Synaptic NMDA receptors in basolateral amygdala principal neurons are triheteromeric proteins: physiological role of GluN2B subunits

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Running Title:
GluN2B / NR2B subunits and LTP in the amygdala

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Abstract

N-Methyl-D-Aspartate (NMDA) receptors are heteromultimeric ion channels that contain an essential GluN1 subunit and two or more GluN2 (GluN2A-GluN2D) subunits. The biophysical properties and physiological roles of synaptic NMDA receptors are dependent on their subunit composition. In the basolateral amygdala (BLA), it has been suggested that the plasticity that underlies fear learning requires activation of heterodimeric receptors composed of GluN1/GluN2B subunits. In this study we investigated the subunit composition of NMDA receptors present at synapses on principal neurons in the BLA. Purification of the synaptic fraction showed that both GluN2A and GluN2B subunits are present at synapses and, co-immunoprecipitation revealed the presence of receptors containing both GluN2A and GluN2B subunits. The kinetics of NMDA receptor-mediated synaptic currents and pharmacological blockade indicate that heterodimeric GluN1/GluN2B receptors are unlikely to be present at glutamatergic synapses on BLA principal neurons. Selective RNAi-mediated knockdown of GluN2A subunits converted synaptic receptors to a GluN1/GluN2B phenotype whereas knockdown of GluN2B subunits had no effect on the kinetics of the synaptically evoked NMDA current. Blockade of GluN1/GluN2B heterodimers with ifenprodil had no effect, but knock down of GluN2B disrupted the induction of CaMKII-dependent long-term potentiation at these synapses. These results suggest that on BLA principal neurons, GluN2B subunits are only present as GluN1/GluN2A/GluN2B heterotrimeric NMDARs. The GluN2B subunit has little impact on the kinetics of the receptor, but is essential for the recruitment of signalling molecules essential for synaptic plasticity.

Key words: LTP, calcium, GluN2B, GluN2A, memory, CaMKII
Introduction

N-methyl-D-aspartate receptors (NMDA) are glutamate-gated ion channels that play a key role in synaptic plasticity that underlies learning and memory formation. These receptors are assembled from three types of subunits, GluN1, GluN2 and GluN3. The GluN1 subunit is the product of a single gene (Cull-Candy et al. 2001) whereas four GluN2 subunits (GluN2A – GluN2D) and two GluN3 (3A and 3B) subunits have been described (Cavara and Hollmann 2008; Cull-Candy et al. 2001). Of these, GluN2C expression is largely confined to the cerebellum and GluN2D, and in most regions, is only present early in development. Although the exact stoichiometry of the NMDA receptor is not fully established (Paoletti and Neyton 2007; Salussolia et al. 2011), it is thought to be a tetramer containing two obligatory GluN1 subunits and two GluN2 and/or GluN3 subunits (Cull-Candy et al. 2001; Furukawa et al. 2005) assembled as a dimer of dimers (Furukawa et al. 2005; Salussolia et al. 2011). GluN3 expression is not widespread, and therefore most receptors in the mammalian forebrain are thought to contain GluN2A and/or GluN2B subunits. These subunits can assemble as GluN1/GluN2A and GluN1/GluN2B heterodimers or as GluN1/GluN2A/GluN2B heterotrimers (Dunah and Standaert 2003; Luo et al. 1997; Sans et al. 2000; Sheng et al. 1994; Tovar and Westbrook 1999). Heterologous expression studies have shown that the subunit composition confers distinct biophysical and pharmacological properties to these receptors (Monyer et al. 1994). Receptors consisting of GluN1/GluN2B subunits have slower deactivation kinetics than those containing GluN1/GluN2A subunits (Erreger et al. 2005; Vicini et al. 1998). GluN1/GluN2B heterodimers are also more sensitive to the non-competitive antagonist ifenprodil and its derivatives than GluN1/GluN2A heterodimers (Hatton and Paoletti 2005; Ogden and Traynelis 2011).

The subunit composition of the NMDA receptors is developmentally regulated, with GluN2B being expressed prenatally whereas GluN2A expression begins shortly after birth and progressively increases (Monyer et al. 1994; Sheng et al. 1994). When first established, glutamatergic synapses express NMDA receptors that are GluN1/GluN2B heterodimers but with development, the GluN2B subunits are replaced by GluN2A (Barria and Malinow 2002; Philpot et al. 2001). This change in subunit composition is mirrored by changes in the kinetics (Carmignoto and Vicini 1992; Hestrin 1992) and pharmacology (Stocca and Vicini 1998; Tovar and Westbrook 1999) of NMDA receptor-mediated synaptic currents and, at mature glutamatergic synapses both GluN2A and GluN2B containing heterodimers are
thought to be present, perhaps with each having distinct functional roles (Yashiro and Philpot 2008).

The amygdala plays a central role in fear conditioning, with both fear learning and extinction thought to result from NMDA receptor-dependent synaptic plasticity (LeDoux 2003; Sah et al. 2003). Both GluN2A and GluN2B subunits are present in the basolateral amygdala (BLA) (Farb and LeDoux 1997; Lopez de Armentia and Sah 2003) and pharmacological studies have suggested that amygdala-dependent learning requires the activation of GluN1/ GluN2B heterodimers (Rodrigues et al. 2001; Walker and Davis 2008). However, although GluN2B subunits are present at synapses, whether they assemble as heterodimers or heterotrimers is not clear (Lopez de Armentia and Sah 2003; Rauner and Kohr 2011). In this study we investigated the subunit composition of synaptic NMDA receptors at glutamatergic synapses on principal neurons in the basolateral amygdala. Our results indicate that at these synapses, NMDA receptors are likely to be GluN1/GluN2A/GluN2B heterotrimers. Receptors composed of GluN1/GluN2B heterodimers do not appear to be present at these synapses.

Methods

All experiments were done using tissue isolated from the brains of Wistar rats. All procedures were approved by the University of Queensland Animal Ethics Committee.

Lysates, PSD fraction purification, western blots and quantification

Tissues from the BLA and hippocampus were micro-dissected from 500 µm coronal brain slices of adult Wistar rats (postnatal day 25-40). The tissues were homogenized with a Teflon-glass potter in Tris buffer (30mM Tris, 1mM EGTA, 4mM EDTA, pH 7.4). All buffers were supplemented with protease inhibitors (Roche). The homogenates were centrifuged at 800 g for 10 min and the supernatants then centrifuged at 100,000 g for 1h at 4°C. The pellets were re-suspended in Tris buffer containing 0.5% Triton-X 100, incubated on ice for 20min and layered over a 1M sucrose gradient. After 1 h centrifugation at 100,000 g, the resulting pellet was denoted as the Triton-insoluble postsynaptic density (PSD) fraction. For total lysates, micro-dissected material was placed in ice-cold phosphate buffered saline containing protease inhibitors and was homogenized using an 18 gauge needle before the addition of sample buffer containing no bromophenol blue. Protein concentrations were then determined using the Pierce BCA kit. PSD fractions were lysed in SDS sample buffer and bromophenol blue was added to the lysates. All samples were denatured with DTT and boiled
for 5-10 min. They were then fractionated on 4-12% Bis-Tris gradient gels (Invitrogen) and transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore) at 150V in 1XMOPS running buffer (Invitrogen). Blots were blocked in TBS containing 5% skim milk powder, and probed with primary antibody, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit or mouse IgG (Biorad) and detection by SuperSignal West Pico or FEMTO chemiluminescent substrate (Pierce). Densitometry analysis was carried out using NIH ImageJ software.

