Synaptic and Extrasynaptic NMDA Receptor NR2 Subunits in Cultured Hippocampal Neurons

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Thomas, Christopher G., Ashleigh J. Miller, and Gary L. Westbrook. Synaptic and extrasynaptic NMDA receptor NR2 subunits in cultured hippocampal neurons. J Neurophysiol 95: 1727–1734, 2006. First published November 30, 2005; doi:10.1152/jn.00771.2005. Early in development, neurons only express NR1/NR2B-containing N-methyl-D-aspartate (NMDA) receptors. Later, NR2A subunits are upregulated during a period of rapid synapse formation. This pattern is often interpreted to indicate that NR2A-containing receptors are synaptic and that NR2B-containing receptors are extrasynaptic. We re-examined this issue using whole cell recordings in cultured hippocampal neurons. As expected, the inhibition of whole cell currents by the NR2B-specific antagonist, ifenprodil, progressively increased from 69.5 ± 2.4% [6 days in vitro (DIV)] to 54.9 ± 2.6% (8 DIV), before reaching a plateau in the second week (42.5 ± 2%, 12–19 DIV). In NR2A−/− neurons, which express only NR1/NR2B-containing NMDA receptors, autaptic excitatory postsynaptic currents (EPSCs; ≥12 DIV) were more sensitive to ifenprodil and decayed more slowly than EPSCs in wild-type neurons. Thus NR2B-containing receptors were not excluded from synapses. We blocked synaptic NMDA receptors with MK-801 during evoked transmitter release, thus allowing us to isolate extrasynaptic receptors. Ifenprodil inhibition of this extrasynaptic population was highly variable in different neurons. Furthermore, extrasynaptic receptors in autaptic cultures were only partially blocked by ifenprodil, indicating that NR2A-containing receptors are not exclusively confined to the synapse. Extrasynaptic NR2A-containing receptors were also detected in NR2A−/− neurons transfected with full-length NR2A. Truncation of the NR2A C terminus did not eliminate synaptic expression of NR2A-containing receptors. Our results indicate that NR2A- and NR2B-containing receptors can be located in either synaptic or extrasynaptic compartments.

INTRODUCTION

N-methyl-D-aspartate (NMDA) receptors are multimers of NR1/NR3 and NR2 subunits. The time- and tissue-specific expression of NR2 subunits affect channel properties as well as the distribution of NMDA receptors (Monyer et al. 1994). Expression of the NR2A subunit in the rodent CNS begins at 6–10 days postnatal (Monyer et al. 1994; Sheng et al. 1994; Zhong et al. 1994), a particularly dynamic period in synapse development. Before that point, hippocampal NMDA receptors are largely NR1/NR2B heterodimers, resulting in excitatory postsynaptic currents (EPSCs) that slowly deactivate and are strongly inhibited by NR2B-specific antagonists such as ifenprodil (Flint et al. 1997; Kew et al. 1998; Kirson and Yaari 1996; Li et al. 1998; Tovar and Westbrook 1999). Although NR2A subunits clearly contribute to synaptic NMDA receptors, the existing data do not completely support the idea that NR2A-containing receptors are exclusively synaptic and NR2B-containing receptors are exclusively extrasynaptic (Mohrmann et al. 2000; Stocca and Vicini 1998; Tovar et al. 2000; Townsend et al. 2003).

Recent experiments suggest that synaptic and extrasynaptic receptors may have distinct roles in synaptic plasticity, coupling to intracellular signaling cascades, and cell death (Hardingham et al. 2002; Liu et al. 2004; Massey et al. 2004). During synaptic development, NMDA receptors become clustered at synapses, but a population of extrasynaptic receptors remains (Cottrell et al. 2000; Pickard et al. 2000; Rosenmund et al. 1995). Synchronous clustering of NMDA receptors is in part controlled by intracellular protein–protein interactions with postsynaptic density proteins (Bolton et al. 2000; Scannevin and Huganir 2000). The sequence of trafficking, targeting, and anchoring of NMDA receptors remains an active area of investigation (Perez-Otano and Ehlers 2004; Wenthold et al. 2003). The underlying mechanisms for localization and signaling may involve differential binding of proteins to the cytoplasmic domains of NR2A and NR2B (Guillaud et al. 2003; Lavezzari et al. 2004; Sans et al. 2000).

