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Double Dissociation of Spike Timing–Dependent Potentiation and Depression by Subunit-Preferring NMDA Receptor Antagonists in Mouse Barrel Cortex

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Spike timing–dependent plasticity (STDP) is a strong candidate for an *N*-methyl-D-aspartate (NMDA) receptor-dependent form of synaptic plasticity that could underlie the development of receptive field properties in sensory neocortices. Whilst induction of timing-dependent long-term potentiation (t-LTP) requires postsynaptic NMDA receptors, timing-dependent long-term depression (t-LTD) requires the activation of presynaptic NMDA receptors at layer 4-to-layer 2/3 synapses in barrel cortex. Here we investigated the developmental profile of t-LTD at layer 4-to-layer 2/3 synapses of mouse barrel cortex and studied their NMDA receptor subunit dependence. Timing-dependent LTD emerged in the first postnatal week, was present during the second week and disappeared in the adult, whereas t-LTP persisted in adulthood. An antagonist at GluN2C/D subunit–containing NMDA receptors blocked t-LTD but not t-LTP. Conversely, a GluN2A subunit–preferring antagonist blocked t-LTP but not t-LTD. The GluN2C/D subunit requirement for t-LTD appears to be synapse specific, as GluN2C/D antagonists did not block t-LTD at horizontal cross-columnar layer 2/3-to-layer 2/3 synapses, which was blocked by a GluN2B antagonist instead. These data demonstrate an NMDA receptor subunit-dependent double dissociation of t-LTD and t-LTP mechanisms at layer 4-to-layer 2/3 synapses, and suggest that t-LTD is mediated by distinct molecular mechanisms at different synapses on the same postsynaptic neuron.

Keywords: development, LTD, LTP, rodent, synaptic plasticity

Introduction

Synaptic plasticity is a strong candidate mechanism for the dynamic changes in sensory cortical maps that are observed in early postnatal development (Foeller and Feldman 2004; Fox 2002; Feldman and Brecht 2005). In the rodent barrel cortex, synaptic and receptive field plasticity have been extensively studied during the first few postnatal weeks (Crair and Malenka 1995; Diamond et al. 1993; Glazewski and Fox 1996; Isaac et al. 1997; Stern et al. 2001). At thalamocortical synapses, activity-dependent synaptic potentiation is restricted to the first postnatal week, during which characteristic "barrel" structures form (Crair and Malenka 1995). It is not known to what extent synaptic plasticity at cortico-cortical connections is also restricted to critical periods of development.

A key hypothesis concerning the development and reorganization of sensory receptive fields is that Hebbian mechanisms of long-term depression (LTD) and long-term potentiation (LTP) underlie cortical map plasticity (Buonomano and Merzenich 1998; Feldman and Brecht 2005). Specifically, spike timing–dependent plasticity (STDP), whereby the temporal order of pre- and postsynaptic neuronal activity is critical for the direction of change in synaptic weights, has been suggested to play an important role in map plasticity of barrel cortex (Feldman 2000; Allen et al. 2003; Celikel et al. 2004; for review, see Caporale and Dan 2008). Both timing-dependent LTP (t-LTP) and timing-dependent LTD (t-LTD) require activation of NMDA receptors (Feldman, 2000; Froemke et al. 2005). Recently, it was demonstrated that postsynaptic *N*-methyl-D-aspartate (NMDA) receptors are necessary for the induction of t-LTP, but not t-LTD (Bender et al. 2006; Nevian and Sakmann 2006), and that presynaptic NMDA receptors are required for t-LTD, but not t-LTP (Rodríguez-Moreno and Paulsen 2008). NMDA receptors are heterotetramers, composed of 2 essential GluN1 subunits and 2 GluN2 subunits (using the subunit nomenclature recently recommended by the International Union of Basic and Clinical Pharmacology (IUPHAR); Collingridge et al. 2009), which confer different functional, kinetic, pharmacological, and intracellular signaling properties to the NMDA receptor (for review, see Cull-Candy et al. 2001; Cull-Candy 2007). A different molecular composition of presynaptic and postsynaptic NMDA receptors during development raises the possibility that different NMDA receptor subunits are required for t-LTD and t-LTP (Corlew et al. 2008). Indeed, it was recently suggested that induction of LTP and LTD depends on different NMDA receptor subunits (Hrabetova et al. 2000; Liu, Wong, et al. 2004; Massey et al. 2004; for review, see Yashiro and Philpot 2008).

Here, we investigated the developmental profile of timing-dependent plasticity at layer 4-to-layer 2/3 synapses of mouse barrel cortex and used subunit-preferring NMDA receptor antagonists to test whether t-LTD and t-LTP at these synapses are differentially dependent upon GluN2A, GluN2B, and GluN2C/D subunits. We found that t-LTD is present in the first and second postnatal weeks, but disappears in adult mice. This presynaptic t-LTD requires the activation of GluN2C/D subunit–containing NMDA receptors. In contrast, t-LTP persists in the adult animal and its induction requires GluN2A subunits. These results provide further evidence that different NMDA receptor subunits have distinct functions in synaptic plasticity during postnatal neocortical development.

Materials and Methods

Animals

C57BL/6 mice were purchased from Harlan (Bicester, UK), and ranged in age from postnatal day (P)6 to P102. Mice were kept on a 12-h light/dark cycle and fed ad libitum. All experiments were performed under the animal care guidelines of the UK Animals (Scientific Procedures) Act 1986.

Thalamocortical Slice Preparation

Thalamocortical slices (350–400 μm) containing the barrel subfield of somatosensory cortex were prepared as previously described (Agmon and Connors 1991; Mierau et al. 2004). Briefly, mice were decapitated under isoflurane anesthesia in accordance with UK Animals (Scientific Procedures) Act 1986. The brain was rapidly removed in ice-cold artificial cerebrospinal fluid containing (in mM): NaCl 126; KCl 3; NaH_2PO_4 1.25; $MgSO_4$ 2; $CaCl_2$ 2; $NaHCO_3$ 26; glucose 10; pH 7.2–7.4; bubbled with carbogen gas (95% O_2/5% CO_2). Slices were cut on a vibrating microtome (VT 1000S; Leica, Wetzlar, Germany) and maintained in a submerged-style recording chamber at room temperature (22–27 °C) until used (1–6 h).

Whole-Cell Recording

Slices containing the barrel subfield were identified under a stereomicroscope by the presence of three to five 200- to 400-μm-wide barrels in layer 4. Whole-cell patch-clamp recordings were made from layer 2/3 pyramidal neurons in one of the barrel columns under visual guidance by infrared differential interference contrast (DIC) microscopy. All recordings were made in a submerged-style recording chamber at 25–29 °C between 1 and 6 h after slice preparation. Current-clamp recordings were made with patch pipettes (5–7 MΩ) pulled from standard-wall borosilicate tubing and filled with a solution containing (in mM): potassium gluconate 110; HEPES (4-[2-hydroxyethyl]piperazine-1-ethanesulfonic acid) 40; NaCl 4; ATP-Mg 4; GTP 0.3, pH 7.2–7.3. In some experiments 5 mg/mL biocytin was included in the pipette solution to enable post hoc identification of the recorded neuron. Voltage-clamp recordings in Supplementary Figure S2 were made with an internal solution containing (in mM): CsCl 140; EGTA (ethylene glycol-bis[β-aminoethyl ether]-N,N,N′,N′-tetraacetic acid) 0.2; HEPES 10; ATP-Mg 2; GTP 0.3; and QX-314 5. All recordings were low-pass filtered at 2 kHz and acquired at 5 kHz using an ITC-16 AD board (Instrutech, Port Washington, NY) and custom-made software procedures programmed in Igor Pro (Wavemetrics, Lake Oswego, OR). Series resistance was monitored by adjusting the bridge balance at regular intervals throughout the experiment. Cells were rejected if series resistance changed by more than 15%.