**Immunoprecipitation**

PSD fractions (250 μg) from the BLA and the hippocampus were solubilized in 2% SDS buffer, then diluted in five volumes of 2% Triton buffer and centrifuged at 100,000 g for 30 min at 4°C. The resulting supernatants were classified as the PSD soluble fraction. Supernatants were pre-cleared for 30 min at 4°C with 20 μl of 50% Protein A sepharose beads (Amersham) slurry and 5 μg rabbit IgG (Sigma, 1mg/ml) then centrifuged for 5 min at 800g. For immunoprecipitation, 3 μg of NMDA2A or NMDA2B antibody (both rabbit polyclonal from Chemicon) was incubated with 20 μl 50% Protein A sepharose bead slurry for 30 min at 4°C and then added to the precleared supernatant overnight at 4°C with rotation. Samples were then spun at 900g for 5 min at 4°C and the pellets recovered. Pellets were washed 3 times in SDS-Triton buffer before elution with sample buffer containing DTT and denatured for 8 min at 70°C. Samples were run together with the PSD fraction input and the supernatant resulting from the immunoprecipitation, transferred as described previously (Faber et al. 2006) and stained for NMDA2A (Millipore, mouse monoclonal), NMDA2B (Watanabe, rabbit polyclonal) and PSD95 (Chemicon, mouse monoclonal).

**Production of shRNA constructs**

A series of 5 siRNA expression cassettes (SECs) were produced using the Ambion Silencer Express siRNA expression cassette kit to knock down rat GluN2A gene expression. These SECs, containing the mouse U6 RNA polymerase III promoter, a hairpin siRNA template and an RNA polymerase III transcription termination site, were ligated into pBluescript (Stratagene) digested with EcoR1 and HindIII. The sequences for these SECs were as follows (GenBank- Rat NR2A NM_012573.3: 1- gacaacagtggacaacagc (786-804); 2- ggacttgtccttcactgag (1260-1278); 3- gtggtctatcaacgagcag (1720-1738 ); 4- gttctcccagggataagat (3554-3572); 5- acactcattgccatcacag(4386-4404)). A scrambled control SEC (SCR) (Ambion) was also used as a negative control. The SEC sequences were tested in HEK293T cells co-transfected with rat GluN2A and GluN2B subunits. Sequence #2 showed the best
knock down and was therefore used for further experiments. To distinguish transfected
versus non-transfected cells in organotypic cultures, an EGFP PCR fragment from
pEF/myc/cyto/GFP (Invitrogen) with the promoter and terminator sequences, was ligated into
the Smal site of the pBluescript SEC construct. A rat GluN2B knockdown construct was also
produced in the same way using an already published siRNA sequence (agctggtcctccaaaagac)
(Tan et al. 2005).

**HEK293T expression of shRNA constructs**

HEK293T cells were plated at a density of $1.5 \times 10^5$ cells per 35 mm well and were
transfected 24h later using FUGENE (Roche) as per the manufacturer’s instructions with
constructs expressing rat GluN2 subunits (GluN2A and GluN2B), and the shRNA EGFP
constructs in a ratio of 1:1:1. Forty-eight hours later, the cells were checked for EGFP
expression (greater than 80% transfection efficiency was typically achieved), washed with
PBS and lysed in 300 µl 1X sample buffer. 30 µl of this suspension was run on an SDS-
PAGE gel, transferred and western blots carried out as described above. For
electrophysiological recordings, cells were transfected with constructs expressing rat GluN2
subunits (GluN2A or GluN2B), GluN1-1a subunits and the shRNA EGFP constructs in a ratio
of 1:1:1, then plated on cover slips that had been previously coated with polyornithine.
Cultures were maintained in d-APV (100 µM, Sigma) to decrease excitotoxic cell death but
this d-APV was washed out prior to electrophysiological recordings.

**Organotypic cultures and biolistic transfection**

Amygdala organotypic cultures were prepared according to the standard interface method
(Stoppini et al. 1991). Coronal brain slices containing the BLA were prepared from P9 rats.
Rats were anesthetized using isoflurane, decapitated, and their brains removed into an ice-
cold artificial cerebral spinal fluid (ACSF) solution containing (in mM) 118 NaCl, 25
NaHCO₃, 10 glucose, 2.5 CaCl₂, 1.2 NaH₂PO₄ and 1.3 MgCl₂. Brains were sliced into 300
µm thick coronal sections using a Leica VT1000S vibratome at 0°C, then transferred to
porous membrane inserts (Millicell inserts, Millipore) using a culture medium containing
50% OptiMEM (Invitrogen), 25% heat-inactivated horse serum and 25% Hanks balanced salt
Solution (Sigma) supplemented with D-glucose (6.5 g/l) and penicillin/streptomycin
(Invitrogen). The cultures were maintained at 37°C in a 5% CO₂ atmosphere for 2-3 days.
Gold particles (9mg, 1.6µm, Biorad) were mixed with 100 µl 0.05 M spermidine and 25 µg
purified plasmid DNA. DNA was precipitated by addition of 100 µl 5% CaCl₂ and the bullets
were washed in 100% ethanol and resuspended in 3 ml of 100% ethanol containing
0.05mg/ml polyvinylpyrrolidone. The mix was taken up in Tefzel tubing and the ethanol was quickly removed before commencing rotation of the tube loading station (Biorad). The particles were allowed to dry using a flow of nitrogen and the bullets were then cut to size. Slices were shot using a Helios Gene Gun (Biorad) with a modified barrel (O’Brien et al, 2001) approximately 3 cm from the slice through a 70 μm nylon mesh (Falcon) at a pressure of 70-80psi approximately 2 h after plating.