We used whole cell recordings in cultured hippocampal neurons to determine the NR2 subunit composition of synaptic and extrasynaptic receptors during development in vitro. We confirmed that NR2A-containing receptors are incorporated into synapses during development. However, the extrasynaptic population included both NR2B- and NR2A-containing receptors. Transfection of an NR2A C-terminal truncation mutant subunit into neurons from NR2A−/− mice showed that the cytoplasmic domain was not essential for synaptic localization.

METHODS

Cell culture

Cell cultures were prepared from hippocampi of newborn wild-type or NR2A−/− mice. As previously described (Jahr and Stevens 1987), hippocampi were removed, incubated with papain (Worthington, Lakewood, NJ), and mechanically dissociated. Neurons (150,000 cells/dish = 300 cells/mm²) were plated onto a confluent layer of glia...
on glass coverslips (Fisher, Pittsburgh, PA). For single neuron (autaptic) cultures, cells (50,000 cells/dish = 100 cells/mm²) were plated onto glial microisolates. Hippocampal glia were grown to confluence on coverslips (25 mm) coated with collagen (Cohesion, Palo Alto, CA) and poly-l-lysine (Sigma, St. Louis, MO). For autapses, coverslips were coated with 0.15% agarose (Sigma) and sprayed with a mixture of collagen, poly-d-lysine (BD Biosciences, Bedford, MA) and 17 mM acetic acid (Bekkers and Stevens 1991). For some experiments, 100 μM AP5 (Tocris, Ellisville, MO) was added to the culture medium from 4 to 6 DIV.

**GENOTYPING** NR2A⁺/− mice were bred on a C57BL/6 wild-type background and genotyped using PCR amplification of genomic DNA. As previously described (Tovar et al. 2000), tissue was incubated in protease K (0.5 mg/ml; Gibco BRL, Carlsbad, CA) at 55°C for ≥12 h. Samples were centrifuged, and genomic DNA was precipitated from supernatants by adding an equal volume of isopropanol. The precipitated DNA was pelleted by centrifugation, washed in ice cold 70% ethanol, and allowed to dry. Genomic DNA was resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and added to the following PCR mixture: Mg²⁺ (2.15 mM), dNTPs (0.2 mM each), oligonucleotide primers (0.01 mg/ml each), Taq polymerase (2.5 units; Promega, Madison, WI), reaction buffer (5 μl), and 2 μl of solubilized genomic DNA (50 μl final volume) in high-performance liquid chromatography (HPLC) water. Two reactions were performed on each DNA sample. The first reaction used primer 1, 5′-TCT-GGGCCCTGTCCTCAACAAATCTGTC-3′; primer 2, 5′-CCTGATGCCGCTGATGCTCCT-3′, and the Neo Primer, 5′-GGCCCTGGTCTTCAACAATTCTGTGC-3′. Reaction products were run on 2% agarose gels and visualized using ethidium bromide.

In the first set of reactions, DNA from NR2A⁺/− mice yielded a single 253-bp product. Gel purification and sequencing of the reaction products yielded the expected DNA sequences (data not shown).

**TRANSFECTION** We transfected cell cultures at 6 DIV using the liposomal Effectene kit (Qiagen USA, Valencia, CA). For most experiments, cultures were incubated in serum free media for 2 h before the reaction solution containing 0.5 μg of full-length NR2A or the truncation mutant (NR2Astop44) subunit plasmid cDNA and 0.5 μg of enhanced green fluorescent protein (EGFP) plasmid cDNA was added to each culture. Transfections were stopped after 90 min by replacing the media with serum containing media. Recordings from transfected neurons were made 1–3 days after transfection (7–9 DIV).

For imaging, 0.5 μg of EGFP-tagged NR2A (GFP-NR2A) was co-transfected with 0.5 μg of carrier DNA (Gibco-BRL). NR2A, NR2Astop44, and GFP-NR2A cDNA were inserted into pcDNA1/AMP provided by Stephen Heinemann (Salk Institute, La Jolla, CA). The EGFP construct was inserted into the JPA7 vector provided by Gary Banker (CROET, OHUS, Portland, OR).

