinemann SF. Assembly with the NR1 subunit is required for surface expression of NR3A-containing NMDA receptors. *J Neurosci*. 2001; 21:1228–1237. [PubMed: 11160393]
- Philpot BD, Sekhar AK, Shouval HZ, Bear MF. Visual experience and deprivation bidirectionally modify the composition and function of NMDA receptors in visual cortex. *Neuron*. 2001a; 29:157–169. [PubMed: 11182088]
- Philpot BD, Weisberg MP, Ramos MS, Sawtell NB, Tang YP, Tsien JZ, Bear MF. Effect of transgenic overexpression of NR2B on NMDA receptor function and synaptic plasticity in visual cortex. *Neuropharmacology*. 2001b; 41:762–770. [PubMed: 11640931]
- Poncer JC, McKinney RA, Gahwiler BH, Thompson SM. Differential control of GABA release at synapses from distinct interneurons in rat hippocampus. *J Physiol*. 2000; 528(Pt 1):123–130. [PubMed: 11018111]

- Poncer JC, McKinney RA, Gahwiler BH, Thompson SM. Either N- or P-type calcium channels mediate GABA release at distinct hippocampal inhibitory synapses. *Neuron*. 1997; 18:463–472. [PubMed: 9115739]
- Prybylowski K, Fu Z, Losi G, Hawkins LM, Luo J, Chang K, Wenthold RJ, Vicini S. Relationship between availability of NMDA receptor subunits and their expression at the synapse. *J Neurosci*. 2002; 22:8902–8910. [PubMed: 12388597]
- Quinlan EM, Olstein DH, Bear MF. Bidirectional, experience-dependent regulation of N-methyl-D-aspartate receptor subunit composition in the rat visual cortex during postnatal development. *Proc Natl Acad Sci U S A*. 1999a; 96:12876–12880. [PubMed: 10536016]
- Quinlan EM, Philpot BD, Hugarir RL, Bear MF. Rapid, experience-dependent expression of synaptic NMDA receptors in visual cortex in vivo. *Nat Neurosci*. 1999b; 2:352–357. [PubMed: 10204542]
- Raman IM, Tong G, Jahr CE. Beta-adrenergic regulation of synaptic NMDA receptors by cAMP-dependent protein kinase. *Neuron*. 1996; 16:415–421. [PubMed: 8789956]
- Ramoas AS, Prusky G. Retinal activity regulates developmental switches in functional properties and ifenprodil sensitivity of NMDA receptors in the lateral geniculate nucleus. *Brain Res Dev Brain Res*. 1997; 101:165–175.
- Roberts EB, Ramoa AS. Enhanced NR2A subunit expression and decreased NMDA receptor decay time at the onset of ocular dominance plasticity in the ferret. *J Neurophysiol*. 1999; 81:2587–2591. [PubMed: 10322092]
- Rosenmund C, Feltz A, Westbrook GL. Calcium-dependent inactivation of synaptic NMDA receptors in hippocampal neurons. *J Neurophysiol*. 1995; 73:427–430. [PubMed: 7714587]
- Sah P, Hestrin S, Nicoll RA. Properties of excitatory postsynaptic currents recorded in vitro from rat hippocampal interneurons. *J Physiol*. 1990; 430:605–616. [PubMed: 1982315]
- Sather W, Johnson JW, Henderson G, Ascher P. Glycine-insensitive desensitization of NMDA responses in cultured mouse embryonic neurons. *Neuron*. 1990; 4:725–731. [PubMed: 2160836]
- Schulz PE, Cook EP, Johnston D. Changes in paired-pulse facilitation suggest presynaptic involvement in long-term potentiation. *J Neurosci*. 1994; 14:5325–5337. [PubMed: 7916043]
- Scimemi A, Fine A, Kullmann DM, Rusakov DA. NR2B-containing receptors mediate cross talk among hippocampal synapses. *J Neurosci*. 2004; 24:4767–4777. [PubMed: 15152037]
- Selby L, Zhang C, Sun QQ. Major defects in neocortical GABAergic inhibitory circuits in mice lacking the fragile X mental retardation protein. *Neurosci Lett*. 2007; 412:227–232. [PubMed: 17197085]
- Sheng M, Cummings J, Roldan LA, Jan YN, Jan LY. Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. *Nature*. 1994; 368:144–147. [PubMed: 8139656]
- Shimamoto K, Lebrun B, Yasuda-Kamatani Y, Sakaitani M, Shigeri Y, Yumoto N, Nakajima T. DL-threo-beta-benzoyloxyaspartate, a potent blocker of excitatory amino acid transporters. *Mol Pharmacol*. 1998; 53:195–201. [PubMed: 9463476]
- Stern EA, Maravall M, Svoboda K. Rapid development and plasticity of layer 2/3 maps in rat barrel cortex in vivo. *Neuron*. 2001; 31:305–315. [PubMed: 11502260]
- Stocca G, Vicini S. Increased contribution of NR2A subunit to synaptic NMDA receptors in developing rat cortical neurons. *J Physiol*. 1998; 507 (Pt 1):13–24. [PubMed: 9490809]
- Sugiyama S, Di Nardo AA, Aizawa S, Matsuo I, Volovitch M, Prochiantz A, Hensch TK. Experience-dependent transfer of Otx2 homeoprotein into the visual cortex activates postnatal plasticity. *Cell*. 2008; 134:508–520. [PubMed: 18692473]
- Sun QQ. Experience-dependent intrinsic plasticity in interneurons of barrel cortex layer IV. *J Neurophysiol*. 2009; 102:2955–2973. [PubMed: 19741102]
- Sun QQ, Dale N. Differential inhibition of N and P/Q Ca²⁺ currents by 5-HT_{1A} and 5-HT_{1D} receptors in spinal neurons of *Xenopus* larvae. *J Physiol*. 1998; 510 (Pt 1):103–120. [PubMed: 9625870]
- Sun QQ, Zhang Z, Jiao Y, Zhang C, Szabo G, Erdelyi F. Differential metabotropic glutamate receptor expression and modulation in two neocortical inhibitory networks. *J Neurophysiol*. 2009; 101:2679–2692. [PubMed: 19244358]