*Viral vectors*

The knock-down hairpin constructs were produced using annealed and kinased oligonucleotides that had been ligated into the pll3.7 vector (courtesy of Luk Van Parijs) digested with Hpa and Xho. All plasmids (pMDG, pMDL g/p RRE, pRSV Rev, and the pll3.7 transfer vector) were prepared using the Qiagen Endofree maxiprep kit and lentivirus was prepared via calcium phosphate transfection of a 60 – 80 % confluent T175 flask of HEK293T cells. Cells were grown in DMEM (Invitrogen) plus 10% foetal bovine serum (FBS) and penicillin/streptomycin (Invitrogen). 13.2 μg of transfer vector together with 6.6 μg of the other 3 plasmids were transfected per T175 flask. Plasmids were diluted to 0.5 μg ml⁻¹ in TE buffer and made up to a volume of 900 μl with sterile distilled water. 100μl of 2.5M CaCl₂ was added together with 1ml of 2XBBS(50 mM BES, 280 mM NaCl, 1.5 mM Na₂HPO₄, pH= 6.95). This was incubated for 15 min at room temperature before adding the mix drop-wise to the cells. The cells were incubated for 3-4 h at 37⁰C in 5% CO₂ before the addition of new medium containing 10 mM sodium butyrate (Sigma). Viral supernatents were taken at 24 and 48 h post transfection and ultracentrifuged at 50,000 g for 90 min. The virus was re-suspended overnight at 4⁰C and then re-suspended in 300 μl PBS. Viral titers were determined by the addition of varying amounts of concentrated virus on known numbers of HEK293T cells. Titers of 10⁸ – 10⁹ IU / ml were routinely produced.

Animals (4-5 weeks of age) were anesthetized with a ketamine (100mg/kg) / xylazine (20 mg/kg) mixture. After animals were placed in a stereotaxic frame an incision was made to expose the skull surface. A small hole was drilled in the skull and the underlying dura removed. A broken micropipette filled with Lentivirus was lowered into the BLA and 1 μl of virus was delivered. The pipette was left in place for 5 min to minimize diffusion of the virus back along the injection tract, after which it was removed, the hole sealed with bone wax, and the incision closed with surgical staples. Animals were scarified after 7 – 10 days recovery and brain slices prepared as described above.

Primary rat cortical neuron cultures
Primary cultures of rat cortical neurons were prepared from freshly isolated embryonic (E18) cortex from Wistar rats. The isolated cortices were disrupted with a blade then transferred to a 15ml falcon tube and gently dispersed in dissection medium (1x HBSS, penicillin, streptomycin, pyruvate, Hepes 10mM, Glucose 30 mM). The supernatant was then taken off and the pellet resuspended with papain (17u/ml, Worthington, suspension 31.3 u/mgP) made up in dissection medium and supplemented with 1% DNase (Sigma) for 20 min at 37°C then the sedimented material was washed 3 times in plating medium (Neurobasal medium supplemented with 5% heat-inactivated bovine fetal serum, pen strep, GlutamaxI and 2% B27). After the last wash, the resulting pellet was resuspended and triturated in 2ml of plating medium using a fire-polished glass Pasteur pipette (up and down passes). The undisrupted material was let to freely settle at the bottom of the tube then the supernatant was collected in a new falcon tube. The remaining pellet was further triturated with 1ml of culture medium and the supernatants pooled. Then the cell suspension has been cleared up from the remaining unsolubilized material by a passage through a 0.2um filter. For cell counts, cells were diluted at 1:10, counted in a Coulter counter and plated at a density of 80 x 10^3 cells/500μl on glass coverslips pre-coated with poly-D-lysine. Cells were cultured in plating medium and maintained at 37°C in 5% CO2. At DIV1 the culture medium was replaced and again at days.

GluN2 knock-down in primary cortical neuron cultures

1μl of lentiviral particles at 10^8 – 10^9 IU / ml and carrying either the GluN2B hairpin construct or the GluN2B scrambled negative control was used to infect cortical neurons at DIV10. At 1 week post-infection cells were processed either for western blot. Western blot analysis: 2 coverslips for each sample were lysed with 150μl/each of 1X sample buffer containing DTT and the protein lysates were denatured for 8 min at 70°C and fractionated on 4-12% Bis-Tris gradient gels (Invitrogen) followed by protein transfer to polyvinylidene difluoride membrane (Immobilon-P, Millipore) at 150V in 1XMOPS running buffer (Invitrogen). Blots were blocked in TBS containing 5% skim milk powder, and probed with primary antibodies, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit or mouse IgG (Biorad) and detection by SuperSignal West Pico or FEMTO chemiluminescent substrate (Pierce). As primary antibodies, NMDA2B (Watanabe, rabbit polyclonal) and α-tubulin (Immuno, MP Biomedical, mouse monoclonal) were used. Samples were run together with non-infected neurons as negative control and with HEK293T cells transfected with NR2B cDNA as control for the NR2B molecular weight.
**Electrophysiological recordings**

All recordings were done in a perfusion chamber attached to an upright microscope (Olympus BX51). Cover slips plated with HEK293 cells, organotypic slices excised from their membrane cartridges, or acute brain slices were placed in the recording chamber and continuously perfused (200 ml/h) with either HEPES buffered saline (HEK293 cells) containing (in mM) 135 NaCl, 5 KCl, 3 CaCl₂, 10 glucose, 10 HEPES and 0.01 glycine or carbogenated ACSF solution (acute brain slices and organotypic slices). Inhibition was blocked by addition of 100 µM picrotoxin to the Ringer solution. Whole cell recordings were made from HEK293 neurons or visually identified pyramidal neurons in the BLA using 3-5 MΩ glass microelectrodes filled with cesium based pipette solution containing (in mM) CsMeSO₄ 135, NaCl 8, HEPES 10, Mg₂-ATP 2, Na₃GTP 0.3, phosphocreatine 7, spermine 0.1, and EGTA 0.3 (pH 7.2 with CsOH, osmolarity 290 mOsm/kg). Signals were amplified using a Multiclamp 700A amplifier (Molecular Devices) controlled by an Apple iMac. Current signals were filtered at 4-8 kHz, digitized at 20 kHz using an Instrutech ITC-18 interface, and acquired on an iMac using Axograph. A concentric bipolar stimulator (WPI, Sarasota, FL) placed over the external capsule was used to electrically stimulate slices. Access resistance was 4-12 MΩ (uncompensated) and monitored throughout the experiments. For LTP experiments, whole cell recordings were made using a potassium based internal solution containing (in mM) 135 KMeSO₄, 8 NaCl, 10 HEPES, 2 Mg₂-ATP, 0.3 Na₃-GTP, 0.1 spermine, 0.3 EGTA (pH 7.3 with KOH, osmolarity 280 - 290 mOsm). Cells were maintained in current clamp and inputs were stimulated at 0.1 Hz. After obtaining a baseline for 10 min, tetanic stimulation (30 Hz 1 sec x 2) was applied and then returned to 0.1 Hz stimulation. KN-62 (3 µM) (1-(Rodrigues et al. 2004)-N-methyl-L-tyrosyl-4-phenylpiperazine) was applied by including it in the perfusing Ringer. All compounds were obtained from Sigma-Aldrich unless otherwise specified.