Timing-Dependent LTD and LTP Induction Protocols

Excitatory postsynaptic potentials (EPSPs) were evoked alternately in 2 input pathways, test and control, each at 0.2 Hz by brief current pulses (50 μs, 5–50 μA) via 2 monopolar stimulation electrodes placed within the base of a barrel in layer 4, vertically aligned to the site of recording. while studying cross-columnar layer 2/3-to-layer 2/3 synapses, one stimulating electrode was placed in layer 2/3, just above the identified barrel structure and another electrode was placed on the opposite side of the recording site and served as control. After a stable EPSP baseline period of 10 min, the test input was paired 100 times with a single postsynaptic spike. The control pathway was not stimulated during the pairing period. To induce t-LTD, the postsynaptic action potential was evoked within 10–15 ms before the onset of the EPSP, whereas the postsynaptic action potential was evoked 10 ms after the onset of the EPSP to induce t-LTP. Both EPSP slopes and peak amplitudes were monitored for at least 20 min after each pairing episode. Presynaptic stimulation frequency remained constant throughout the experiment. Interleaved control t-LTD and t-LTP experiments were performed for each pharmacological blocker tested.

Data Analysis

The slope of the EPSP was measured as a linear fit between time points on the rising phase of the EPSP corresponding to 25–30% and 70–75% of the peak amplitude during control conditions. For statistical comparisons, the mean EPSP slope was calculated from 60 consecutive sweeps immediately before the start of pairing (baseline) and compared with 60 sweeps corresponding to 25–30 min after pairing. Data analysis was carried out using Igor Pro software. Data are given as mean ± SEM, unless otherwise stated. Statistical comparisons were made using one-sample or 2-sample 2-tailed Student's *t*-test as appropriate. One-sample tests are reported in the text, 2-sample tests in figure legends. *P* values less than 0.05 were considered statistically significant.

Drugs

D-2-amino-5-phosphonopentanoic acid (D-AP5), (–)-bicuculline methiodide, 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide (NBQX) disodium salt, 2-(4-benzylpiperidino)-1-(4-hydroxyphenyl)-1-propanol (ifenprodil) hemitartrate, (α*R*,β*S*)-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidinepropanol (Ro 25-6981) maleate, (2*S**,3*R**)-1-(phenanthren-2-carbonyl)piperazine-2,3-dicarboxylic acid (PPDA), and *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM251) were purchased from Tocris Bioscience (Bristol, UK). (2*R**,3*S**)-1-(Phenanthrenyl-3-carbonyl)piperazine-2,3-dicarboxylic acid (UBP141) was purchased from Ascent Scientific (Weston-Super-Mare, UK). (*R*)-[(*S*)-1-(4-bromophenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid (NVP-AAM077) was a gift from Novartis Pharma AG (Switzerland). All drugs were bath applied.

Cytochrome Oxidase Staining

Cortical slices were flat mounted between glass slides separated by 1- to 1.5-mm spacers and post fixed in 4% paraformaldehyde (PFA) for about 6 h. They were then transferred to 30% sucrose in phosphate buffer (PB) and left overnight. Slices were cut into 80-μm sections using a freezing microtome. Sections were mounted on double-subbed slides and dried overnight at room temperature. Slides were then placed for one hour in PFA at room temperature. Slides were rinsed with PB, transferred into cytochrome oxidase staining solution, containing 15 mg cytochrome *c* (Sigma, Dorset, UK), 50 mg diaminobenzidine (Sigma), and 4 g sucrose per 100 mL of 0.1 M PB (Wong-Riley 1979; Land and Simons 1985), and incubated at 37 °C in the dark for 24 h. The reaction was stopped by placing the sections in PB. They were then rinsed in distilled water and dehydrated through graded alcohols (50%, 70%, 95%, and 100%), and finally cleared in xylene and mounted in DPX.

Immunohistochemistry for Biocytin-Filled Cells

Barrel cortex slices (400 μm) with biocytin-filled cells were first fixed with 4% PFA and 0.1% glutaraldehyde in PB and resectioned at 30–40 μm. They were then incubated overnight at 4–8 °C in Alexa Fluor–conjugated streptavidin (1:1000; Molecular Probes, Eugene, OR) in phosphate-buffered saline (PBS), washed with PBS, and mounted using Vectashield fluorescence mounting medium (Vector Laboratories, Burlingame, CA).

Results

Input-Specific Timing-Dependent Plasticity at Vertical layer 4-to-layer 2/3 Synapses in Mouse Barrel Cortex

Barrels were clearly visible in unstained mouse thalamocortical slices (Fig. 1*A*; Agmon and Connors 1991; Feldmeyer et al. 2002). We also stained and identified barrels in some slices using cytochrome oxidase staining (Fig. 1*B*). Whole-cell recordings were made from layer 2/3 pyramidal neurons immediately above barrel structures. A few cells were filled with biocytin and processed histochemically afterward to confirm their identity and location in layer 2/3 (Fig. 1*C*). To study the induction of timing-dependent plasticity at excitatory layer 4-to-layer 2/3 synapses, we recorded EPSPs in current-clamp mode from layer 2/3 pyramidal neurons elicited by an extracellular stimulation electrode in layer 4 of the corresponding barrel column (Fig. 1*A*,*D*). After a stable baseline period of 10 min, timing-dependent plasticity was induced by pairing synaptic responses with a single action potential evoked by a brief current pulse

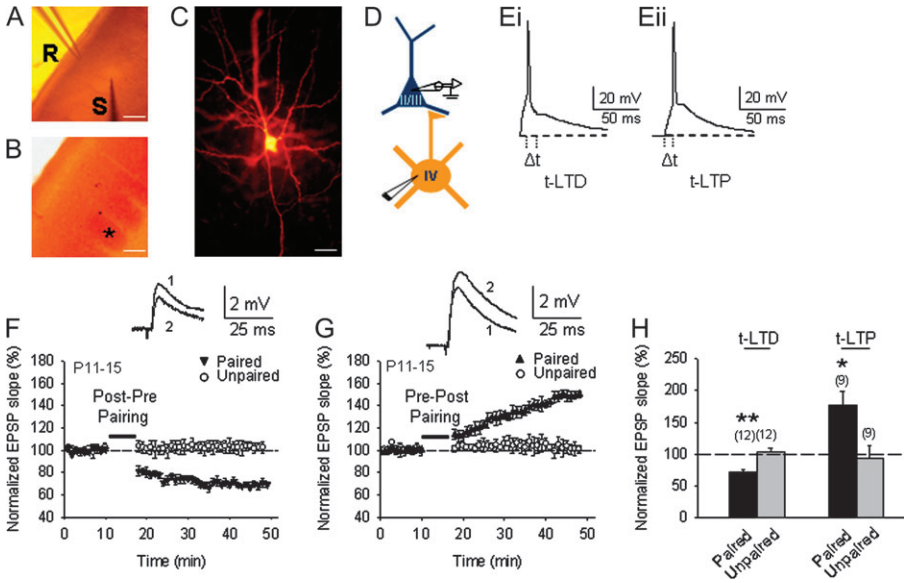

Figure 1. Input-specific timing-dependent plasticity in mouse barrel cortex. (*A*) Light-microscopic view of thalamocortical slice showing positioning of stimulation electrode (S) and recording pipette (R). Scale bar, 100 μm. (*B*) Cytochrome oxidase-stained thalamocortical slice showing barrels (*) in layer 4. Scale bar, 150 μm. (*C*) Biocytin-filled pyramidal neuron in layer 2 stained with 1:1000 dilution of Streptavidin Alexa Fluor 594. Scale bar, 20 μm. (*D*) Schematic diagram of a layer 2/3 pyramidal neuron with patch pipette at the soma and an extracellular stimulation electrode in layer 4. (*E*) Diagram of pairing paradigm. (*Ei*) Post-before-pre pairing protocol induces t-LTD. Δt is the time between peak of spike and EPSP onset. (*Eii*) Pre-before-post pairing protocol induces t-LTP. Δt is the time between EPSP onset and peak of spike. (*F*) A post-before-pre pairing protocol induces t-LTD. EPSP slope monitored in paired experimental (downward black triangles) and unpaired control pathway (open circles). *Inset*, Traces show EPSP amplitude from a sample cell before 1) and 30 min after 2) post–pre pairing. (*G*) A pre-before-post pairing protocol induces robust t-LTP. EPSP slope monitored in paired experimental (upward black triangles) and unpaired control pathway (open circles). Stimulation electrode of unpaired pathway was placed in the same barrel column. *Inset*, Traces show EPSP amplitude from a sample cell before 1) and 30 min after 2) pre–post pairing. (*H*) Summary of results. Error bars are SEM. $*P < 0.05$, $**P < 0.01$, Student's *t*-test. The number of slices used for each protocol is indicated in parentheses at the top of each error bar.