**ELECTROPHYSIOLOGY** Whole cell voltage-clamp recordings were made at a holding potential of −70 mV. The superfused extracellular bath solution contained (in mM) 162 NaCl, 2.4 KCl, 10 HEPES, 10 glucose, 1 CaCl₂, 0.0005 TTX (Alomone Labs, Jerusalem, Israel), 0.01 bicuculline methiodide (Sigma), 0.002 strychnine (Sigma), and 0.1 glycine, pH 7.2, 320 mOsm. For autapses, TTX was omitted, and calcium was increased to 2 mM. Patch pipettes (2–5 MΩ) were filled with (in mM) 145 Cs-glucuronate, 1 MgCl₂, 10 HEPES, 1.1 EGTA, and 5 CsBAPTA, pH 7.2, 310 mOsm. For autapses, we used (in mM) 150 K-glucuronate, 1.418 CaCl₂, 2 MgCl₂, 10 EGTA, 10 HEPES, 2 Na₂ATP, and 0.2 GTP, pH 7.2, 310 mOsm. Quartz flow pipes for solution exchanges were placed 50–100 μm from the cell and mounted to a piezoelectric bimorph driven by a stimulus isolation unit (Winston Electronics, San Francisco, CA). Step depolarizations (+10 mV, 0.5 ms) were used to evoke autaptic EPSCs. Currents were recorded using an Axopatch 1C (Axon Instruments, Foster City, CA) amplifier and Axograph acquisition software. Currents were filtered at 2 kHz and digitized at 5 kHz.

**IMMUNOFLUORESCENCE** Four days after transfection, GFP-NR2A- transfected NR2A⁺/− neurons (10 DIV) were live stained (anti-GFP, rabbit, Molecular Probes, Eugene, OR, 1:200) in serum-free medium for 5 min at 37°C. Neurons were washed in PBS solution containing 1 mM MgCl₂ and 0.1 mM CaCl₂ and fixed in 4% paraformaldehyde and 4% sucrose in PBS for 20 min at 37°C. Cells were permeabilized in 0.3% Triton-X-100 in PBS for 5 min, blocked in 0.5% fish gelatin in PBS for 30 min, incubated in primary antibody (anti-Synapsin 1, mouse, Synaptic Systems, Goettingen, Germany, 1:500 in blocking solution) for 2–3 h and then in secondary antibodies (goat anti-mouse Alexa A633, Molecular Probes, 1:1000 and biotinylated goat anti-rabbit, Jackson ImmunoResearch Laboratories, West Grove, PA, 1:1000 in blocking solution) for 30 min. All steps excluding live staining and fixation were performed at room temperature. A Leica DM-RXA (Bannockburn, IL) microscope equipped with a ×63, 132 NA, Plan Apo objective and a Princeton Instruments Micromax (Trenton, NJ) CCD camera were used for immunofluorescence imaging of stained neurons. Digital images were acquired and pseudocolored with MetaMorph (Universal Imaging, Buckinghamshire, UK) imaging software and prepared for presentation with Adobe Photoshop (San Jose, CA).

**DATA ANALYSIS** Currents were analyzed with Axograph software (Axon Instruments). Ifenprodil inhibition was expressed as percent inhibition of the peak NMDA receptor–mediated current [1 - I(NMDA+Iifenprodil)/I(NMDA)] × 100. Miniature EPSC recordings were analyzed with a variable template function. The template was set up to detect AMPA receptor–mediated events that were five to six times greater than the SD of the background noise. Each event was verified by eye and averaged with a minimum of 50 events per recording condition. Autaptic EPSCs and mEPSCs were averaged for each drug condition. Ifenprodil inhibition of EPSCs was expressed as percent inhibition of the NMDA receptor–mediated component, measured 10–20 ms after the beginning of an event. The decay of NMDA receptor–mediated autaptic EPSCs, recorded in the presence of NBQX, was fit with the sum of two exponentials and presented as a weighted average decay. The larger amplitude of EPSCs at 12–19 DIV allowed a more accurate estimate of the slow time constant than in our earlier studies on neurons at 6 DIV (Tovar and Westbrook 1999). Pooled data are presented as means ± SE. For statistical comparisons, paired and unpaired t-tests or ANOVAs with subsequent Bonferroni/Dunn test for multiple comparisons were used as appropriate. Statistical significance was set at P < 0.05.