**Model of BLA neuron**

Computational modelling of NMDA currents in BLA neurons was performed with the NEURON simulation program (version 7.0) (Hines 1989). A realistic model of a BLA projection neuron was constructed based on morphological measurements from fluorescence Z-stacks of BLA projection neurons recorded in previous studies. This stylized neuron had an axon, soma, single apical dendrite and 5 basal dendrites. The axon was 300 µm in length and 0.7 µm in diameter. The soma was spherical with a 15 µm diameter. The diameter of the apical dendrite prior to the first branch point (75 µm) was 1.5 µm. and this dendrite split into
secondary, tertiary, and quaternary branches each 60 μm in length and 0.5 μm in diameter. As the diameter of basal-like dendrites was found to be constant throughout the dendritic tree, we simplified the structure of each dendrite using the 3/2 power rule, collapsing the branches into a single equivalent cylinder (length 700 μm, width 2 μm).

The resting membrane potential was set to 0 mV. Passive electrical parameters were: $R_m$ 165,000 Ω cm$^{-2}$, $C_m$ 1 μF cm$^{-2}$ in the somatic compartment. In the dendrites, $R_m$ was reduced to 110,000 Ω cm$^{-2}$, and capacitance was doubled to account for the dendritic spines. The axial resistivity $R_i$ was 150 Ωcm except in the axon where it was 50Ω cm. A single electrode voltage-clamp point process (SEClamp; $R_s$ 13 MΩ) was placed on the soma. These settings yielded an input resistance that closely matched the experimental data (400 MΩ).

Synaptic inputs were distributed throughout the apical dendrite by placing a single NMDA conductance on the midsection of each apical dendrite branch. The NMDA conductance was modeled as having a reversal potential of 0 mV, an exponential rise, and a dual exponential decay. The decay was implemented using the following formula: $g_{max}*(1-(frac*e^{-tau_1} + (1-frac)*e^{-tau_2})$, where $g_{max}$ is the maximum conductance, $tau_1$ is the fast decay time constant, $tau_2$ is the slow decay time constant and $frac$ is the relative contribution of the $tau_1$ to the peak current amplitude. $tau_1$, $tau_2$ and $frac$ were 16.5 ms, 123.5 ms, and 0.90 for GluN2A synapses, and 35.5 ms, 269 ms, and 0.42 for GluN2B-containing synapses. These deactivation time constants correspond to values measured in recombinant expression systems (Vicini et al. 1998), which have been adjusted to approximate 32°C recording conditions in the present study. The rise time constant was 0.2 ms for both subtypes.

**Statistics**

Statistical significance was determined using a two-tailed student’s t-test or ANOVAs as appropriate.

**Results**

**NMDA receptor subunit protein expression**

NMDA receptors are heteromultimers composed of two obligatory GluN1 subunits and two NR2 (GluN2A-NR2D) or GluN3 (GluN3A, GluN3B) subunits. GluN2C expression is confined to the cerebellum, whereas GluN2D is largely present early in development (Cull-Candy et al. 2001). Both GluN2A and GluN2B subunits are expressed in the adult BLA (Farb et al. 1995; Lopez de Armentia and Sah 2003). Although GluN3 subunits are expressed in the amygdala, receptors containing these subunits show greatly reduced magnesium
sensitivity (Chatterton et al. 2002) and are unlikely to be present at synapses in BLA neurons as synaptic NMDA receptors in both principal neurons (Lopez de Armentia and Sah 2003; Mahanty and Sah 1999) and interneurons (Polepalli et al. 2010) show normal magnesium sensitivity. To understand the subunit composition of synaptic NMDA receptors in the BLA we first determined the expression of GluN1, GluN2A and GluN2B subunits using subcellular fractionation and western blot. As the development and subunit composition of NMDA-receptors in hippocampal neurons is well understood (Monyer et al. 1994; Sans et al. 2000; Sheng et al. 1994), we used tissue from adult and neonatal (P2) hippocampus as controls.

Tissue lysates from the adult BLA contained both GluN2A and GluN2B subunits (Fig. 1A). Expression levels were similar to that in the hippocampus, and as shown previously (Monyer et al. 1994; Sans et al. 2000; Sheng et al. 1994), expression of GluN2A subunits was low in P2 hippocampus (Fig. 1A). Isolation of the synaptic fraction (see Methods) showed that, together with PSD95, both GluN2A and GluN2B subunits were clearly detectable (Fig. 1B), showing that both subunits were present in the postsynaptic density. Overall, expression of both subunits was similar in synaptic fractions from the hippocampus and BLA (Fig. 1C). Immunoprecipitation of NMDA receptors using GluN1 antibodies revealed that both GluN2A and GluN2B subunits were present in functional receptors (Fig. 1D). In the adult brain, heterotrimeric receptors containing GluN1/GluN2A/GluN2B subunits are also present (Al-Hallaq et al. 2007; Sheng et al. 1994).

Indeed, in some regions, it has been suggested that trimeric receptors may be the dominant receptor present at mature synapses (Dunah and Standaert 2003; Luo et al. 1997). We therefore tested whether the GluN2A and GluN2B subunits formed heterotrimeric assemblies in the BLA (Sans et al. 2000; Sheng et al. 1994). Synaptic membrane fractions from adult hippocampus were immunoprecipitated using either GluN2A or anti-GluN2B antibodies. Probing the immunoprecipitated GluN2A protein with anti-GluN2B clearly revealed the presence of di-GluN2A/GluN2B complexes in the hippocampus and BLA (Fig. 1D). Similarly, immunoprecipitated GluN2B protein showed the presence of GluN2A subunits (Fig 1D). Together, these results show that as in the hippocampus tri-heteromers containing GluN2A and GluN2B subunits are present at excitatory synapses in the BLA.

\textit{RNAi knockdown of NMDA receptor subunit expression}

As currently available antagonists do not distinguish between heterodimers and heterotrimers, we used short hairpin RNAi constructs to reduce the expression of individual subunits in projection neurons, allowing us to investigate the role of these subunits in synaptic
transmission. Short hairpin RNAi constructs targeting GluN2A and GluN2B subunits were prepared (see Methods), and the efficacy and specificity of these constructs was first tested in HEK293T cells. Cells transfected with GluN2A, GluN2B and RNAi vectors were compared to those expressing scrambled sequences. As shown in Fig 2, RNAi vectors against GluN2A and GluN2B subunits selectively reduced expression of the targeted subunit (Fig. 2A, B; p < 0.05, n = 3) with no effect on the non-targeted subunit.