through the patch pipette at 0.2 Hz, repeated 100 times, following which the EPSP was monitored for a further 25–30 min. The last 5 min were used to estimate change in synaptic efficacy compared with baseline. A post-before-pre pairing protocol (with a postsynaptic spike occurring within 10–15 ms before EPSP onset; Fig. 1*Ei*) elicited robust t-LTD in P11–15 mice. Significant t-LTD occurred following a post-before-pre single-spike pairing protocol (slope, 72 ± 3%; amplitude, 71 ± 4%; mean ± SEM; both $P < 0.01$, *t*-test, $n = 12$; Fig. 1*F,H*), whereas an unpaired pathway remained unchanged, or, in the case of amplitude, was also reduced (slope, 103 ± 6%, $P > 0.05$; amplitude, 87 ± 2%, $P < 0.01$, *t*-test, $n = 12$; Fig. 1*F,H*). Conversely, a pre-before-post pairing protocol (with a postsynaptic spike occurring ~10 ms after presynaptic stimulation; Fig. 1*Eii*) induced robust t-LTP. Overall, in slices taken at the end of the second postnatal week (P11–15), significant t-LTP occurred following a pre-before-post single-spike pairing protocol (slope, 163 ± 6%; amplitude, 155 ± 3%; both $P < 0.01$, *t*-test, $n = 9$; Fig. 1*G,H*), whilst an unpaired pathway remained unchanged or was slightly reduced (slope, 94 ± 20%, $P > 0.05$; amplitude, 84 ± 6%, $P < 0.05$, *t*-test, $n = 9$; Fig. 1*G,H*). Thus, input-specific timing-dependent LTD and LTP could both be induced at excitatory layer 4-to-layer 2/3 synapses in mouse barrel cortex in the second postnatal week of development (Fig. 1*H*).

Developmental Profile of Timing-Dependent LTD at Layer 4-to-Layer 2/3 Synapses in Barrel Cortex

LTD plays an important role in the refinement of cortical circuitry during development. We therefore investigated the developmental profile of t-LTD. A significant depression was observed at P6–8 (slope, 72 ± 4%; amplitude, 71 ± 3%; both $P < 0.01$, *t*-test, $n = 6$; Fig. 2*A*), P11–15 (slope, 71 ± 5%; amplitude, 72 ± 5%; both $P < 0.05$, *t*-test, $n = 4$; Fig. 2*B*) as well as P19–25 (slope, 70 ± 6%, $P < 0.05$; amplitude, 76 ± 9%, $P = 0.08$, *t*-test, $n = 4$; Fig. 2*C*). However, the single-spike post-before-pre pairing protocol was unable to induce t-LTD in P25–42 mice (slope, 99 ± 2%; amplitude, 96 ± 4%; both $P > 0.05$, *t*-test, $n = 5$; Fig. 2*D*). This is in agreement with previous reports that LTD is not readily induced in adult animals (Bear and Abraham 1996). The failure to induce t-LTD with a single-spike pairing protocol in P25–42 mice contrasts starkly with the robust t-LTP that was seen at all ages tested after the end of the second postnatal week. Timing-dependent LTP was induced with a pre-before-post single-spike pairing protocol, with potentiation observed at both ~P30 (P19–45; slope, 156 ± 3%; amplitude, 140 ± 10%; both $P < 0.05$, *t*-test, $n = 4$; Fig. 2*E*) and ~P90 (P81–102; slope, 141 ± 8%, $P < 0.05$; amplitude, 154 ± 18%; $P < 0.05$, *t*-test, $n = 5$; Fig. 2*F*). Thus, t-LTD induced by a post-before-pre single-spike pairing paradigm is present at the end of the first postnatal week (P6) and persists till the end of the third postnatal week, but then disappears toward adulthood (Fig. 2*G*) whereas t-LTP induced by a pre-before-post single-spike pairing paradigm persists in the adult barrel cortex (Fig. 2*G*).

Dissociation of NMDA Receptor Subunit Dependence of Timing-Dependent Plasticity at Vertical Layer 4-to-layer 2/3 Synapses in Barrel Cortex

Both t-LTP and t-LTD depend on NMDA receptors

We first confirmed that NMDA receptors are necessary for both t-LTD and t-LTP, using the general NMDA receptor antagonist D-AP5. Brief application of D-AP5 (beginning 10 min before the