- Tamamaki N, Yanagawa Y, Tomioka R, Miyazaki J, Obata K, Kaneko T. Green fluorescent protein expression and colocalization with calretinin, parvalbumin, and somatostatin in the GAD67-GFP knock-in mouse. *J Comp Neurol*. 2003; 467:60–79. [PubMed: 14574680]
- Tong G, Jahr CE. Regulation of glycine-insensitive desensitization of the NMDA receptor in outside-out patches. *J Neurophysiol*. 1994; 72:754–761. [PubMed: 7983533]
- Tong G, Shepherd D, Jahr CE. Synaptic desensitization of NMDA receptors by calcineurin. *Science*. 1995; 267:1510–1512. [PubMed: 7878472]
- Toth K, McBain CJ. Target-specific expression of pre- and postsynaptic mechanisms. *J Physiol*. 2000; 525(Pt 1):41–51. [PubMed: 10811723]
- Tovar KR, Westbrook GL. The incorporation of NMDA receptors with a distinct subunit composition at nascent hippocampal synapses in vitro. *J Neurosci*. 1999; 19:4180–4188. [PubMed: 10234045]
- Tsukada S, Iino M, Takayasu Y, Shimamoto K, Ozawa S. Effects of a novel glutamate transporter blocker, (2S, 3S)-3-[3-[4-(trifluoromethyl)benzoylamino]benzyloxy]aspartate (TFB-TBOA), on activities of hippocampal neurons. *Neuropharmacology*. 2005; 48:479–491. [PubMed: 15755476]
- Ulbrich MH, Isacoff EY. Rules of engagement for NMDA receptor subunits. *Proc Natl Acad Sci U S A*. 2008; 105:14163–14168. [PubMed: 18779583]
- van Brederode JF, Helliesen MK, Hendrickson AE. Distribution of the calcium-binding proteins parvalbumin and calbindin-D28k in the sensorimotor cortex of the rat. *Neuroscience*. 1991; 44:157–171. [PubMed: 1770994]
- Vicini S, Wang JF, Li JH, Zhu WJ, Wang YH, Luo JH, Wolfe BB, Grayson DR. Functional and pharmacological differences between recombinant N-methyl-D-aspartate receptors. *J Neurophysiol*. 1998; 79:555–566. [PubMed: 9463421]
- Vyklicky L Jr, Vlachova V, Krusek J. The effect of external pH changes on responses to excitatory amino acids in mouse hippocampal neurones. *J Physiol*. 1990; 430:497–517. [PubMed: 1707970]
- Washbourne P, Liu XB, Jones EG, McAllister AK. Cycling of NMDA receptors during trafficking in neurons before synapse formation. *J Neurosci*. 2004; 24:8253–8264. [PubMed: 15385609]
- Watanabe M, Inoue Y, Sakimura K, Mishina M. Developmental changes in distribution of NMDA receptor channel subunit mRNAs. *Neuroreport*. 1992; 3:1138–1140. [PubMed: 1493227]
- Williams K. Ifenprodil discriminates subtypes of the N-methyl-D-aspartate receptor: selectivity and mechanisms at recombinant heteromeric receptors. *Mol Pharmacol*. 1993; 44:851–859. [PubMed: 7901753]
- Woo TU, Whitehead RE, Melchitzky DS, Lewis DA. A subclass of prefrontal gamma-aminobutyric acid axon terminals are selectively altered in schizophrenia. *Proc Natl Acad Sci U S A*. 1998; 95:5341–5346. [PubMed: 9560277]
- Wulff P, Schonewille M, Renzi M, Viltono L, Sassoe-Pognetto M, Badura A, Gao Z, Hoebeek FE, van DS, Wisden W, Farrant M, De Zeeuw CI. Synaptic inhibition of Purkinje cells mediates consolidation of vestibulo-cerebellar motor learning. *Nat Neurosci*. 2009; 12:1042–1049. [PubMed: 19578381]
- Yan YH, Winarto A, Mansjoer I, Hendrickson A. Parvalbumin, calbindin, and calretinin mark distinct pathways during development of monkey dorsal lateral geniculate nucleus. *J Neurobiol*. 1996; 31:189–209. [PubMed: 8885200]
- Zaitsev AV, Povysheva NV, Lewis DA, Krimer LS. P/Q-type, but not N-type, calcium channels mediate GABA release from fast-spiking interneurons to pyramidal cells in rat prefrontal cortex. *J Neurophysiol*. 2007; 97:3567–3573. [PubMed: 17329622]
- Zhang Y, Behrens MM, Lisman JE. Prolonged exposure to NMDAR antagonist suppresses inhibitory synaptic transmission in prefrontal cortex. *J Neurophysiol*. 2008; 100:959–965. [PubMed: 18525022]
- Zhang ZJ, Reynolds GP. A selective decrease in the relative density of parvalbumin-immunoreactive neurons in the hippocampus in schizophrenia. *Schizophr Res*. 2002; 55:1–10. [PubMed: 11955958]