To verify that the reduction in GluN2 subunits resulted in reduced functional receptors, we tested these vectors using electrophysiological recordings of NMDA receptor-mediated currents. HEK293 cells were co-transfected with GluN1/GluN2 subunits and either the scrambled RNAi (Scr-RNAi), GluN2A-RNAi or GluN2B-RNAi. NMDA (50 µM) was co-applied with 1 µM glycine. Whole-cell current amplitudes were measured relative to total cell capacitance, calculated from a 10 mV voltage step response (Fig. 2C). Expression of GluN2A-RNAi significantly reduced the NMDA current in GluN1/GluN2A-transfected cells compared to Scr-RNAi-transfected control cells (5.3 ± 2.3 pA/pF control Vs 2.3 ± 1.9 pA/pF in 2A-RNAi ; p < 0.05, n = 9 and 13 respectively, Fig. 2D), whereas expression of GluN2B-RNAi had no significant effect (p > 0.05, n = 7, Fig. 2D). Similarly, only the GluN2B-RNAi construct reduced NMDA currents in GluN1/GluN2B-transfected HEK293T cells (p < 0.05, n = 8, 11 and 9 respectively, Fig. 2D). These results show that the RNAi constructs were specific for their targeted subunit, and could efficiently reduce the expression of the subunit protein and the number of membrane-bound functional receptors containing this subunit. To confirm that these RNAi vectors could effectively reduce expression in neurons we used viral transfection in cultured neurons (Methods). As in HEK293 cells, viral expression of RNAi vectors against GluN2A or GluN2B in cultured effectively reduced expression of each subunit (Fig 2).

NMDA receptor subunit knockdown in BLA neurons

To test the effects of knocking down specific subunits on synaptic currents in the BLA, two methods were used. Initially we used organotypic slice cultures prepared from P9 rat pups to transf ect neurons with hairpin constructs. To verify that organotypic slice cultures represented an appropriate model to study differential NMDA receptor subunit expression in the amygdala, we compared the kinetics and pharmacology of synaptic NMDA receptor currents from cells in organotypic cultures after three days in culture, with synaptic currents recorded from acute slices from age-matched P12-14 pups and previously reported data from adult rats (Lopez de Armentia and Sah 2003). NMDA-receptor EPSCs were fit with a dual
exponential as described previously (Lopez de Armentia and Sah 2003). Weighted time constants for each condition are given below and the values for the fast and slow time constants and the relative contribution of the fast component are presented in table 1. Synaptic NMDA receptor currents recorded from BLA principal neurons in organotypic culture had average weighted decay time constants not significantly different from those recorded from BLA principal neurons in P13 acute slices. The average weighted decay time constant ($\tau_w$) of the NMDA receptor-mediated excitatory synaptic current (EPSC), measured at +40 mV, was 90 ± 8 ms (Fig. 3A; n = 14) in organotypic slices, compared to 102 ± 11 ms (n = 4) in acute slices (p = 0.42), and similar to that reported in adult rats (Lopez de Armentia and Sah 2003). Delivery of GluN2A-RNAi constructs by biolistic transfection of BLA neurons (Fig. 3A upper traces) significantly slowed the NMDA receptor-EPSC decay ($\tau_w$ : 154 ± 10 ms, n = 9) as compared to that of cells transfected with scrambled RNAi ($\tau_w$ : 99 ± 11 ms, n = 5, p < 0.05). In contrast, delivery of GluN2B-RNAi (Fig. 3B, lower traces) had no significant effect on decay kinetics ($\tau_w$ : 81 ± 15 ms; p > 0.05, n = 6). These results show that in organotypic brain slices, inputs to principal neurons in the BLA maintain the properties of synapses during normal development and, reducing GluN2A expression at mature synapses slows the NMDA receptor-mediated EPSC while reducing GluN2B expression has little or no effect. However, there remains a possibility that conditions in culture do not accurately mimic conditions in situ. To confirm these observations, RNAi vectors were therefore moved into a lentivirus to test the results of knockdown in mature animals. Lentivirus containing RNAi vectors was delivered into the BLA at ~P21, an age where these synapses have reached mature conditions (Lopez de Armentia and Sah 2003). Delivery of RNAi vectors in vivo reproduced the results from organotypic cultures (Fig 3B,C). The weighted time constant of decay of NMDA receptor-mediated EPSCs (measured at +40 mV) from neurons infected with scrambled vectors was 96 ± 19 ms (n=5). Delivery of GluN2A RNAi slowed this to 163 ± 14 ms (n=9) whereas in neurons expressing GluN2B RNAi the decay time constant was 93 ± 7 ms (n=5), not significantly different from that obtained with scrambled vectors.

These results suggest that there are very few if any GluN1/GluN2B heterodimers present at synapses on BLA principal neurons. We next sought to confirm this by testing the effects of the specific antagonist ifenprodil (Williams 1993). NMDA receptor-mediated EPSCs recorded in organotypic cultures were pharmacologically similar to those of P14 acute slices. In heterologous expression systems ifenprodil, at a concentration of 5 µM provides selective and maximal block of GluN1/GluN2B heterodimeric receptors (Hatton and Paoletti
and possibly also block triheromeric receptors by ~20 % (Hatton and Paoletti 2005). However, at this concentration ifenprodil had little effect on NMDA receptor-mediated EPSCs (Fig. 4A). Ifenprodil also had no significant effect on AMPA receptor-mediated EPSCs that were fully blocked by the AMPA/kainate receptor antagonist NBQX (Fig. 4A, right) showing that it is selectively acting at NMDA receptors (Delaney et al. 2012). In contrast, in neurons transfected with GluN2A-RNAi, NMDA receptor-mediated EPSCs were blocked by ifenprodil (49 ± 7 %, p < 0.05, n = 6, Fig. 4B,C) whereas scrambled RNAi transfected cells (n=5) and GluN2B-RNAi-transfected cells (n=4) remained insensitive (2 ± 4 % and 8 ± 11 % respectively, p > 0.05, Fig. 4C). These results in organotypic slices were also replicated by viral transfection of BLA neurons in adult animals. In neurons transfected with GluN2A-RNAi, NMDA receptor-mediated EPSCs were blocked by ifenprodil (5 µM) (63 ± 5 % block, p < 0.05, n=8; Fig 4B, C) whereas scrambled RNAi transfected cells (n=4) and GluN2B-RNAi-transfected cells (n=4) remained insensitive (0.4 ± 11.9 % and 8 ± 7 % respectively; Fig. 4C).

Interestingly, the ratio of AMPA receptor-mediated EPSC (recorded at -60 mV), to that of the NMDA receptor-mediated EPSC (recorded at + 40 mV, measured 50ms post-stimulation) was unaffected by any of these manipulations, the ratio being 0.8 ± 0.2 (n = 11) in GluN2A-RNAi transfected BLA neurons compared to 0.7 ± 0.1 (n = 5) in cells with scrambled-RNAi and 1.0 ± 0.2 (n = 14) in non-transfected control cells. For GluN2B-RNAi transfected cells, the AMPA/NMDA-EPSC ratio was 0.6 ± 0.3, not significantly different to control non-transfected cells or scrambled-RNAi-transfected cells (p > 0.05, n = 6). These results show that in mature BLA neurons, when GluN2A expression is reduced, synaptic GluN2A subunits are replaced by GluN2B subunits. The fact that the AMPA:NMDA ratio is unchanged by this manipulation suggests that, proportionately, total synaptic NMDA receptor expression remains constant.