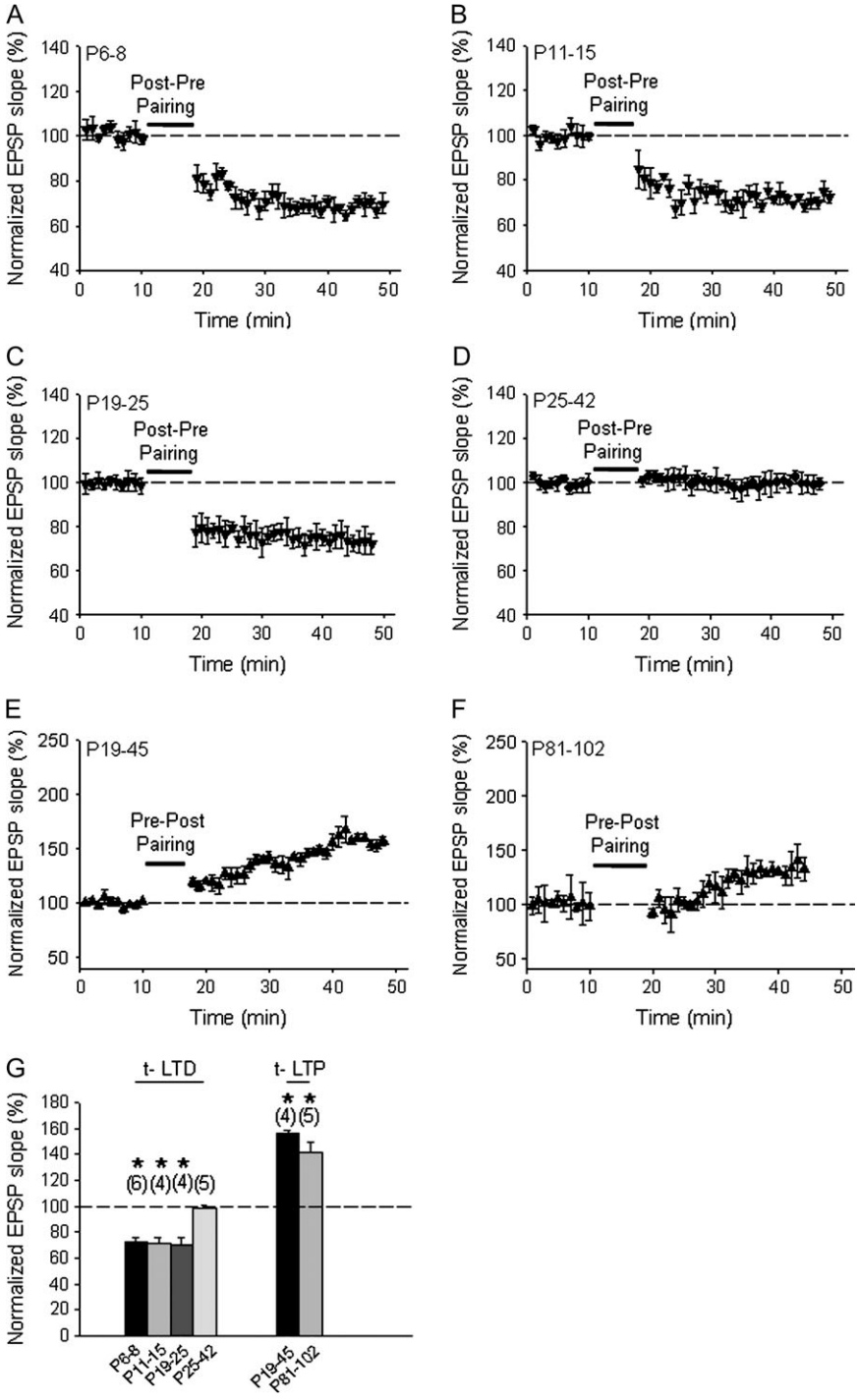

Figure 2. Developmental profile of timing-dependent LTD and timing-dependent LTP at layer 4-to-layer 2/3 synapses in mouse barrel cortex. Synaptic efficacy was monitored over time following post-before-pre single-spike pairing protocol in (*A*) P6–8, (*B*) P11–15, (*C*) P19–25 (black triangles), and (*D*) P25–42 animals (black circles). Developmental profile of timing-dependent LTP at layer 4-to-layer 2/3 synapses in mouse barrel cortex was observed using pre-before-post protocol. Synaptic efficacy was monitored over time following pre-before-post single-spike pairing protocol in (*E*) P19–45, and (*F*) P81–102 animals (black triangles). (*G*) Summary of results. Error bars are SEM. $^*P < 0.05$, Student's *t*-test. The number of slices used for age group is indicated in parentheses at the top of each error bar.

start of pairing) blocked the induction of t-LTD (slope, 95 ± 3%, $P > 0.05$; amplitude, 96 ± 1%, $P < 0.05$, *t*-test, $n = 4$; Fig. 3*A*). Similarly, brief D-AP5 application completely blocked t-LTP (slope, 92 ± 7%; amplitude, 97 ± 10%; both $P > 0.05$, *t*-test, $n = 5$; Fig. 3*B*) at layer 4-to-layer 2/3 synapses in P11–15 mice. These results show that NMDA receptors are necessary for both the induction of t-LTD and t-LTP in the mouse barrel cortex following post-before-pre and pre-before-post pairing protocols, respectively (Fig. 3*C*).

t-LTP but not t-LTD depends on GluN2A subunit–containing NMDA receptors

We next asked whether the different pre- and postsynaptic NMDA receptor requirement of t-LTD and t-LTP might be

reflected in different NMDA receptor subunit involvement. To test whether t-LTD is dependent upon GluN2A subunit–containing receptors, we used the GluN2A subunit-preferring antagonist, NVP-AAM077 (Auberson et al. 2002). We tested whether NVP-AAM077 has any effect on the induction of t-LTD. Timing-dependent LTD induced by a post-before-pre paradigm was not affected by bath application of 100 nM NVP-AAM077 at layer 4-to-layer 2/3 synapses in P11–15 mice (slope, 72 ± 3%; amplitude, 76 ± 2%; both p < 0.01, *t*-test, n = 5; Fig. 4*A*), but NVP-AAM077 (100 nM) completely blocked the induction of t-LTP in P11–15 mice (slope, 109 ± 6%; amplitude, 102 ± 6%; both $P > 0.05$, *t*-test, $n = 6$; Fig. 4*B*). In P6–8 mice, NVP-AAM077 also failed to affect the induction of t-LTD (slope, 75 ± 2%; amplitude, 77 ± 2%; both $P < 0.01$, *t*-test, $n = 6$; Fig. 4*C*), confirming that the GluN2A subunit is not necessary for the induction of t-LTD at both P6–8 and P11–15 layer 4-to-layer 2/3 synapses. Thus, NVP-AAM077 dissociated the NMDA receptor subunit requirement of plasticity at layer 4-to-layer 2/3 synapses during postnatal development (Fig. 4*D*).

Neither t-LTP nor t-LTD is Blocked by an Antagonist at GluN2B Subunit–Containing NMDA Receptors

We then investigated whether GluN2B subunit–containing NMDA receptors are necessary for the induction of t-LTD and

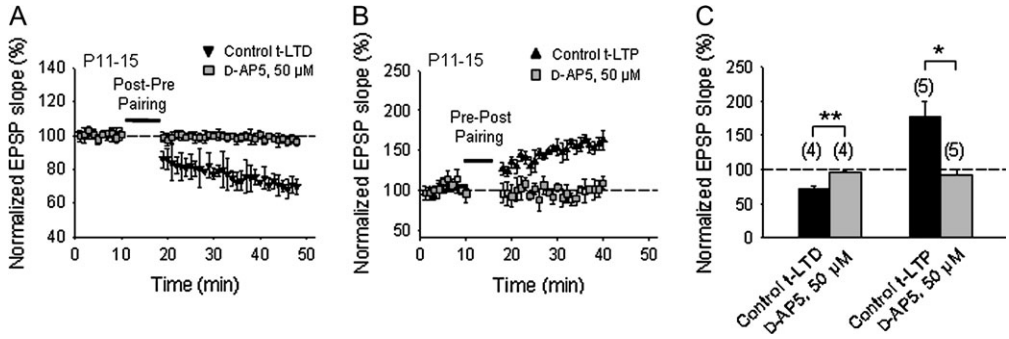

Figure 3. NMDA receptor dependence of timing-dependent plasticity in mouse barrel cortex. Control t-LTD and t-LTP (black triangles) were induced using a post-before-pre and a pre-before-post protocol, respectively, in P11–15 mice. Induction of both t-LTD (*A*) and t-LTP (*B*) was completely blocked following bath application of 50 μM D-AP5 (gray squares). (*C*) Summary of results. Error bars are SEM. $^*P < 0.05$, $^{**}P < 0.01$, Student's *t*-test. The number of slices for each condition is indicated in parentheses at the top of each error bar.