Figure 1.

EPSC_{S_{NMDAR}} in GABAergic interneurons of layer 2/3 barrel cortex. **A1**, Experimental designs. **A2**, Schematic of recording paradigms in layer 2/3 barrel cortex. Interneurons were represented with cells with roundish soma. Dashed lines demarcate different layers of barrel cortex. **B1**, **B2**, Neurobiotin filled GAD67-GFP positive neurons in a P6 (**B1**) and a P24 (**B2**) mouse, respectively. Scale bar, 20 μm. The stimulating electrodes were placed 20–50 μm from recorded cells. **C**, 5 consecutive EPSCs evoked at +40 mV holding potential at indicated stimulus intensities (in V) in a representative P9 (left) and a P24 (right) GABAergic interneuron, respectively. The threshold intensity was indicated by (*). **D**, The input (stimulus intensity) and output (response amplitude) relationship curve. Note the required minimal stimulus intensity for evoking reliable EPSCs were smaller in the P24 neuron; the amplitude of evoked EPSCs was larger in the P24 neuron.

Figure 2.

Voltage-dependent properties of pharmacologically isolated EPSCs_{NMDAR}. **A1, A2**, The averaged traces of EPSCs_{NMDAR} from a representative P8 (A1) and P28(A2) neuron, respectively, recorded at indicated holding potentials ranged from -80 to +40 mV. Note that the amplitudes of inward and outward currents were larger in the P28 neuron. **B**, The average *I-V* curve for the neurons from preCP (n=6) and postCP (n=13). **C**, Normalized *g-V* data showing a leftward shift in *V*_{0.5} for the postCP group (*V*_{0.5 postCP} = -13.8 ± 2.0 mV vs. *V*_{0.5 preCP} = -6.3 ± 5.7 mV, *p* < 0.01). The solid line is the best-fitting Boltzmann equation, $I/I_{\max} = \{1 + \exp [(V + V_{1/2})/K]\}^{-1}$, where *V*_{1/2} = -5.1 ± 3.9 mV, *K* = 0.015 in preCP and *V*_{1/2} = -6.7 ± 2.1 mV, *K* = 0.004 in postCP, respectively.