**RESULTS**

NR2A subunits are incorporated into NMDA receptors during synaptic maturation in vitro

We used the noncompetitive, NR2B-selective inhibitor, ifenprodil, to examine the contribution of NR2A and NR2B subunits to whole cell and synaptic currents. At a concentration of 3 μM, ifenprodil blocked 73.8 ± 2.4% of whole cell NMDA-induced currents recorded in hippocampal neurons from NR2A⁺/− mice (n = 14, Fig. 1, A and B) that only express NR1/N2B heterodimeric NMDA receptors. This concentration of ifenprodil is the highest that can be used without affecting NR2A-containing receptors, and the degree of block...
we observed is consistent with block of NR1/2B recombinant receptors (Tovar and Westbrook 1999). NR2A expression develops as synaptic activity gradually increases during the first week in vitro (Monyer et al. 1994; Sheng et al. 1994). Thus to synchronize NR2A expression (Chavis and Westbrook 2001; Hoffmann et al. 2000), we blocked synaptic activity with AP5 (100 μM) between days 4 and 6 in vitro. Ifenprodil inhibited 69.5 ± 2.4% (n = 25) of the whole cell current recorded in wild-type neurons at 6 DIV, consistent with prior evidence that early in development NMDA receptors are primarily NR1/NR2B heteromers. The slightly higher ifenprodil inhibition in NR2A−/− neurons than in wild-type neurons at 6 DIV presumably reflects a small amount of NR2A expression triggered by residual synaptic activity between days 4 and 6. Ifenprodil inhibition decreased from 61.4 ± 2% (n = 29) at 7 DIV and 54.9 ± 2.6% (n = 26) at 8 DIV to 42.5 ± 2% (n = 21) in synthetically mature neurons (12–19 DIV). This is consistent with an increase in NR2A-containing receptors, but also indicates that both NR2A- and NR2B-containing receptors contribute to the whole cell current even in synthetically mature cells.

Whole cell currents include both synaptic and extrasynaptic components. To examine synaptic NMDA receptors, we evoked EPSCs in autaptic cultures. EPSCs in wild-type (12–19 DIV) and NR2A−/− neurons (12–19 DIV) had a fast AMPA receptor component followed by an AP5-sensitive NMDA receptor component (Fig. 1C). Ifenprodil blocked 86.5 ± 5.7% (n = 4) of NR2A−/− EPSCs, showing that NR1/2B receptors can be located at synapses after synaptic maturation. Ifenprodil inhibition was partial in wild-type neurons (55.8 ± 9.9%, n = 5), indicating NR2B-containing receptors are also present at wild-type synapses. NR1/2B receptors have much slower deactivations than NR1/2A receptors (Vicini et al. 1998). Thus as expected, NMDA receptor–mediated EPSCs in wild-type neurons decayed significantly faster (435 ± 31 ms, n = 6) than NR2A−/− EPSCs (723 ms, n = 4).

Isolation of extrasynaptic NMDA receptors

It is difficult to separate synaptic and extrasynaptic receptors in most preparations. However, extrasynaptic NMDA receptors can be studied in isolation in autapses by first irreversibly blocking synaptic NMDA receptors with MK-801 (Rosenmund et al. 1995). We developed an analogous protocol using high potassium–evoked release of glutamate to block synaptic NMDA receptors in multi-cell cultures. We first measured the whole cell NMDA current (Fig. 2A, left). Then the cell was...
NR2A is present in extrasynaptic NMDA receptors

If NR2A-containing receptors are synaptic and NR2B-containing receptors are extrasynaptic, the extrasynaptic population should be highly sensitive to ifenprodil. To follow the distribution of newly expressed NR2A subunits during maturation, we measured ifenprodil inhibition of NMDA receptor currents before and after MK-801 treatment. As seen in Fig. 1, ifenprodil inhibition of total whole cell current, including both synaptic and extrasynaptic receptors, was less in synaptically mature neurons (43.4 ± 2.6%, n = 14) than at 6 DIV (64.4 ± 3.6%, n = 12; Fig. 3A, left). The ifenprodil inhibition of the extrasynaptic population as measured after MK-801 treatment (Fig. 3A, right) was highly variable between neurons. At 6 DIV, the ifenprodil inhibition of the extrasynaptic population was 63.8 ± 3.8% (range, 39–63%, n = 12; Fig. 3A, top right). In synaptically mature neurons, the ifenprodil inhibition was 49.1 ± 3.5% (range, 19–61%, n = 13), which was marginally greater than that for the total whole cell current (Fig. 3A, bottom). The variability in ifenprodil inhibition of the extrasynaptic population suggested that, at least in some cells, NR2A-containing receptors were extrasynaptic. Consistent with this idea, the total and extrasynaptic population of NMDA receptors in mature autapses showed similar ifenprodil inhibition (Fig. 3B). Before MK-801, the inhibition was 42.5 ± 6.9% (n = 4) compared with 37.5 ± 5.5% (n = 4) after blocking synaptic NMDA receptors. Furthermore, in inhibitory neuron autapses that lack any synaptic NMDA receptors, ifenprodil inhibition of this purely extrasynaptic population was 40.1 ± 5.1% (n = 9). Thus under several different experimental conditions, the ifenprodil sensitivity of the extrasynaptic population was less than predicted for NR1/NR2B receptors.