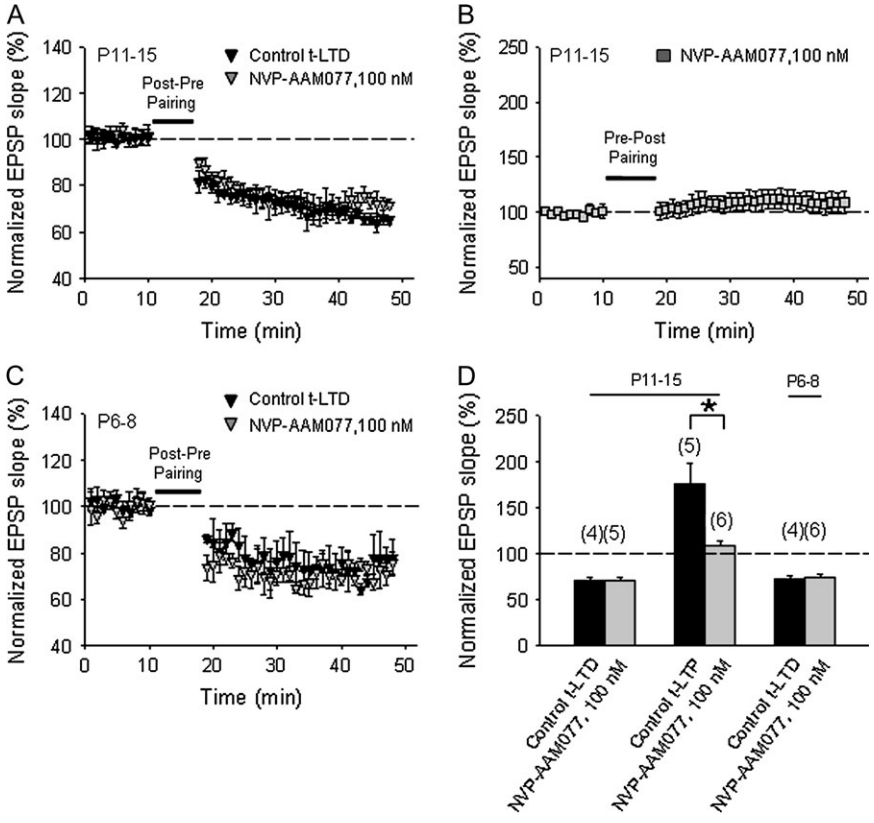

Figure 4. GluN2A subunit dependence of timing-dependent LTP. (*A–C*) t-LTD induction following a post-before-pre pairing paradigm (*A*; black triangles) was unaffected by 100 nM NVP-AAM077 (gray triangles) in P11–15 mice, whereas t-LTP induction was blocked (*B*; gray squares). NVP-AAM077 also did not block t-LTD in P6–8 mice (*C*; gray triangles). (*D*) Summary of results. Error bars are SEM. $^*P < 0.05$, Student's *t*-test. The number of slices used for each condition is indicated in parentheses at the top of each error bar.

t-LTP at layer 4-to-layer 2/3 synapses, using the GluN2B subunit-selective antagonist Ro 25-6981 (Fischer et al. 1997). Ro 25-6981 (0.5 μM) did not affect the induction of t-LTD at layer 4-to-layer 2/3 synapses in P11–15 mice (slope, 69 ± 6%; amplitude, 71 ± 4%; both $P < 0.01$, *t*-test, $n = 5$; Fig. 5*A,C*); neither did it affect the induction of t-LTP at layer 4-to-layer 2/3 synapses in P11–15 mice (slope, 139 ± 8%; amplitude, 137 ± 11%; both $p < 0.01$, *t*-test, n = 6; Fig. 5*B,C*). We also tested the effect of another GluN2B receptor antagonist, ifenprodil (Willams 1993). Ifenprodil (3 μM) only partially reduced t-LTD induced by a post-before-pre pairing protocol in P11–15 (slope, 83 ± 4%; amplitude, 84 ± 3%; both $P < 0.01$, *t*-test, $n = 6$; Fig. S1*A,C*) and P6–8 mice (slope, 91 ± 19%; amplitude, 96 ± 18%; both $P > 0.05$, *t*-test, $n = 6$). A pre-before-post pairing protocol in the presence of ifenprodil still showed t-LTP (slope, 149 ± 15%; amplitude, 156 ± 16%; both $P < 0.05$, *t*-test, $n = 8$; Fig. S1*B,C*) in P11–15 mice. Together, these data reveal no effect of Ro 25-6981, and only a small effect of ifenprodil on t-LTD or t-LTP in P11–15 mice, suggesting that GluN2B subunit might not be essential for timing-dependent plasticity at layer 4-to-layer 2/3 synapses. This raises the question of whether other NMDA receptor subunits might be involved in induction of t-LTD. We therefore investigated the possible involvement of GluN2C/D subunits, which are expressed during early development (Monyer et al. 1994) and by layer 4 neurons (Binshtok et al. 2006).

t-LTD but not t-LTP Depends on GluN2C/D Subunit–Containing NMDA Receptors

The GluN2C/D subunit is expressed postnatally in neocortex, and this expression peaks around the first week of postnatal development (Monyer et al. 1994). To test whether GluN2C/D subunit is involved in timing-dependent plasticity at layer 4-to-layer 2/3 synapses in mouse barrel cortex, we used PPDA, a moderately selective, competitive antagonist at GluN2C/D subunit–containing NMDA receptors. PPDA has >60-fold higher affinity for GluN2C and GluN2D and shows a 3- to 5-fold selectivity for GluN2C/GluN2D versus GluN2A/GluN2B (Morley et al. 2005). Bath application of PPDA (10 μM) completely blocked t-LTD at P11–15 synapses (slope, 110 ± 3%, $P < 0.05$; amplitude, 99 ± 1%, $P > 0.05$, *t*-test, $n = 9$; Fig. 6*A*). In contrast, bath application of PPDA (10 μM) did not block t-LTP induced by a pre-before-post pairing protocol (slope, 153 ± 9%; amplitude, 132 ± 6%; both $P < 0.01$, *t*-test, $n = 5$; Fig. 6*B*). A less potent but more selective GluN2C/D blocker, UBP141, which shows 5- and 7-fold selectivity for GluN2D versus GluN2A and GluN2B, respectively (Morley et al. 2005), also selectively blocked t-LTD in P11–15 mice (slope, 96 ± 3%, $p < 0.05$; amplitude, 89 ± 2%, $P > 0.05$, *t*-test $n = 6$; Fig. 6*C*) with no effect on t-LTP (slope, 195 ± 8%, $P < 0.05$; amplitude, 174 ± 4%, $P < 0.05$, *t*-test $n = 4$; Fig. 6*D*). PPDA also blocked t-LTD in young, immature synapses (P6–8; slope, 119 ± 6%, $P = 0.05$; amplitude, 105 ± 9%, $P > 0.05$, *t*-test, $n = 4$; Fig. 6*E*). Thus, 2 selective antagonists at GluN2C/D subunit–containing NMDA receptors, PPDA (10 μM) and UBP141 (3 μM), selectively block t-LTD without affecting t-LTP at layer 4-to-layer 2/3 synapses during barrel cortex development (Fig. 6*F*).

t-LTD Requires GluN2C/D Subunit–Containing NMDA Receptors at Vertical Intracolumnar but not Horizontal Cross-Columnar Connections

Because t-LTD requires presynaptic NMDA receptors at layer 4-to-layer 2/3 synapses (Rodriguez-Moreno and Paulsen 2008), and GluN2C/D subunits have been reported to be selectively expressed in layer 4 neurons (Binshtok et al. 2006), we predicted that t-LTD should be sensitive to PPDA at vertical layer 4-to-layer 2/3 synapses but not at horizontal layer 2/3-to-layer 2/3 synapses. Indeed, we found that even a 20-fold lower concentration of PPDA (500 nM) completely blocked t-LTD at layer 4-to-layer 2/3 synapses (slope, 97 ± 7%; amplitude, 90 ± 13%; both $P > 0.05$, *t*-test, $n = 5$; Fig. 7*A*), without affecting t-LTD at layer 2/3-to-layer 2/3 synapses. We investigated t-LTD at layer 2/3-to-layer 2/3 synapses by positioning a stimulation electrode in layer 2/3 of a neighboring barrel column. A post-before-pre protocol successfully induced t-LTD at these connections (slope, 75 ± 6%, $P < 0.01$; amplitude, 77 ± 6%, $P < 0.05$, *t*-test, $n = 6$; Fig. 7*B*). But strikingly, although PPDA blocked layer 4-to-layer 2/3 intracolumnar t-LTD, this cross-columnar layer 2/3-to-layer 2/3 t-LTD was unaffected by 10 μM PPDA (slope, 76 ± 8%, $P = 0.06$; amplitude, 73 ± 7%, $P < 0.05$, *t*-test, $n = 4$; Fig. 7*B*). In contrast, t-LTD at horizontal layer 2/3-to-layer 2/3 synapses was blocked by the general NMDA receptor antagonist, 50 μM D-AP5 (slope, 95 ± 3%; amplitude, 93 ± 3%; both $P > 0.05$, *t*-test, $n = 4$) as well as the GluN2B subunit-selective antagonist Ro 25-6981 (slope, 99 ± 6%, $n = 6$ vs. control 75 ± 3%, $n = 4$; amplitude, 94 ± 2.5% vs. control 71 ± 5%; both $P < 0.05$, *t*-test; Fig. 7*B,C*). These results suggest a synapse-specific requirement of GluN2C/D subunit for induction of t-LTD at layer 4-to-layer 2/3 synapses (Fig. 7*C*).