Figure 3. Separation of FS and RSNP neurons in postCP group. **A**, Representative action potential (AP) traces from a FS (top) and a RSNP (bottom) neuron. Inset: the normalized traces of APs from the FS and RSNP neurons indicated by the squares. **B1**, **B2**, Separation of FS and RSNP neurons based on the firing frequency, half-width and weighted τ_{decay} of APs. **C1**, 10 consecutive EPSCs_{NMDAR} in a representative P7 (top), P23 RSNP (middle) and P23 FS (bottom) neuron, respectively. **C2**, The coefficient of variation (CV) in preCP and postCP RSNP and postCP FS neurons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: no significance, as in this and the following graphs.

Figure 4.

Developmental changes in the properties of EPSCs_{NMDAR}. **A1**, The averaged traces of EPSCs_{NMDAR} in a representative P7 (left), P23 RSNP (middle) and P23 FS (right) neuron, respectively. **A2**, The normalized traces of A1. The arrows indicated the values of fast and slow τ_{decay} for each traces. **B**, The comparison of the amplitude of EPSCs_{NMDAR} in preCP (white bar), postCP RSNP (grey bar) and postCP FS (black bar) neurons. Both RSNP and FS neurons of the postCP group had larger peak amplitude than preCP neurons ($***p < 0.001$). No significant difference in the amplitude of EPSCs_{NMDAR} between RSNP and FS neurons. **C**, The comparison of the half-width of EPSCs_{NMDAR} in preCP (white bar), postCP RSNP (grey bar) and postCP FS (black bar) neurons ($***p < 0.001$). **D**, The comparison τ_{rise} of the EPSCs_{NMDAR} in preCP (white bar), postCP RSNP (grey bar) and postCP FS (black bar) neurons ($**p < 0.01$, $***p < 0.001$). **E**, The comparison of the τ_{decay} of EPSCs_{NMDAR} in preCP (white bar), postCP RSNP (grey bar) and postCP FS (black bar) neurons. ($***p < 0.001$).

Figure 5.

The pharmacological experiments showing developmental shift in NR2B/NR2A ratio. **A1**, **B1**, Representative EPSCs_{NMDAR} traces in the absence (black) and presence of NVP-AAM077 (0.5 μ M, dark gray) and NVP-AAM077 (0.5 μ M) plus Ro 25-6981 (0.5 μ M, light gray) in a P8 neuron (A1) and P25 neuron (B1), respectively. **A2**, **B2**, Representative EPSCs_{NMDAR} traces in the absence (black) and presence of Ro 25-6981 (0.5 μ M, dark gray) and the mixture of NVP-AAM077 (0.5 μ M) plus Ro 25-6981 (0.5 μ M, light gray) in a P8 neuron (A2) and P 28 neuron (B2), respectively. **A3**, **B3**, Representative EPSCs_{NMDAR} traces in the absence (black) and presence of ifenprodil (3 μ M, dark gray) and NVP-AAM077 (0.5 μ M) plus ifenprodil (3 μ M, light gray), and PPDA (0.5 μ M, light black) in a P7 neuron (A3) and P24 neuron (B3), respectively. **C**, The effects of NVP-AAM077, Ro 25-6981 and ifenprodil on the amplitudes of EPSCs_{NMDAR}. **D**, The effects of NVP-AAM077 and ifenprodil on the amplitude of EPSCs_{NMDAR} in postCP RSNP and postCP FS neurons.

Figure 6.

A1–A3, B1–B3, Representative traces from preCP (A1–A3) and postCP (B1–B3) groups, showing the effects of NR2A and NR2B subunit-specific antagonists on the amplitude of EPSC_{NMDAR}. **C,** The dose-response curve of NVP-AAM077 fitted with Hill equation. The arrows indicated the IC_{50} values of the fitted curves with (black dots) and without (grey dots) ifenprodil (3 μ M) in the bath perfusion solution. **D, E,** The effect of NR2A and NR2B subunit-specific antagonists in all GABAergic cells regardless their age group. **D,** The effects of NVP-AAM077, Ro 25-6981 and ifenprodil on the amplitudes of EPSC_{NMDAR}. **E,** Plot showing 'NR2B remaining EPSCs' (n=48, light gray bar), comparing with Ro 25-6981 (n=28, dark gray bar) and ifenprodil (n=51, black bar). 'NR2A remaining EPSCs 1' (from Ro 25-6981 experiments, n=28, light gray bar), and 'NR2A remaining EPSCs 2' (from ifenprodil experiments, n=51, dark gray bar) comparing with NVP-AAM077 (n=48, black bar) in all neurons examined, regardless their age group.