Transfected NR2A is incorporated into synaptic NMDA receptors of NR2A−/− neurons

The intracellular domains of NMDA receptors subunits contain protein interacting domains that are thought to influence their targeting, location, and function (Perez-Otano and Ehlers 2004; Wenthold et al. 2003). To examine the role of the cytoplasmic domain of NR2A in synaptic localization, we used NR2A−/− neurons as a background into which we introduced...
full-length or mutant recombinant NR2A subunits. We transfected NR2A⁻/⁻ cultured neurons at 6 DIV with an N-terminally, EGFP-labeled NR2A (GFP-NR2A) subunit. Four days later (10 DIV), the neurons were live-immunostained for GFP, fixed, permeabilized, and immunostained for synaptophysin. Puncta representing cell-surface GFP-NR2A were scattered throughout the dendrites (Fig. 4A, red pseudocolor). These cultures containing both excitatory and inhibitory neurons, the presynaptic marker synaptophysin identified synapses, some of which were co-localized with GFP-NR2A (Fig. 4A). These results suggest that transfected NR2A subunits were functionally expressed at synapses.

To examine the synaptic receptors in the transfected neurons, we recorded mEPSCs in cells that had been transfected with full-length NR2A subunit cDNA. GFP-NR2A has low fluorescent intensity, thus we co-transfected the neurons with EGFP as a screen. Ensemble average mEPSCs from mature wild-type (12–19 DIV), mature untransfected NR2A⁻/⁻ (12–19 DIV), and NR2A-transfected NR2A⁻/⁻ (7–14 DIV) neurons all had fast AMPA receptor-mediated components followed by a slow NMDA receptor-mediated component (Fig. 4B). In each case, the slow component was completely blocked by AP5. The NR1/2B receptors of the NR2A⁻/⁻ mEPSCs were more inhibited by ifenprodil (71.8 ± 6.2%, n = 8 cells) than either wild-type neurons (57.3 ± 8.3%, n = 6 cells) or NR2A⁻/⁻ neurons transfected with NR2A (24.1 ± 12.3%, n = 4). Thus transfected full-length NR2A subunits were successfully incorporated into functional synaptic NMDA receptors.

NR2A subunits lacking the cytoplasmic domain can be located at synapses

Steigerwald et al. (2000) reported that mice expressing a C-terminal truncation mutant of NR2A have less synaptic NMDA receptors. We tested the role of the NR2A C terminus using cultured NR2A⁻/⁻ neurons transfected with either a full-length NR2A or a C-terminal truncation mutant, NR2Astop844 (Fig. 5). Using the protocol shown in Fig. 3, we measured ifenprodil inhibition of the total whole cell NMDA current and the isolated extrasynaptic component. Compared with ifenprodil inhibition of untransfected NR2A⁻/⁻ neurons (73.79 ± 2.4%, n = 14), expression of either NR2A subunit significantly reduced inhibition both before and after MK-801 treatment. For neurons transfected with full-length NR2A ifenprodil inhibition was 32.8 ± 5.9%, (n = 9) before MK-801 treatment and 24.4 ± 5.6%, (n = 9) after the treatment. For neurons transfected with NR2Astop844, ifenprodil inhibition was 56.2 ± 4.3%, (n = 12) before MK-801 treatment and 53.9 ± 3.8% (n = 12) after the treatment. There was no effect on the average amplitude of the whole cell current (NR2A⁻/⁻, 4.2 ± 1.1 nA, n = 14; NR2A⁻/⁻ + NR2A, 6.6 ± 0.9 nA, n = 9; NR2A⁻/⁻ + NR2Astop844, 6.6 ± 1.2 nA, n = 12). Thus transfected NR2A subunits lacking the C-terminal domain can be incorporated into synaptic as well as extrasynaptic NMDA receptors. Consistent with this idea, ifenprodil inhibition of the ensemble average mEPSC from NR2A⁻/⁻ neurons transfected with NR2Astop844 was 22.0 ± 5.9% (n = 7), similar to mEPSCs in neurons transfected with full-length NR2A.