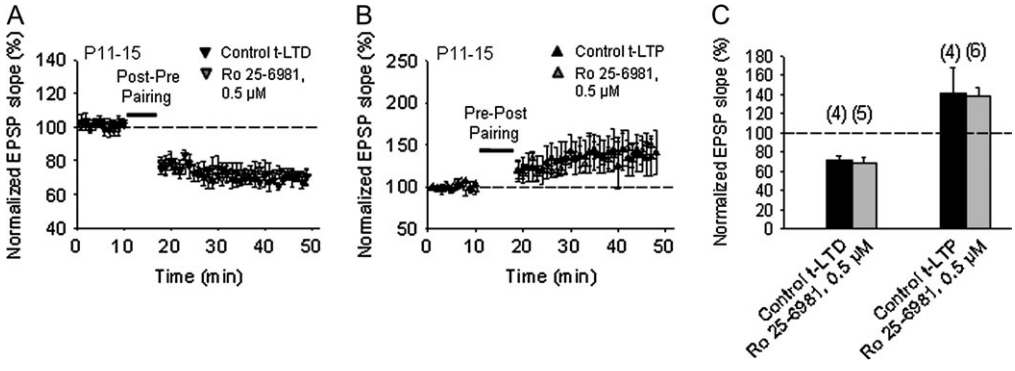

Figure 5. GluN2B subunit in timing-dependent plasticity. (*A, B*) Ro 25-6981 (0.5 μM) did not affect t-LTD (*A;* gray triangles) or t-LTP (*B;* gray triangles) in P11–15 mice. (*C*) Summary of results. Error bars are SEM. The number of slices used for each condition is indicated in parentheses at the top of each error bar.

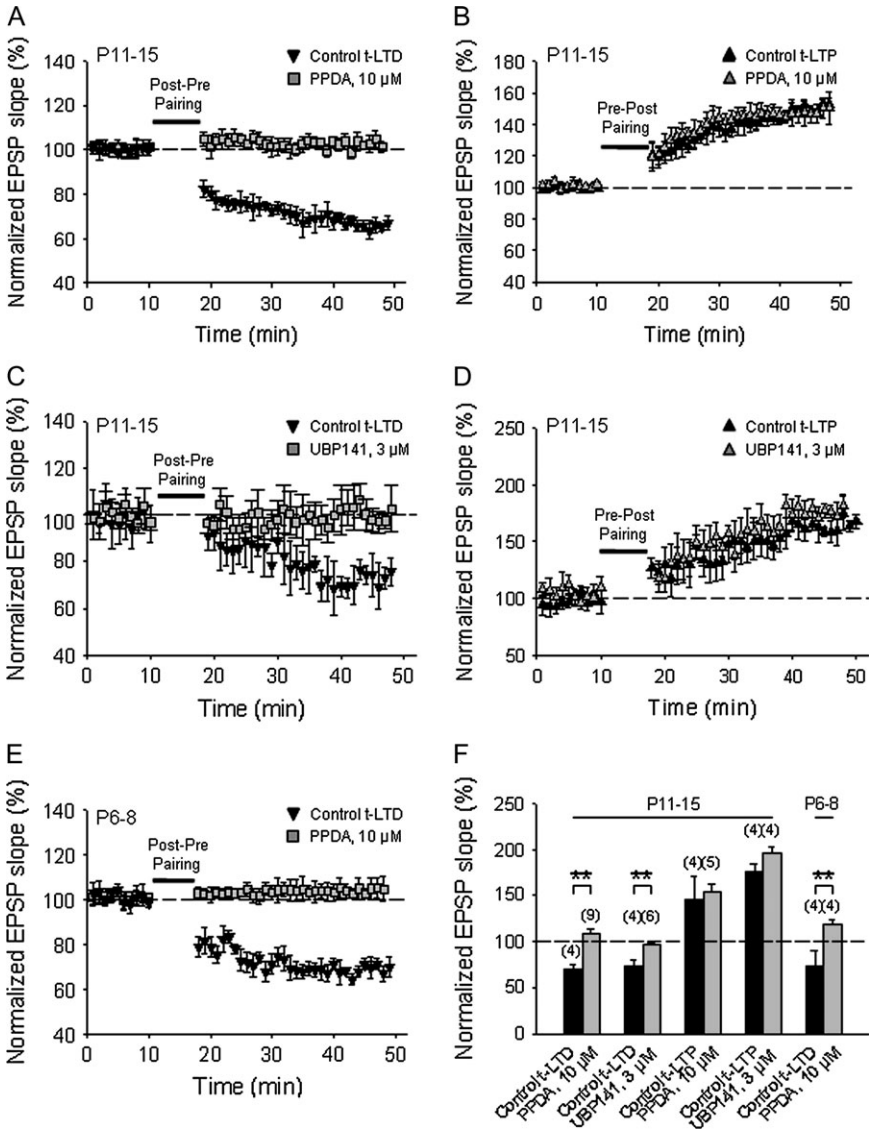

Figure 6. GluN2C/D subunit dependence of timing-dependent LTD. (*A*) PPDA (10 μM) blocked t-LTD following post-before-pre pairing in P11–15 mice (gray squares). (*B*) PPDA (10 μM) did not block t-LTP following pre-before-post pairing in P11–15 mice (gray triangles). (*C*, *D*) A more selective GluN2C/D blocker, UBP141, also blocked t-LTD (*C*; gray squares) in layer 4-to-layer 2/3 synapses but had no effect on t-LTP (*D*; gray triangles). (*E*) PPDA also blocked t-LTD in young, immature synapses (gray squares). (*F*) Summary of results. Error bars are SEM. $^{**}P < 0.01$, Student's *t*-test. The number of slices used for each condition is indicated in parentheses at the top of each error bar.

t-LTD Requires CB1 Receptors at Horizontal Cross-Columnar but not Vertical Intracolumnar Connections

Recent studies have implicated endocannabinoid signaling through CB1 receptors in intracortical LTD induction (Sjöström et al. 2003; Bender et al. 2006). To further dissociate t-LTD at vertical layer 4-to-layer 2/3 and horizontal layer 2/3-to-layer 2/3 synapses, we investigated whether these forms of t-LTD require activation of CB1 receptors. Preincubation (60 min) and bath application of the CB1 receptor antagonist, AM251 (3 μM), did not affect t-LTD at layer 4-to-layer 2/3 synapses (slope, 68 ± 13%, $P < 0.05$; amplitude, 72 ± 13%, $P < 0.05$, *t*-test, $n = 9$; Fig. 7*D*,*F*). This result is different from that reported at layer 4-to-layer 2/3 synapses in rat barrel cortex (Bender et al. 2006), but consistent with another recent report in mice (Hardingham et al. 2008). In contrast, AM251 completely blocked t-LTD in cross-columnar horizontal layer 2/3-to-layer 2/3 synapses (slope, 104 ± 8%, $P > 0.05$; amplitude, 94 ± 4%, $P > 0.05$, *t*-test, $n = 5$; Fig. 7*E*,*F*). Thus, vertical intracolumnar synapses and horizontal cross-columnar synapses on layer 2/3 neurons appear to have distinct molecular properties and different requirements for the induction of t-LTD.

In summary, both t-LTD and t-LTP could be induced at excitatory layer 4-to-layer 2/3 synapses in the second week of postnatal development in mouse barrel cortex. However, these forms of plasticity showed different developmental profiles, and different NMDA receptor subunit requirement. Whereas t-LTD requires the activation of GluN2C/D subunit–containing NMDA receptors, t-LTP requires GluN2A subunit–containing NMDA receptors. The GluN2C/D subunits are localized presynaptically, and appear to contribute to t-LTD specifically at the layer 4-to-layer 2/3 synapse.