Figure 7. Effects of TBOA on EPSC_{S_{NMDAR}}. **A1, B1**, The representative traces from preCP (A1) and postCP (B1) in the absence (black) and presence of TBOA (30 μ m, gray). The traces were the average of 10 consecutive EPSCs. The insets show the normalized traces. Note the change in the half-width and τ_{decay} in both groups. Insets: normalized traces of A1 & B1.

Figure 8. Effects of NVP-AAM077 and Ro25-6981 injection on PV expression in barrel cortex of GAD67-GFP mouse (TC sections). **A, B&C**, Photomicrographs of double-immunofluorescence-stained TC section (40 μ m thickness) from a control mouse (A), a NVP-AAM077 injected (from P7–P25) mouse (B) and a Ro25-6981 injected (from P7–P25) mouse (C). Photomicrographs of PV (**A1–C1**), GFP (**A2–C2**), and merged images of PV +GFP (**A3–C3**). Scale bars, 50 μ m. Dashed white lines outline different layers throughout the barrel cortex. **D1–F1**, Statistical comparison of cell density of PV-positive (D1), GFP-positive (E1) cells, and the ratio of PV/GFP-positive (F1) neurons in control (white bars, n=6 mice), NVP-AAM077 treated (grey bars, n=6 mice) and Ro25-6981 treated (black bars, n=6 mice) mice at age P25 (injection from P7 to P25). **D2–F2**, Statistical comparison of cell density of PV-positive and GFP-positive cells in control (white bars, n=3), NVP-AAM077 treated (grey bars, n=3) and Ro25-6981 treated (black bars, n=3) mice at age P18. No

significant difference in the density of PV-positive (D2), GFP-positive (E2) and the ratio of PV/GFP-positive (F2) neurons between the control and NVP-AAM077 treated groups.

Figure 9.

Characterization of evoked IPSCs from layer 4 pyramidal neurons in control and NVP-AAM077 injected mice. **A**, Representative traces of evoked IPSCs under minimal stimulation intensity before and after the application of agatoxin in both control and NVP-AAM077 injected neurons. **B–F**, The characterization of evoked IPSCs. **B**, The amplitude (measured by the first IPSC); **C**, HWs; **D**, τ_{decay} ; **E**, CV of the amplitude; **F**, The paired-pulse ratio (IPSC2/IPSC1).

Figure 10.

Characterization of spontaneous IPSCs from layer 4 pyramidal neurons in control and NVP-AAM077 injected mice. **A1, A2**, Representative traces of spontaneous IPSCs before and after the application of agatoxin in both control (A1) and NVP-AAM077 injected (A2) neurons. **B–E**, The characterization of spontaneous IPSCs. **B**, The amplitude; **C**, The instant frequency; **D**, HWs and **E**, τ_{decay} .

Figure 11.

Effects of chronic blockage of NR2A on EPSC_{NMDAR}. **A1, A2**, The representative traces from control (A1) and NVP-injected (A2) in the absence (black) and presence of ifenprodil (3 μ m, gray). The traces were the average of 10 consecutive EPSCs. **B, C**, The percentage of inhibition of the peak amplitude of EPSC_{NMDAR} (B) and the area of EPSC_{NMDAR} (C) provided by ifenprodil (3 μ m) in control and NVP-AAM077 injected mice.

Table 1
The effects of NR2A and NR2B antagonists on the fast and slow τ_{decay} of EPSC_{NMDAR} between the pre- and postCP interneurons.

	PreCP						PostCP					
	Fast decay tau (ms)			Slow decay tau (ms)			Fast decay tau (ms)			Slow decay tau (ms)		
	Before	After	n	Before	After	n	Before	After	n	Before	After	n
NVP-AAAM077	124.4 ± 12.1	135.3 ± 14.0	491.1 ± 60.80	790.0 ± 143.1 *	52.7 ± 8.5	88.2 ± 26.0 *	287.4 ± 65.2	455.7 ± 90.6 *	n=11			
Ifenprodil	101.5 ± 4.5	64.4 ± 6.5 ***	455.1 ± 50.4	281.5 ± 44.5 **	40.1 ± 2.8	39.9 ± 2.6	286.2 ± 28.1	214.0 ± 22.3 *	n=35			
Ro 25-6981	110.9 ± 15.7	47.4 ± 10.5 **	543.7 ± 72.3	254.2 ± 40.7 ***	45.0 ± 4.5	38.5 ± 3.9	250.3 ± 49.8	216.8 ± 47.8	n=12			

Data expressed in mean ± S.E.M. Paired Student's *t* test was performed for comparisons of before and after antagonists' applications. Significance was placed at *P* < 0.05.