Although the NR2A C-terminal truncation mutant was expressed at synapses, the surface expression of this construct appeared to be less than the full-length construct because whole cell currents in NR2Astop844-transfected neurons were significantly more sensitive to ifenprodil than those transfected with full-length NR2A. The vector for both constructs was the same, suggesting that truncation of the C terminus affects receptor trafficking.

**Discussion**

We re-examined the NR2 subunit composition of synaptic and extrasynaptic NMDA receptors using cultured hippocampal neurons. Our results suggest that NR2A- and NR2B-containing receptors are not exclusively segregated into synaptic and extrasynaptic compartments. This conclusion was supported by analysis of the extrasynaptic complement of NMDA receptors in whole cell currents, analysis of miniature and evoked EPSCs, and transfection of NR2A constructs into NR2A⁻/⁻ neurons.

Validity of our approach

Our analysis relied on comparing the ifenprodil inhibition of whole cell NMDA receptor currents before and after irreversible block of synaptic receptors with MK-801. The existence of
triheteromeric receptors containing NR2A and NR2B (Sheng et al. 1994; Tovar and Westbrook 1999) complicates the analysis of NMDA receptor subunit composition based on selective NR2A and NR2B antagonists. We used ifenprodil because it has been characterized on NR1/2B as well as NR1/2A/2B receptors and involves binding of the antagonist to two NR2B subunits for full inhibition (Hatton and Paolietti 2005; Kew et al. 1998; Tovar and Westbrook 1999). Even on NR1/2B receptors, ifenprodil at selective concentrations only blocks ~75–80% of NR1/2B current because of its noncompetitive kinetics. Using this degree of block as a reference, most whole cell current or EPSCs in mature hippocampal neurons are partially inhibited by ifenprodil (Tovar and Westbrook 1999) reflecting the contribution of NR2A-containing receptors. Another complication with the use of ifenprodil is that it increases receptor affinity for glutamate (Kew et al., 1996). Thus at low glutamate concentrations, ifenprodil can potentiate NMDA responses. We avoided this issue by using high agonist concentrations.

It is also essential to our analysis that we were able to isolate a pure population of extrasynaptic receptors. For whole cell recording, we used a modified protocol based on irreversible block of synaptic NMDA receptors by MK-801 after evoked release (Rosenmund et al. 1995). The protocol we used eliminated the NMDA receptor component of mEPSCs as well as the evoked EPSC in autaptic neurons without affecting transmitter release, confirming that the remaining whole cell NMDA responses were exclusively mediated by extrasynaptic NMDA receptors. The high potassium stimulation could have resulted in some spillover to extrasynaptic receptors, but this would not affect our interpretations of the remaining extrasynaptic receptor current. The high potassium stimulation might also have led to activation of “perisynaptic” receptors that could affect the analysis if this hypothetical population of receptors has a distinct NMDA receptor subunit composition.

We used transfection of NR2A constructs into NR2A−/− neurons to examine whether the cytoplasmic domain was essential for the presence of NR2A-containing receptors at synapses. Inappropriate localization caused by overexpression is always a concern with such experiments. However, the surface expression of NMDA receptors is limited by endogenous NR1, and the whole cell current amplitudes were not increased in transfected neurons. In fact, the surface expression of the truncation mutant was less than the full-length construct. Thus it seems unlikely that overexpression was the reason that NR2A subunits lacking cytoplasmic domains were present in synapses. We cannot exclude that receptors containing some exogenous NR2 subunits were inappropriately expressed in extrasynaptic locations. However, NR2A-containing receptors were also present in extrasynaptic receptors of wild-type neurons.