Discussion

Our data reveal that timing-dependent depression at layer 4-to-layer 2/3 synapses in the mouse barrel cortex emerges during the first postnatal week and disappears in adulthood. This form

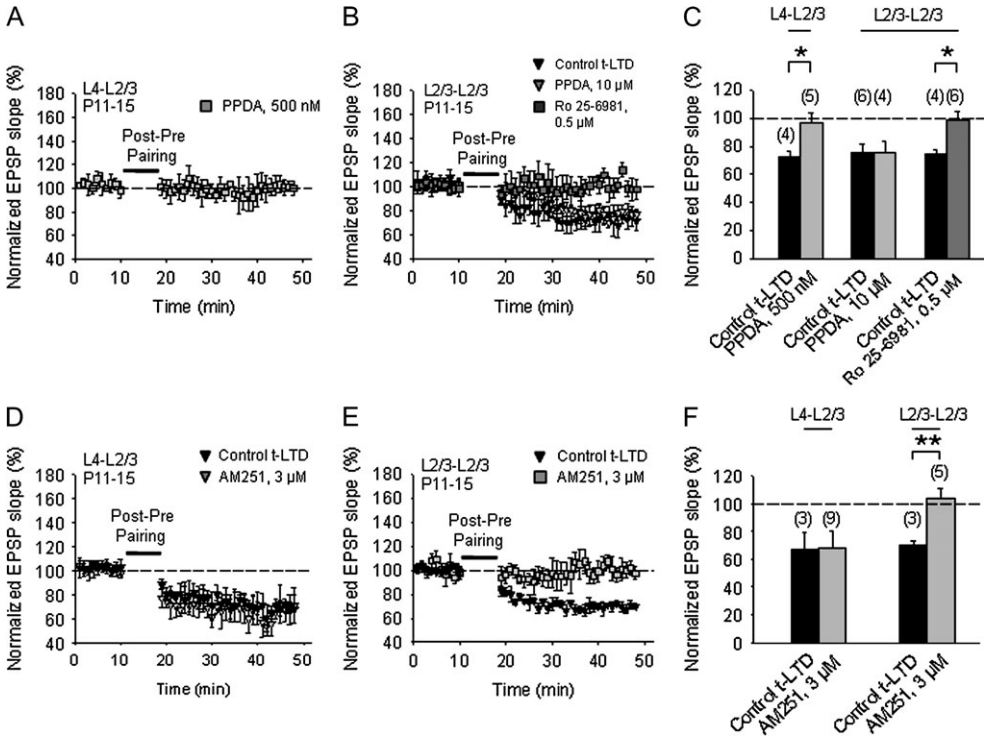

Figure 7. Different induction requirements for timing-dependent LTD at vertical layer 4-to-layer 2/3 and horizontal layer 2/3-to-layer 2/3 synapses. (*A*) t-LTD is blocked by 500 nM PPDA at layer 4-to-layer 2/3 synapses in P11–15 mice (gray squares). (*B*) t-LTD induction at layer 2/3-to-layer 2/3 synapses is unaffected by 10 µM PPDA (gray triangles) but is blocked by 0.5 µM Ro 25-6981 (dark gray squares). (*C*) Summary of results. Error bars are SEM. $*P < 0.05$, Student's *t*-test. The number of slices used for each condition is indicated in parentheses at the top of each error bar. Control t-LTD at layer 4-to-layer 2/3 synapses is the same data as presented in Figure 6*A*,*F*. (*D*, *E*) Preincubation and bath application of CB1 receptor antagonist AM251 did not block t-LTD at layer 4-to-layer 2/3 synapses (*D*; gray triangles), but blocked t-LTD at layer 2/3-to-layer 2/3 synapses (*E*; gray squares). (*F*) Summary of results. Error bars are SEM. $**P < 0.01$, Student's *t*-test. The number of slices used for each condition is indicated in parentheses at the top of each error bar.

of LTD was blocked by a GluN2C/D subunit-selective antagonist at NMDA receptors. By contrast, from the second postnatal week, these synapses show timing-dependent potentiation which persists in adulthood. This form of potentiation was selectively blocked by a GluN2A subunit-preferring antagonist. Thus, at these synapses, t-LTD and t-LTP are developmentally dissociated and differentially dependent upon GluN2C/D and GluN2A NMDA receptor subunits, respectively.

LTD and LTP in Sensory Cortices

LTD has been suggested to play major roles in map plasticity during development (for review, see Buonomano and Merzenich 1998; Feldman and Brecht 2005). Even after cortical maps have been formed, depending on sensory input, LTD is thought to weaken excitatory synapses which are underused or behaviorally irrelevant. In our experiments, we did not observe t-LTD in layer 4-to-layer 2/3 synapses after ~P25, consistent with earlier reports that the capacity for synaptic depression in cortical synapses declines with age (Dudek and Bear 1993; Bear and Abraham 1996), although pairing-induced LTD was reported to persist in mouse visual cortex (Jiang et al. 2007).

Our results extend the developmental period of timing-dependent LTP previously reported at layer 4-to–layer 2/3 synapses in barrel cortex (Feldman 2000) into young adulthood, in contrast to potentiation at the thalamocortical input, which is restricted to the first postnatal week (Crair and Malenka 1995). In both visual and barrel cortices, STDP was seen at horizontal connections in slices from 2- to 5-week-old rats (Froemke and Dan 2002; Nevian and Sakmann 2004). It has been suggested previously that synaptic plasticity at this cortico-cortical level remains in adulthood (Fox 2002), consistent with our finding that t-LTP persists in adult barrel cortex and in agreement with previous studies showing tetanically induced LTP in adult rat motor cortex (Aroniadou and Keller 1995), rat barrel cortex (Glazewski et al. 1998), and similar to pairing-induced plasticity in mouse visual cortex (Jiang et al. 2007; but see Yoshimura et al. 2003).

NMDA Receptor Dependence of Synaptic Plasticity

NMDA receptors figure prominently in both learning and developmental plasticity. Their 2 key properties, the voltage dependent magnesium block at resting membrane potential and calcium permeability, have made them strong candidate coincidence detectors for Hebbian plasticity (Paulsen and Sejnowski 2000; Bi and Poo 2001). Previous studies in barrel cortex have shown timing-dependent plasticity to be NMDA receptor dependent, using the antagonist D-AP5 (Feldman 2000; Bender et al. 2006). The composition of NMDA receptors changes during the second and third postnatal weeks of development in somatosensory cortical synapses with an increase in the expression of the GluN2A receptor subunit, and is accompanied by a quickening of excitatory postsynaptic NMDA current decay times and a decrease in the sensitivity to the GluN2B subunit-selective antagonist, ifenprodil (Liu, Murray, et al. 2004; Mierau et al. 2004). Our results show that t-LTP is sensitive to GluN2A subunit-preferring antagonists at

NMDA receptors and is present at a time when the GluN2A subunit is expressed (Monyer et al. 1994). Conversely, t-LTD was dependent on the GluN2C/D subunit, and was downregulated with age, reflecting, perhaps, the downregulation of these NMDA receptor subunits with development (Monyer et al. 1994).