* *p* < 0.05,
** *p* < 0.01 and
*** *p* < 0.001

Table 2
The effects of Inhibition of plasmalemmal glutamate transporter activity on the properties of EPSC_{NMDAR}.

	CV	Amplitude (pA)	Area (pA [*] ms)	τ _{rise} (ms)	HW's (ms)	τ _{decay-Fast} (ms)	τ _{decay-Slow} (ms)	n
Pre-CP	Control	36.5 ± 2.3	5588.1 ± 551.5	5.1 ± 2.0	99.1 ± 32.8	106.9 ± 6.9	472.7 ± 29.7	65
	TBOA	24.5 ± 2.5 **	9119.1 ± 1382.6	5.7 ± 0.8	269.5 ± 35.4 ***	390.8 ± 107.9 **	2228.6 ± 672.2 **	18
Post-CP	Control	81.0 ± 5.5	6604.7 ± 759.7	4.1 ± 0.3	49.5 ± 2.7	47.7 ± 3.5	281.0 ± 21.2	65
	TBOA	46.2 ± 1.5 ***	3061.2 ± 317.5 ***	4.9 ± 0.4	68.1 ± 9.8 *	119.7 ± 16.0 ***	651.5 ± 165.2 *	12

Data expressed in mean ± S.E.M. Paired Student's *t* test was performed for comparisons of before and after antagonists' applications. Significance was placed at *P* < 0.05.

* *p* < 0.05,

** *p* < 0.01 and

*** *p* < 0.001

Table 3

The calbindin and calretinin antibody staining in control and NVP-AAM077-injected groups.

		Calbindin			Calretinin		
		Calbindin (cell/cm ²)	GFP (cell/cm ²)	Calbindin/GFP	Calretinin (cell/cm ²)	GFP (cell/cm ²)	Calretinin/GFP
Control	Layer 2/3	3000 ± 500	600 ± 60	5.5 ± 0.9	100 ± 20	600 ± 50	0.2 ± 0.03
	Layer4	3000 ± 500	700 ± 40	4.2 ± 0.5	60 ± 1	700 ± 50	0.09 ± 0.01
	Layer5	1000 ± 200	600 ± 40	1.9 ± 0.2	80 ± 4	600 ± 50	0.1 ± 0.05
NVP-injected	Layer 2/3	3000 ± 900	600 ± 40	5.1 ± 0.8	200 ± 50	600 ± 40	0.3 ± 0.02
	Layer4	3000 ± 400	700 ± 70	4.7 ± 0.7	60 ± 8	700 ± 50	0.1 ± 0.007
	Layer5	1000 ± 200	600 ± 50	1.8 ± 0.3	80 ± 3	600 ± 80	0.1 ± 0.03

No significant difference in the density of calbindin and calretinin expression in the layer II/III, IV and V barrel cortex between control and NVP-AAM07 treated groups.

Table 4

The effects of chronic NVP-AAM077 injection on the properties of EPSC_{NMDAR}.

	CV	Amplitude (pA)	Area (pA * ms)	τ_{rise} (ms)	HWs (ms)	$\tau_{decay-fast}$ (ms)	$\tau_{decay-slow}$ (ms)	n
Control	0.2 ± 0.0	92.6 ± 9.4	6506.9 ± 948.9	3.6 ± 0.2	48.8 ± 0.8	44.8 ± 3.9	274.9 ± 31.6	29
NVP-injected	0.4 ± 0.0 **	59.6 ± 6.4 **	4987.4 ± 343.9 ***	3.5 ± 0.3	57.8 ± 3.4 *	55.8 ± 3.8 *	402.5 ± 47.0 *	22

Data expressed in mean ± S.E.M. Paired Student's *t* test was performed for comparisons of before and after antagonists' applications. Significance was placed at *P* < 0.05.

* *p* < 0.05,
** *p* < 0.01 and
*** *p* < 0.001