Subunit stoichiometry of NMDA receptors and EPSC kinetics

The kinetics of NMDA receptors is determined by their subunit stoichiometry with the offset kinetics of heterodimeric GluN1/GluN2A receptors being substantially faster than that of GluN1/GluN2B heterodimers (Erreger et al. 2005; Stocca and Vicini 1998; Tovar et al. 2000; Vicini et al. 1998). In both expression systems and cultured neurons, NMDA receptors containing GluN1/GluN2A subunits have offset time constants of 30-40 ms, whereas GluN1/N2B heterodimers have offset kinetics of ~ 200 ms. In agreement with these results,
early in development, NMDA receptors mediated EPSCs on principal neurons have decay
time constants close to 200 ms, consistent with the presence of GluN1/GluN2B subunits
(Lopez de Armentia and Sah 2003) whereas at mature synapses the decay time constant is
significantly faster (80 - 90 ms) suggesting replacement of GluN2A by GluN2B (Lopez de
Armentia and Sah 2003). This EPSC decay time constant at mature synapses is significantly
slower than the offset time constant of expressed GluN1/GluN2A heterodimers (30-40 ms;
Vivini et al., 1998), raising the possibility that GluN2B subunits make a significant
contribution to the EPSC (Lopez de Armentia and Sah 2003). However, as shown above,
application of ifenprodil at a concentration that clearly blocks synaptic GluN1/GluN2B
heterodimeric receptors (Delaney et al. 2012), has no effect on the NMDA-receptor mediated
EPSC. Moreover, reducing GluN2B expression in mature neurons was without effect on the
kinetics of NMDA receptor mediated EPSCs (Fig. 3) suggesting that these synapses do not
express GluN1/GluN2B heterodimers. One possible explanation for this discrepancy is that
the slow EPSC kinetics measured at the soma results from electrotonic filtering of currents
generated in the distal dendritic tree (Williams and Mitchell 2008). To test this possibility we
constructed a computational model of BLA projection neurons (Fig. 5). GluN1/GluN2A and
GluN1/GluN2B heterodimer conductances incorporated into the model were given
deactivation time constants derived from recombinant expression systems (Vicini et al. 1998).
When synapses containing GluN1/GluN2A heterodimers were placed on the soma, the
resultant EPSC, at the soma, had a weighted decay time constant of 53 ms. Distributing these
receptors along the dendrite (> 60 µm from the soma) yielded a weighted decay time constant
of ~93 ms, strikingly similar to the experimentally measured time-constant (90 ± 8 ms in
organotypic slices; 102 ± 11 ms acute slices). The kinetics of the GluN1 / GluN2A / GluN2B
heterotrimer is not known; however, it is likely that the offset time constant of these
receptors is dominated by the properties of GluN2A containing receptors (Santucci and
Raghavachari 2008).

As expected, modelling shows that dendritic filtering also slowed the time-course of
GluN1 / GluN2B conductances (Fig. 5C). The decay time constant of a somatically recorded
pure GluN1/GluN2B current was predicted to be 277 ms, whereas a synaptic conductance
comprising equal proportions of GluN1/GluN2A and GluN1/GluN2B heterodimers predicted
a current with a weighted decay time constant of 225 ms, considerably slower than the
experimentally recorded EPSC decay. Furthermore, our modelling shows that dendritic
filtering would not obscure the reduction of the synaptic current’s decay time expected from
ifenprodil’s selective attenuation of GluN1/GluN2B receptors (Fig. 5D). If dihetromeric
GluN1/GluN2B receptors provided half of the synaptic conductance, an 80% reduction of the GluN2B conductance by ifenprodil would enhance the decay of the synaptic current by 43% (from 224.5 ms to 129.1 ms). These results support our molecular and pharmacological data, indicating that in mature BLA projection neurons, synaptic NMDA receptors are unlikely to contain GluN1/GluN2B heterodimers.

Functional role of synaptic GluN2B subunits

Our results show that at mature synapses in BLA projection neurons, the postsynaptic density contains both GluN2A and GluN2B subunits. However, the kinetic and pharmacological properties of the NMDA receptor-mediated EPSC indicate that GluN1/GluN2B heterodimeric receptors make little or no contribution at these synapses. Thus, the GluN2B subunits present in the PSD must assemble as a heterotrimeric GluN1/GluN2A/GluN2B receptor. These receptors appear to have kinetic properties similar to those of GluN1/GluN2A heterodimers. What then is the functional role of the GluN2B subunit? Glutamatergic synapses on BLA principal neurons are well known to undergo NMDA receptor-dependent LTP (Pape and Pare 2010; Sah et al. 2008). In CA1 hippocampal pyramidal neurons, where NMDA receptor-dependent LTP has been extensively studied, LTP induction requires activation of postsynaptic CaMKII (Malenka and Bear 2004), and the long cystolic C-terminal end of GluN2B is thought to be essential in this activation (Barria and Malinow 2005; Kohr et al. 2003). Thus, at these hippocampal synapses, the presence of GluN2B subunits is essential for LTP. We therefore tested if a similar interaction may be required in the BLA. As shown previously (Faber et al. 2005), tetanic stimulation (1sec 30 Hz x 2) of these inputs robustly evoked LTP (161 ± 19%; n=5). However, when GluN2B subunit expression was reduced using RNAi, it was not possible to evoke LTP (87 ± 37%; n=6) (Fig. 6A). In contrast, application of ifenprodil (5 µM) had no effect on LTP (160 ± 10 % ; n=4) (Fig 6B). NMDA receptor-dependent LTP results from influx of calcium via NMDA receptors, activation of CaMKII and activation of a signalling cascade that ultimately results in LTP. This CaMKII is thought to be anchored at excitatory synapses by binding to GluN2B subunits (Barria and Malinow 2005). In agreement with this possibility, LTP at BLA synapses was also blocked by incubation of slices in the CaMKII inhibitor KN-62 (88 ± 13 %; n=5; Fig. 6C).

Discussion
We have examined the subunit composition of NMDA receptors present at glutamatergic synapses on principal neurons in the BLA. Our results show that (1) both GluN2A and GluN2B subunits are present at excitatory synapses on these neurons (2) NMDA receptors present at mature synapses are most likely heterotrimeric proteins containing GluN1/GluN2A/GluN2B subunits and, the kinetics and pharmacology of these receptors are dominated by the properties of GluN2A (3) GluN2B subunits are required for activation of CaMKII-dependent LTP induced by tetanic stimulation at these synapses and (4) GluN1/GluN2B heterodimers are either not present at these synapses or make a minor contribution.