NMDA Receptor Subunits in Timing-Dependent Plasticity

The presence of distinct subpopulations of NMDA receptors at different ages and in different brain regions raises the attractive possibility that different receptor subtypes play different roles in brain function (Cull-Candy and Leszkiewicz 2004). Using subunit-preferring pharmacological agents, it was reported that LTP and LTD induced by high-frequency and low-frequency afferent stimulation, respectively, could be dissociated, with LTP being dependent on GluN2A, but not GluN2B, subunit–containing receptors, and LTD requiring GluN2B, but not GluN2A, subunit–containing receptors, in the hippocampus (Liu, Wong, et al. 2004) as well as perirhinal cortex (Massey et al. 2004). In contrast, other reports suggested that both receptor subtypes are involved in the induction of LTP in the hippocampus (Berberich et al. 2005) and in the induction of LTD in the anterior cingulate cortex (Toyoda et al. 2005). Moreover, it has been reported that the NR2A subunit is not required for LTP in the dorsolateral bed nucleus of the stria terminalis (Weitlauf et al. 2005) and that the NR2B subunit is not required for LTD in the hippocampus (Morishita et al. 2007), suggesting that the situation is more complex than the first studies appeared to suggest. Finally, truly subunit-selective antagonists are currently available only for the NR2B subunit, and caution must be exercised when interpreting data obtained with less selective subunit-preferring drugs (Neyton and Paoletti 2006). Nevertheless, in our experiments, the GluN2A-preferring antagonist NVP-AAM077 at 100 nM concentration completely abolished t-LTP at layer 4-to-layer 2/3 synapses in barrel cortex in the second postnatal week, without affecting t-LTD, whereas the GluN2C/D-preferring antagonists PPDA and UBP141 both completely blocked t-LTD without affecting t-LTP, thus presenting an experimental double dissociation. Moreover, the GluN2B antagonist ifenprodil (Williams 1993), at a concentration that blocks a similar fraction of the postsynaptic NMDA receptor–mediated current (see Fig. S2), did not block t-LTP, suggesting a preferential involvement of GluN2A subunit–containing NMDA receptors in the induction of t-LTP at this synapse, although a contribution by GluN2B subunit–containing NMDA receptors to the induction of t-LTP can not be excluded.

Previously, the GluN2B subunit has been linked specifically to the induction of LTD in the hippocampus (Liu, Wong, et al. 2004), perirhinal cortex (Massey et al. 2004), visual cortex (Sjöström et al. 2003) as well as barrel cortex (Bender et al. 2006). Two GluN2B-selective antagonists failed to block t-LTD in our experiments, prompting our search for a different subunit involved in t-LTD at layer 4-to-layer 2/3 synapses in mouse barrel cortex. A moderately selective antagonist at GluN2C/D subunit–containing receptors, PPDA (10 μM and 500 nM), and also a more selective blocker, UBP141 (3 μM), both blocked t-LTD without affecting t-LTP at P6–8 and P11–15 synapses indicating that GluN2C/D subunit might be necessary for t-LTD at these synapses. Interestingly, based on the potency of PPDA in blocking LTD relative to LTP in the stratum radiatum of CA1 of hippocampal slices from P16 to 21 rats, compared with other NMDA receptor antagonists, it was concluded that receptors containing GluN2C/D subunits are critical for LTD also at these synapses (Hrabetova et al. 2000).

GluN2C/D Subunits and Synaptic Plasticity

The GluN2C/D NMDA receptor subunits are interesting in relation to cortical plasticity because both their mRNA (Monyer et al. 1994) and protein are expressed postnatally in the cortex in a developmentally regulated way (Dunah et al. 1996, 1998; Binshtok et al. 2006). We found that 2 GluN2C/D subunit–selective NMDA receptor antagonists, PPDA (500 nM and 10 μM) and UBP141 (3 μM), selectively block t-LTD at layer 4-to-layer 2/3 synapses. Strikingly, neither PPDA nor UBP141 had any effect on the induction of t-LTP at these same synapses, and did not block t-LTD at layer 2/3-to-layer 2/3 synapses, which was instead blocked by a GluN2B subunit–selective antagonist. Similar to the presynaptic NMDA receptor requirement for t-LTD in mouse visual cortex before P20 (Corlew et al. 2007), it was recently shown that t-LTD at layer 4-to-layer 2/3 synapses in mouse barrel cortex requires presynaptic rather than postsynaptic NMDA receptors (Rodríguez-Moreno and Paulsen 2008). The present data suggest that these presynaptic NMDA receptors contain GluN2C/D subunits. Thus, the differential NMDA receptor subunit requirement for the induction of LTP and LTD might reflect compartment-specific expression of different NMDA receptor subunits (Duguid and Sjöström 2006). It remains to be determined whether the presynaptic NMDA receptors are located in the axon or might be expressed in the dendrites of the presynaptic neuron and influence the axon terminals via passive propagation of a somatodendritic depolarization, as recently reported in cerebellar stellate cells (Christie and Jahr 2008).

Functional presynaptic NMDA receptors were first reported in the entorhinal cortex (Berretta and Jones 1996), and have since been found in many other brain regions, including neocortical layer 4-to-layer 2/3 synapses (Corlew et al. 2007; Brasier and Feldman 2008; for review see Corlew et al. 2008). In addition to t-LTD, presynaptic NMDA receptors have been implicated in other forms of plasticity at both excitatory and inhibitory synapses, including heterosynaptic associative LTP at cortical afferent synapses in the amygdala (Humeau et al. 2003), depolarization-induced potentiation of inhibition in the cerebellum (Duguid and Smart 2004), and LTD at GABAergic synapses in the tadpole optic tectum (Lien et al. 2006), as discussed in 2 recent reviews (Duguid and Sjöström 2006; Corlew et al. 2008).

The involvement of presynaptic GluN2C/D subunits in t-LTD at layer 4-to-layer 2/3 synapses is particularly interesting because the deactivation time constant of GluN2C/D subunit–containing receptors is slow (Momiyama et al. 1996; Brothwell et al. 2008; Wyllie 2008), which might be relevant for the particularly broad time window for induction of t-LTD at this synapse (Feldman 2000). The presence of NMDA receptors with low conductance and reduced susceptibility to Mg^{2+} block in the presynaptic layer 4 spiny stellate cells was reported earlier using transgenic mice expressing beta-galactosidase under the GluN2C promoter (Binshtok et al. 2006). The unavailability of selective pharmacological blockers that distinguish between GluN2C and GluN2D subtype did not allow us to specifically investigate whether it is GluN2C and/or

GluN2D subunits that are important in t-LTD. Nevertheless, considering their interesting kinetic properties, both are potential candidates for mediating input-specific t-LTD at layer 4-to-layer 2/3 synapses.

Endocannabinoid Involvement in Timing-Dependent Depression

Previous studies have identified an NMDA receptor and endocannabinoid-dependent form of LTD at synapses between layer 5 pyramidal neurons in rat visual cortex (Sjöström et al. 2003). Although CB1 receptors were necessary for induction of t-LTD at horizontal layer 2/3-to-layer 2/3 synapses, we found no evidence for a requirement of CB1 receptors at mouse layer 4-to-layer 2/3 synapses. This result is different from that reported at layer 4-to-layer 2/3 synapses in rat barrel cortex (Bender et al. 2006), but consistent with a recent report from mouse barrel cortex (Hardingham et al. 2008), suggesting a possible species difference. Our result indicates that endocannabinoids are not obligatory for all forms of timing-dependent synaptic depression and suggests that at least 2 distinct forms of presynaptic NMDA receptor-dependent LTD can be dissociated, one dependent on endocannabinoid signaling and the GluN2B subunit (Sjöström et al. 2003), and another independent of endocannabinoids and dependent on the GluN2C/D subunit. This result supports the suggestion that different excitatory synapses onto the same postsynaptic neurons can have different molecular requirements for induction of synaptic plasticity (Duguid and Sjöström 2006).

In conclusion, this study has revealed the developmental profile and NMDA receptor subunit requirement of a timing-dependent form of synaptic depression at layer 4-to-layer 2/3 synapses. Timing-dependent LTD was present at the end of the first postnatal week, disappeared after the third postnatal week, and was dependent on the GluN2C and/or GluN2D subunit of the NMDA receptor. In contrast, a timing-dependent form of potentiation at layer 4-to-layer 2/3 synapses was seen in the second postnatal week, persisted into adulthood, and was sensitive to GluN2A subunit-preferring antagonists. Together, these results demonstrate a developmental and NMDA receptor subunit-dependent double dissociation of plasticity at vertical layer 4-to-layer 2/3 synapses in the mouse barrel cortex.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/

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Notes

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