**Distribution of NR2-containing receptors**

Our results are consistent with some aspects of prior studies on NMDA receptor subunit distribution, but not with others. Specifically, the expression of both synaptic and extrasynaptic receptors early in cell culture consisted of NR1/2B receptors (Li et al. 1998; Tovar and Westbrook 1999). Also consistent with prior data, NR2A-containing receptors appeared soon after synapse formation and contributed to synaptic responses. However, the consensus across studies breaks down over whether NR2B-containing receptors are expressed at synapses and whether NR2A-containing receptors are expressed in extrasynaptic locations. Both of these occurred in our experiments. These discrepancies may be caused by difficulties in isolating extrasynaptic receptors and an underappreciation of triheteromeric receptors. However, we cannot exclude that there may also be differences based on cell types or preparations. It is perhaps not surprising that NR2B-containing receptors are expressed at synapses in NR2A−/− neurons, an effect that could be attributed to compensation. However, miniature NMDA receptor–mediated EPSCs disappear during development in collicular neurons (Townsend et al. 2003), suggesting that compensation does not occur in that preparation. There is strong evidence that NR2B subunits contribute to triheteromeric synaptic receptors in wild-type neurons (Kew et al. 1998; Luo et al. 1997; Sheng et al. 1994; Tovar and Westbrook.
NR2A intracellular C-terminal domain

The intracellular domains of ionotropic glutamate receptor subunits provide binding motifs for many interacting proteins that function in the trafficking and signaling of receptor complexes (Wenthold et al. 2003). Interactions between the C-terminal domains of NMDA receptor subunits and intracellular proteins are thought to control the synaptic and extrasynaptic localization of NMDA receptors (Perez-Otano and Ehlers 2004). The intracellular domains of NR2A and NR2B can differentially interact with proteins involved in trafficking and anchoring, thus providing a potential mechanism for differential distribution of NR2A– and NR2B-containing receptors (Wenthold et al. 2003). Consistent with an important role of the NR2A cytoplasmic domain in synaptic localization, Steigerwald et al. (2000) reported smaller NMDA receptor–mediated EPSCs and less clustering of receptors at synapses in hippocampal neurons from transgenic mice expressing an NR2A truncation mutant. However, they also noted an overall decrease in total NR2A expression.

Given this evidence, it was somewhat surprising to us that truncated NR2A subunits were present in synaptic NMDA receptors of transfected NR2A–/– neurons. NMDA current amplitudes in transfected neurons were not increased compared with wild-type neurons, suggesting that overexpression did not cause inappropriate targeting to the synapse. Prybylowski et al. (2002) also did not see increased synaptic current when NR2A was overexpressed in wild-type cerebellar granule cells. Our results by no means eliminate the possibility that the intracellular domain of NR2 subunits influences targeting because both NR1 and NR2B subunits were intact and therefore NR1/NR2A heterodimeric or NR1/NR2A/NR2B heterotrimetric receptors could be compensatory.

Perhaps more interestingly, truncation of the cytoplasmic domain reduced the surface expression of NR2A-containing receptors as also was observed by Steigerwald et al. (2000). This suggests that the cytoplasmic domain is important in receptor trafficking. For example, elimination of the PDZ binding motif, which occupies the last four amino acids (ESDV) of the NR2A and NR2B C termini (Kim and Sheng 2004), reduces the contribution of NR2A and NR2B to synaptic NMDA receptors (Barria and Malinow 2002). In addition, NR2A and NR2B may be trafficked differently (Guillaud et al. 2003; Lavezzari et al. 2004; Sans et al. 2000). NR2A, NR2B, synaptic, and extrasynaptic

The categorization of NR2A-containing receptors as synaptic and NR2B-containing receptors as extrasynaptic has taken on additional significance because extrasynaptic receptors seem to have important functions. For example, extrasynaptic receptors have been reported to influence synaptic plasticity (Massey et al. 2004) and neuronal cell death (Hardingham et al. 2002). It has also been suggested that NR2A-containing receptors are required for long-term potentiation (Liu et al. 2004; Massey et al. 2004), whereas two recent studies have come to different conclusions (Berberich et al. 2005; Weitlauf et al. 2005). These potential distinctions between synaptic and extrasynaptic receptors may involve not only different access to released glutamate but also different intracellular signaling cascades. Our results indicate that the segregation of NR2A
and NR2B is not absolute. Although the NR2-selective antagonists are handy tools, they cannot always be equated with selective block of synaptic or extrasynaptic receptors. Thus separation of these receptor populations using methods such as MK-801 block may be preferable.

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