NMDA receptors are present at most glutamatergic synapses in the mammalian central nervous system where they co-localise with AMPA receptors (Traynelis et al. 2010). At mature synapses, these receptors play a key role in the synaptic plasticity that is thought to underlie learning and memory formation (Cull-Candy et al. 2001; Yashiro and Philpot 2008). These receptors are tetrameric proteins that contain two obligatory GluN1 subunits and either GluN2A, GluN2B or both subunits (Traynelis et al. 2010). In the mammalian forebrain, GluN2B subunit expression is high when synapses are first formed (Monyer et al. 1994; Sheng et al. 1994; Tovar and Westbrook 1999), and the activity of receptors containing GluN1/GluN2B subunits is thought to play a central role in synapse formation and maturation (Cull-Candy et al. 2001). With maturation, GluN2A expression increases and there is a switch in subunit expression, with GluN2A replacing GluN2B subunits (Monyer et al. 1994). This switch is activity dependent (Barria and Malinow 2002; Philpot et al. 2001) and requires binding of glutamate to GluN1/GluN2B heterodimers (Barria and Malinow 2002).

Synapses on BLA principal neurons follow this developmental sequence and, when first formed the NMDA receptor-mediated EPSCs have relatively slow kinetics and are blocked by GluN2B selective antagonists (Lopez de Armentia and Sah 2003). At mature synapses, the NMDA receptor-mediated EPSC kinetics is faster, consistent with the presence of GluN2A subunits. However, the subunit composition of synaptic NMDA receptors is not clear. As well as the obligatory GluN1 subunit, both GluN2A and GluN2B subunits are present in the synaptic fraction purified from the BLA (see also (Lopez de Armentia and Sah 2003; Miwa et al. 2008). Although these subunits can form heterodimeric receptors, heterotrimeric receptors containing both GluN2A and GluN2B subunits are also present.

Biochemical approaches using membrane fractionation and co-immunoprecipitation have consistently demonstrated the presence of heterotrimeric GluN1/GluN2A/GluN2B NMDA receptors in mature neurons, but their relative abundance, and location has been very variable.
Al-Hallaq et al. 2007; Hawkins et al. 1999; Luo et al. 1997; Sheng et al. 1994). This no doubt results largely from the poor solubility of synaptic NMDA receptors, as extensively discussed in a recent study (Al-Hallaq et al. 2007), and as also evident from our purification (Fig. 1).

Previous studies have used pharmacological approaches to determine the subunit composition of NMDA receptors. However, these studies have relied on antagonists tested in recombinant systems that can discriminate receptors containing GluN2A from those containing GluN2B, but do not allow separation of heterodimeric from heterotrimeric receptors.

Four lines of evidence indicate that NMDA receptors at mature synapses are likely to be trimeric, containing GluN1/GluN2A/GluN2B subunits. Firstly, immunoprecipitation of NMDA receptors using the synaptic fraction shows that the GluN2A and GluN2B subunits are present as heterotrimers in the BLA. Secondly, neither NMDA receptor-mediated EPSCs, nor LTP at these synapses was affected by ifenprodil at a concentration that maximally and selectively blocks GluN1/GluN2B receptors, suggesting that such receptors are not present at these synapses. Thirdly, when GluN2A expression is reduced using RNAi, the NMDA receptor-mediated EPSC displays slower kinetics and becomes sensitive to ifenprodil, showing that GluN1/GluN2B heterodimers can replace receptors in the postsynaptic density. In contrast, when GluN2B subunits expression is reduced, there is no effect on the kinetics of NMDA receptor-mediated EPSCs, whereas modelling indicates that such a change would be detectable. Lastly, when GluN2B expression is reduced, LTP is blocked showing that these subunits are present, and have a functional role at the synapse. In hippocampal pyramidal neurons, NMDA receptor-dependent LTP requires activation of postsynaptic CaMKII which is recruited to active synapses by binding to the C-terminus of GluN2B (Barria and Malinow 2005). At excitatory synapses on BLA principal neurons, CaMKII is also required for LTP (Rodrigues et al. 2004) (Fig 5). The most parsimonious explanation of these data is that postsynaptic receptors at BLA excitatory synapses are largely GluN1/GluN2A/GluN2B heterotrimers. Interestingly, a similar conclusion has recently been reached for excitatory synapses on mature hippocampal pyramidal neurons (Rauner and Kohr 2011), where the properties of NMDA receptor-mediated EPSCs share the same kinetic and pharmacological properties as those in BLA neurons (Lopez de Armentia and Sah 2003).

In BLA principal neurons, postsynaptic NMDA receptor activation has been found to be required for the synaptic plasticity thought to underlie fear conditioning (Miserendino et al. 1990). More recently, it has been suggested that this plasticity is selectively mediated by activation of GluN2B-containing receptors (Laurent et al. 2008; Rodrigues et al. 2001; Walker and Davis 2008). Our results show that at mature synapses in the BLA,
GluN1/GluN2B hetrodimeric receptors are unlikely to be present. It is therefore unclear how GluN1/GluN2B-containing receptors participate in the acquisition of fear conditioning. One possibility is that the effects of selective blockers of this type of receptor result from activation of extrasynaptic NMDA receptors, as has been suggested in the entorhinal cortex (Massey et al. 2004). Alternatively, drugs delivered in vivo might diffuse to the central amygdala where GluN1/GluN2B heterodimer are present at synapses in the adult (Lopez de Armentia and Sah, 2003) and NMDA-receptors are required for synaptic plasticity (Lopez de Armentia and Sah 2007).

Acknowledgements
We thank Rowan Tweedale for comments on the manuscript. Robert Sullivan, Victor Aggono and Juergen Gotz for help with immunoprecipitation experiments. Supported by grants from the National Health and Medical Research Council of Australia, the Australian Research Council and Queensland Smart State Fellowship. Correspondence should be addressed to P. Sah at Pankaj.sah@uq.edu.au

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Figure 1. GluN2A and GluN2B subunits are both present at synapses in the adult basolateral amygdala. A. Representative immunoblots showing protein expression level of GluN2A and GluN2B protein in whole cell lysates of BLA and hippocampal tissue, with α-tubulin shown as a loading control. B. Representative immunoblots from a purified synaptic fraction, showing protein levels for GluN2A and GluN2B subunits in the BLA and hippocampus. The postsynaptic protein PSD-95 is shown as a loading control. C. Histogram showing relative synaptic GluN2A and GluN2B protein expression in the BLA and hippocampus normalized to PSD-95 expression. D. Hetrotrimeric GluN1/GluN2A/GluN2B receptors are present in BLA synaptic fraction. Upper blots show immunoprecipitation of the solubilised synaptic fraction with GluN1 antibody and probed with GluN2A or GuN2B antibodies. The middle panel shows the results from immunoprecipitation using either GluN2A or GLuN2B antibody. Blots were probed for GluN2A or GluN2B as marked, showing the presence of Glu2A/GluN2B containing complexes in both the hippocampus and BLA. PSD-95 expression is shown in the solubilised synaptic fraction as a loading control.

Figure 2. Knock down of NMDA receptor subunit expression by RNAi constructs in HEK293T cells. A. Total GluN2A subunit protein levels in HEK293T cells over-expressing GluN2A subunits co-transfected with scrambled RNAi, GluN2A RNAi or GluN2B RNAi constructs (normalized to α-tubulin for loading). B. Total GluN2B subunit protein levels in HEK293T cells over-expressing GluN2B subunits that were co-transfected with scrambled RNAi, GluN2B RNAi or GluN2A RNAi constructs (normalized to α-tubulin for loading). (* denotes p < 0.05, n = 3 for each) C. Whole cell response from HEK293T cells transfected with GluN1/GluN2B, in response to bath application of saturating concentrations of NMDA (50µM NMDA + 1µM glycine). The lower traces show average whole-cell response to 10mV voltage steps, used to calculate the membrane capacitance of recorded HEK293T cells. D, Average NMDA whole-cell currents in HEK293T cells transfected with GluN1/GluN2A or GluN1/GluN2B subunits in cells expressing GluN2A-RNAi or GluN2B-RNAi or untransfected controls (n = 9, 13,7,8,11 and 9 cells respectively; * denotes p < 0.05). E. Knockdown of GluN2B (upper blots) and GluN2A subunits (lower blots) in cultured neurons. Blots show GluN2B expression from untransfected cortical neuronal cultures (CTR), cultures transfected with virus carrying scrambled (Scr-RNAi) or RNAi vectors (2B RNAi, upper and
2A RNAi, lower). For comparison are shown blots from HEK293 cells transfected with GluN2B and control cells.

Figure 3. GluN1/GluN2B heterodimeric receptors do not contribute to NMDA-receptor mediated EPSCs in BLA principal neurons. NMDA receptor-mediated EPSC recorded from principal neurons in the BLA at +40 mV in organotypic culture (A) or acutely prepared coronal brain slices (B). Upper traces show normalized EPSCs recorded from neurons expressing scrambled RNAi (grey) or GluN2A RNAi vector. EPSCs from neurons expressing GluN2A RNAi have a slower decay. Lower traces show normalized EPSCs recorded from neurons expressing either scrambled RNAi (grey) or GluN2B RNAi. Knocking down GluN2B subunits has no effect on the EPSC kinetics. C. Bar graph plotting the weighted time constants of NMDA receptor mediated EPSCs from organotypic slices (left) or acutely prepared coronal brain slices (right). Plotted are decay time constants from control untransfected neurons (organotypic slices only), cells transfected with scrambled vectors (Scr RNAi) or RNAi vectors for GluN2A and GluN2B.

Figure 4. Synaptic NMDA receptors on BLA principal neurons do not contain GluN1/GluN2B heterodimers. A. Traces show the NMDA receptor-mediated EPSC recorded at +40 mV (left) or AMPA receptor-mediated EPSC, at -70 mV (right) recorded from a BLA principal neuron transfected with scrambled RNAi vector in organotypic slices. 5 µM ifenprodil has no effect on either EPSC. Application of 10 µM NBQX on washout of ifenprodil fully blocked the AMPA receptor-mediated EPSC. B. Effect of ifenprodil (5 µM) on the NMDA receptor mediated EPSC recorded from principal neurons in the BLA at +40 mV in organotypic culture (left) or acutely prepared coronal brain slices (right). The neurons were transfected with GluN2A RNAi constructs using either biolistic transfection (organotypic slices) or viral transfection (acute slices). Ifenprodil reduces the EPSC in both organotypic and acute slices. C. Bar graphs show mean data for the effects of ifenprodil (5 µM) on the NMDA receptor-mediated EPSC from neurons transfected with scrambled RNAi (Scr RNAi), GluN2A RNAi or GluN2B RNAi in organotypic slices (left; n= 5, 6, 4) or acutely prepared brain slices (right; n = 4, 8, 4 respectively). * denotes p < 0.05.

Figure 5. Computational model demonstrates that dendritic filtering slows the decay of NMDA currents measured in the soma. Cartoon illustrating the model neuron’s topology is shown in A. Synapses (filled circles) were distributed along the single dendritic tree as
indicated. Five additional dendrites that were represented by single equivalent cylinders also project from the soma.  

B, shows the normalized synaptic currents measured in the soma (Vm +40 mV) when GluN1/GluN2A heterodimeric conductances were distributed along the dendrite (GluN2A dendrite) and when the channels were located on the soma (GluN2A soma). Numbers indicate the weighted decay time constant for each distribution. C shows the somatic currents calculated when only GluN2A-containing or only GluN2B-containing heterodimeric NMDA receptors are activated, as well as when the GluN2A only and GluN2B only mediated conductances are equally represented (GluN2A / GluN2B). The action of ifenprodil on synaptic current comprising equal proportions of GluN1/GluN2A and GluN1/GluN2B heterodimeric NMDA-Rs (GluN2A / GluN2B) is modelled in D. Ifenprodil’s 80% attenuation of the GluN2B only conductance hastens the decay of the synaptic current.

Figure 6. GluN2B subunits but not GluN1/GluN2B heterodimers are required for LTP in BLA principal neurons.  

A. Plots comparing LTP generated in control cells and cells expressing GluN2B RNAi vectors. Tetanic stimulation was delivered at the arrow. LTP was absent in neurons expressing GluN2B RNAi vector (n = 6). Representative traces from baseline and LTP are show on the right.  

B, Plots comparing LTP generated in control slices and slices bathed in the GluN1/GluN2B receptor blocker ifenprodil (5 µM). LTP is unaffected by the presence of ifenprodil. Representative traces from baseline and LTP are show on the right.  

C, LTP in BLA principal neurons requires CaMKII. Plots show LTP in control slices and slices bathed in the CaMKII blocker KN-62. LTP was blocked in the presence of KN62 (n=5). Representative traces from baseline and LTP are show on the right.
**Figure 2; Delaney et al.**

- **A**
  - Bar graphs showing normalized integrated optical density for GluN1/GluN2A and GluN1/GluN2B under different RNAi treatments.
  - Scr-RNAi, 2A-RNAi, and 2B-RNAi conditions are compared.

- **B**
  - Graphs depict normalized integrated expression levels of GluN1/GluN2B under RNAi treatments.

- **C**
  - Graph illustrating NMDA responses with current traces at 100 pA, 300 pA, and 10 mV.

- **D**
  - Comparative graph showing normalized expression levels of GluN1/GluN2A and GluN1/GluN2B under RNAi treatments.

- **E**
  - Western blot analysis for GluN2B, α-TUBULIN, GluN2A, and GluN2A in NEURONS and HEK cells.
  - Molecular weights: 180 kDa for GluN2B, 50 kDa for α-TUBULIN, and 177 kDa for GluN2A.
  - Normalized expression levels are indicated.
A. organotypic slices

B. viral transfection

C.

Figure 3: Delaney et al
Table 1. Kinetic properties of NMDA receptor mediated EPSC measured at +40 mV, and with a sum of two exponentials. All values are mean ± SEM

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